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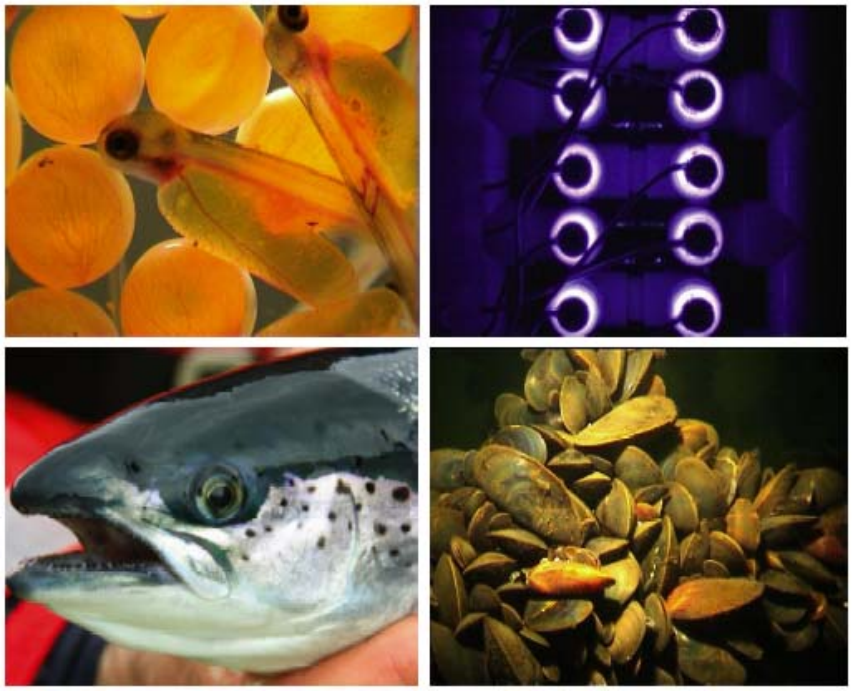
Evaluation Of Sensitivity To Chemotherapeutants In Successive Generations Of Lepeoptheirus Salmonis From A Resistant Population.



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Evaluation of sensitivity to chemotherapeutants in successive generations of *Lepeoptheirus salmonis* from a resistant population.

1. INTRODUCTION

1.1 Study Background

There are currently reports of reduced sensitivity to certain lice treatments in different parts of Scotland and world-wide, and research is on-going into the extent and mechanisms of resistance to different treatments (Denholm *et al*., 2002; Sevatdal & Horsberg, 2003; Sevatdal *et al*., 2005). In particular, increasing evidence of resistance of *Lepeophtheirus salmonis* to the chemotherapeutant emamectin benzoate (Lees *et al*., 2008; Espedal *et al*., 2010) poses a serious problem to commercial farms because there are few licensed and effective treatments available.

In order to address the heritability of this trait we assessed the sensitivity of successive generations of emamectin-resistant (RS) and emamectin-sensitive (naïve, NV) *L. salmonis*. Machrihanish Experimental Research Laboratory (MERL) was in a unique position to be able to conduct this study as we had maintained two verified strains of *L. salmonis* on site in isolation and in the absence of treatment. The naïve strain was originally taken from a farm site where the only treatment that had been used was hydrogen peroxide. Lice from this site were collected in approximately 2001 and had been cultured since without exposure to any lice treatment. The resistant strain was established in 2008 for the purposes of a separate experimental study. These lice had been shown to be five to seven times less sensitive to emamectin benzoate than the naïve strain using bioassays and *in vivo* treatments (unpublished data). The strain had also been shown to have reduced sensitivity to other licensed treatments (unpublished data).

Through producing multiple generations of both lice strains in the absence of treatments and through the hybridisation of the lice strains under controlled conditions, this study aimed to investigate if resistance to lice treatments could be reduced at the population level and thus make the lice treatments effective again at farms where resistance occurs.

The study ran from February 2010 up to February 2011.

1.2 Objectives

The main objectives of the study as set out in the original application were as follows:

1) To maintain populations of naïve and resistant lice in culture through multiple generations.
2) To monitor sensitivity levels to a given treatment over a period of 12 months and up to 6 subsequent generations in a population of resistant lice in the absence of any treatments.
3) To measure the fecundity and successful hatching and development of lice from each generation compared to a naïve control population of lice.
4) To determine the sensitivity to a given treatment of hybrids of resistant and naïve lice strains.

2. MATERIALS AND METHODS

2.1 Objective 1. Maintenance of naïve and resistant lice strains through multiple generations

Prior to the start of this study, the naïve lice strain (NV) had been cultured in laboratory conditions through at least 40 generations since being collected from a farm site. The resistant strain (RS) had been cultured through approximately eight generations since it was first established. As the results of this study appertain to the sensitivity of each subsequent generation of resistant lice since the last emamectin treatment, the results of bioassays conducted prior to the start of this study are also included in the results that follow.

2.1.1 Culturing of Lice

Both strains of lice were maintained onsite under a Home Office project licence, by infecting fish, allowing lice to develop to adults and produce eggs which were then collected and used to re-infect fish.

For the collection of lice eggs, ovigerous female lice were removed from host fish and incubated at ambient tank temperatures in 10L culture vessels. Eggs were left to hatch

and develop to the infective copepodid stage. Aeration and daily partial water changes were performed to maintain the cultures. When copepodids were present, aliquots of culture water from each vessel were examined under a dissecting microscope to estimate the total number of copepodids present.

Experimental fish were then challenged with lice copepodids in tank water. Challenges were conducted at the ambient temperature of the inflowing water except when this fell below 10ºC and water was heated to maintain this level from the day prior to until the day following challenge. During the challenge, water inlets were switched off and levels reduced. Aeration and mixing was provided with air stones and oxygen levels monitored. When necessary, oxygen was diffused into tank water to keep levels above 6mg/L. The number of copepodids used was selected to provide an expected settlement rate of at least 20 lice per fish. Following challenge, a minimum of two fish were selected from each tank and examined under anaesthesia with 2-phenoxyethanol to determine the level of lice settlement. The fish were returned to the tanks. Where lice settlement was below the level required, a repeat challenge using lice from the same cohort of copepodids was conducted as soon as possible where louse numbers allowed.

2.1.2 Fish Stock

Mixed sex Atlantic salmon (*Salmo salar* L.), with an initial mean weight of 200-500g, were used for the sea lice culture. The numbers of fish used for infection of each generation of lice ranged from 20 to 50 depending on the numbers of lice copepodids available to infect the fish.

2.1.3 Fish Holding Conditions

Fish were held in circular glass reinforced plastic (GRP) tanks (1m and 3m diameter). Tanks were provided with a continuous supply of seawater. Light was supplied to each tank on a photoperiod that corresponded with the natural conditions of sunrise and sunset and was adjusted each week. Water recirculation was not used. All tanks were cleaned, disinfected and rinsed before stocking with fish.

2.2 Objective 2. Stability of sensitivity to Emamectin benzoate in multiple generations of naïve and resistant lice

Bioassays were used to test the sensitivity of the NV and RS strains of lice to emamectin benzoate (EB). The estimated EC_{50} values from the bioassays were compared for the two strains. EC_{50} was defined as the effective concentration at which 50% of lice in a population were affected.

2.2.1 Test Material

The test material for the bioassays was technical grade emamectin benzoate (EB). Test material was supplied by Intervet/Schering-Plough and stored dry and in the dark according to label conditions.

2.2.2 Bioassay Methods

Fish were netted and killed by cervical dislocation. Lice were removed using fine forceps. Adult male lice were transferred to plastic bags containing 8-10 litres of clean seawater from the collection site at ambient salinity and temperature. When enough lice were collected, the remaining fish were left until the female lice had become gravid. These were later collected the same way and transferred to the MERL culturing system to incubate eggs for infecting fish with the next generation. It was decided to only conduct assays on adult male lice as removing female lice for this purpose may have endangered the continuity of the cultures by having insufficient eggs for the subsequent generation. The adults males used in each bioassay were of a similar age, all being tested 1-2 weeks after moulting to the adult stage.

Bags containing lice for bioassays were transferred to an incubator set at 12ºC until temperatures had equilibrated. Live lice were added to deep-sided 100 mL plastic Petri dishes containing 70mL of test solution (see 2.2.3) as soon as possible after water temperatures had reached 12ºC.

Lice were held in test solutions in a dark incubator for 24 hours at 12ºC. No additional water or aeration was supplied.

There were eight test doses for each louse strain within each generation. Doses were tested in duplicate. Doses were as follows:

1) Placebo control containing Polyethylene glycol (PEG) 300
2) EB in PEG 300 diluted in sea water. EB concentration 2000ppb
3) EB in PEG 300 diluted in sea water. EB concentration 1000ppb
4) EB in PEG 300 diluted in sea water. EB concentration 500ppb
5) EB in PEG 300 diluted in sea water. EB concentration 250ppb
6) EB in PEG 300 diluted in sea water. EB concentration 125ppb
7) EB in PEG 300 diluted in sea water. EB concentration 62.5ppb
8) EB in PEG 300 diluted in sea water. EB concentration 31.25ppb

In later assays of the hybrid strains, both adult male and adult female lice were tested.

2.2.3 Preparation of Test Solutions

Test doses were prepared not more than 2 hours before the start of each assay. Technical grade emamectin benzoate (EB) was dissolved in PEG300 at 5 mg/mL. 1 mL was further diluted in 999 mL of filtered seawater to make 5 mg/L. 250 mL of each test dose was made up by taking different amounts of the 5 mg/L stock and making up to 250 mL with seawater and also PEG300 (Table 1). The PEG300 was added to each dose so that each contained 0.1 mL, this was equivalent to the volume of PEG300 used to make up the highest test dose.

Table 1: Preparation of emamectin benzoate (EB) test solutions. Volume of 5 mg/L EB solution, seawater and PEG300 required to make 250 mL of a test solution at different doses

EB Test Dose (ppb)	Vol. of 5 mg/L stock added (mL)	Vol. seawater added (mL)	Vol. of PEG300 added (mL)
2000	100.000	150.000	-
1000	50.000	199.950	0.050
500	25.000	224.925	0.075
250	12.500	237.413	0.088
125	6.250	243.656	0.094
62.50	3.125	246.778	0.097
31.25	1.563	248.339	0.098
0	0	249.900	0.100

Sea lice were randomly allocated to the experimental Petri dishes so that each contained 5 male lice.

Petri dishes were removed from the incubator after 24 hours of the exposure period. Lice were examined and recorded as live, dead or moribund. The number of affected lice were those recorded as dead or moribund vs. those live (still able to swim normally and re-attach when detached from the vessel surface). Examinations were not performed on a blinded basis. This could be considered in future studies.

2.2.4 Data Analysis

Probit analysis was used to generate EC_{50} data for male lice within each strain for each bioassay. The software used for this was Minitab v.13 (Minitab Inc).

2.3 Objective 3. Fecundity and egg hatching

Following a review of literature it became evident that data on egg string length and hatchability could not be expected to demonstrate differences in fitness between naïve and resistant sea lice. More complex studies are necessary to identify the 'fitness cost' of emamectin resistance in sea lice.

2.4 Objective 4. Determination of sensitivity to EB of F1 hybrids between naïve and resistant lice strains

2.4.1 Generation of hybrid strains

Gravid female lice were collected from the resistant strain (generation 12) and naïve strain of lice and eggs were hatched and developed to copepodid stage. Two tanks, each containing 70 salmon were infected using the methods previously described. When adult males and pre-adult females had developed, fish were killed by cervical dislocation and lice removed with forceps to petri dishes. Under a dissecting microscope, lice were sorted into male and female stages using the shape of the genital segments as the criterion. Where it was uncertain of the sex of the louse, it was discarded.

A further two tanks of 20 fish each were set up and infected with the collected lice as follows:

1) To the first tank were added adult male lice from the RS strain and pre-adult female (virgin) lice from the NV strain.
2) To the second tank of fish were added adult male lice from the NV strain and pre-adult female (virgin) lice from the RS strain.

The water flows were switched off for 3 hours until most of the lice added to the tanks had attached to the fish.

The fish were maintained until lice had mated and females had produced egg strings. The eggs were collected and incubated and used to infect a further two tanks with the methods previously described. These lice were F1 hybrids, described thereafter as F1a (RS male + NV female parents) and F1b (RS female + NV male parents).

When the F1 hybrids had developed to adult stages (pre-gravid), lice were removed and bioassays performed. The remaining lice were left until eggs were being produced, then they were collected, pooled as a single F1 strain, incubated and used to infect fish in the *in vivo* study described below (section 2.4.3). The pooled F1 strain is referred to as the HB strain (hybrid strain) in later sections of the report.

2.4.2 Bioassay for two strains of F1 hybrid lice (pre in vivo trial)

Two bioassays were set-up on the 7th July 2010, one for the F1a hybrid strain and one for the F1b hybrid strain. The bioassay procedure was in accordance with the set-up described in section 2.2. Five adult males and five adult females (pre-gravid) were allocated to each dose.

2.4.3 In vivo testing of hybrid Lice

For the experimental lice challenge, three 2m diameter tanks each containing 70-160 Atlantic salmon (100-150g) previously marked with a passive transponder chip (PIT tag) were set-up. Each tank of salmon were each challenged with either NV, HB or RS lice. Lice were allowed to develop to chalimus III/IV stages. The pre-treatment sample, Time-point 1, was conducted on the 11-12th December 2010 (Days -7, -8). Fish were anaesthetised with 2-phenoxyethanol, weighed, examined for lice, PIT tag recorded and allocated to 1m diameter tanks so that there were 6 tanks in total, each containing 25 fish, with 2 tanks for each strain of lice.

Fish were then allowed time to recover from the handling and resume normal feeding behaviour. Lice continued to develop during this period to motile stages. Fish were then starved for one day on the 19th January 2011 (Day 0) and experimental feeds were supplied to the fish for seven days (Day 1 to 7). The test material was Slice® premix (Intervet/Schering-Plough Animal Health), containing 0.2% emamectin benzoate, prepared as medicated feed. This was stored dry and in the dark according to label conditions. The groups and diets presented are shown in Table 2.

Table 2: Summary of experimental groups including experimental group name, lice strain and treatment.

Group	Lice strain	Diet for 7 days
NV Control	Naïve	Control
NV Treated	Naïve	Slice to deliver 50µg EB/kg/day
HB Control	F1 Hybrid	Control
HB Treated	F1 Hybrid	Slice to deliver 50µg EB/kg/day
RS Control	Resistant	Control
RS Treated	Resistant	Slice to deliver 50µg EB/kg/day

Sampling Time-point 2 was on the 27th January 2011 (Day 8, 1 day post-treatment) when 5 fish per tank were killed by cervical dislocation, weighed, examined for lice and pit tag removed and recorded.

Sampling Time-point 3 was on the 16th February 2011 (Day 28, 21 days post-treatment), three weeks after the last day of experimental feeding. The remaining fish in each tank were killed and sampled as described above. Lice were removed from fish at this stage and used for further bioassays.

During the experimental feeding phase, three meals per day were presented to the fish and uneaten pellets were collected in fine screens in each tank outflow. The number of pellets collected was converted to a dry weight of uneaten feed in order to estimate the mean dosage administered for each tank.

The basal feed used throughout the study was Biomar Pearl 3 mm. The mean weight of fish estimated on Day 0, based on the weights recorded at allocation plus an estimated gain in the interim was 142g. Experimental diets were prepared by weighing out the appropriate amount of feed into a polythene bag, adding the necessary amount of Slice, turning the pellets for 5 minutes until the powder appeared homogenous and then top coating with 1% v/v of pure cod liver oil (Seven Seas Healthcare, Marfleet, UK) and turning for a further 5 minutes. No premix was visibly left in the bag, all appeared to be adhered to the pellets. Control diets were prepared in the same way using a clean new polythene bag with only the addition of the fish oil.

2.4.4 Bioassay for NV, HB and RS strains of lice (post in vivo trial)

Lice removed at Time-point 3 were further tested in bioassays using the procedure previously described. Lice from the NV control, HB control and RS control tanks were each tested in a bioassay. Around 3-5 adult males and 3-5 adult females (pre-gravid) were allocated to each dose. There were not always 5 individuals of each sex available, after the *in vivo* trial, to be added to each dose.

2.4.5 Data Analysis

Regarding the bioassays, Probit analysis was used to generate EC_{50} data for male and female lice separately within each strain for each bioassay.

For the *in vivo* trial, sea lice count data were summarised for each tank. Mean number, standard deviation and median number of lice per fish were calculated for tanks. Due to low sample sizes and sea lice infections typically being over dispersed, non-parametric statistics were used to compare lice counts between tanks based on sample median lice infections. Significant differences in median lice count between all tanks at Time-point 1 were compared using a Kruskal-Wallis test. For Time-points 2 and 3, significant differences in median lice count between the treatment tank and control tank for each lice strain were compared using Mann-Whitney tests.

To determine the efficacy of EB treatment for each strain in the *in vivo* trial, a modified Henderson-Tilton formula was applied as follows:

Corrected % = (1-(mean of control before treatment*mean of treated after treatment)/(mean of control after treatment*mean of treated before treatment))*100

The Henderson-Tilton formula was applied to data for both Time-point 2 and 3.

All statistical analysis was conducted using either Minitab version 13 or GraphPad InStat software. In all cases a significance level of $p<0.05$ was set.

3. RESULTS

3.1. Maintenance of naïve and resistant lice strains through multiple generations

The lice from the NV and RS strains were successfully cultured through 16 generations in laboratory conditions with no apparent loss of viability or changes in morphology or behaviour.

3.2. Stability of sensitivity to EB in multiple generations of naïve and resistant lice

Table 3 shows the results of the bioassays of naïve and resistant strains of lice before and during this study. Results are included from prior to the study (generations 0-7 inclusive) in order to show stability since the resistant strain were first cultured in the laboratory. The data is graphically illustrated in Figure 1.

Table 3: EC_{50} values for successive generations of pure NV and RS strains of *L. salmonis* cultured separately in the absence of lice treatments. Lice were exposed to emamectin benzoate at 12 ºC for 24 hours in dark conditions. Data are presented for adult male lice only. All values are parts per billion (ppb), with 95% confidence intervals reported. Lw 95% = lower 95% confidence interval, Up 95% = upper 95% confidence interval.

		Adult male RS lice			Adult male NV lice		
Date	**Generation**	**EC_{50}**	**Lw 95%**	**Up 95%**	**EC_{50}**	**Lw 95%**	**Up 95%**
11/12/2008	Gen 0	715	550	1048	141	104	190
10/03/2009	Gen 1	1211	766	2442	145	106	207
10/07/2009	Gen 3	732	601	989	145	106	207
25/10/2009	Gen 7	823	744	960	121	94	192
02/03/2010	Gen 9	814	700	990	131	87	299
06/04/2010	Gen 10	853	683	999	101	72	171
17/06/2010	Gen 12	732	622	921	114	85	170
24/11/2010	Gen 15	620	535	796	189	121	398
16/12/2010	Gen 16	940	754	1220	122	91	189

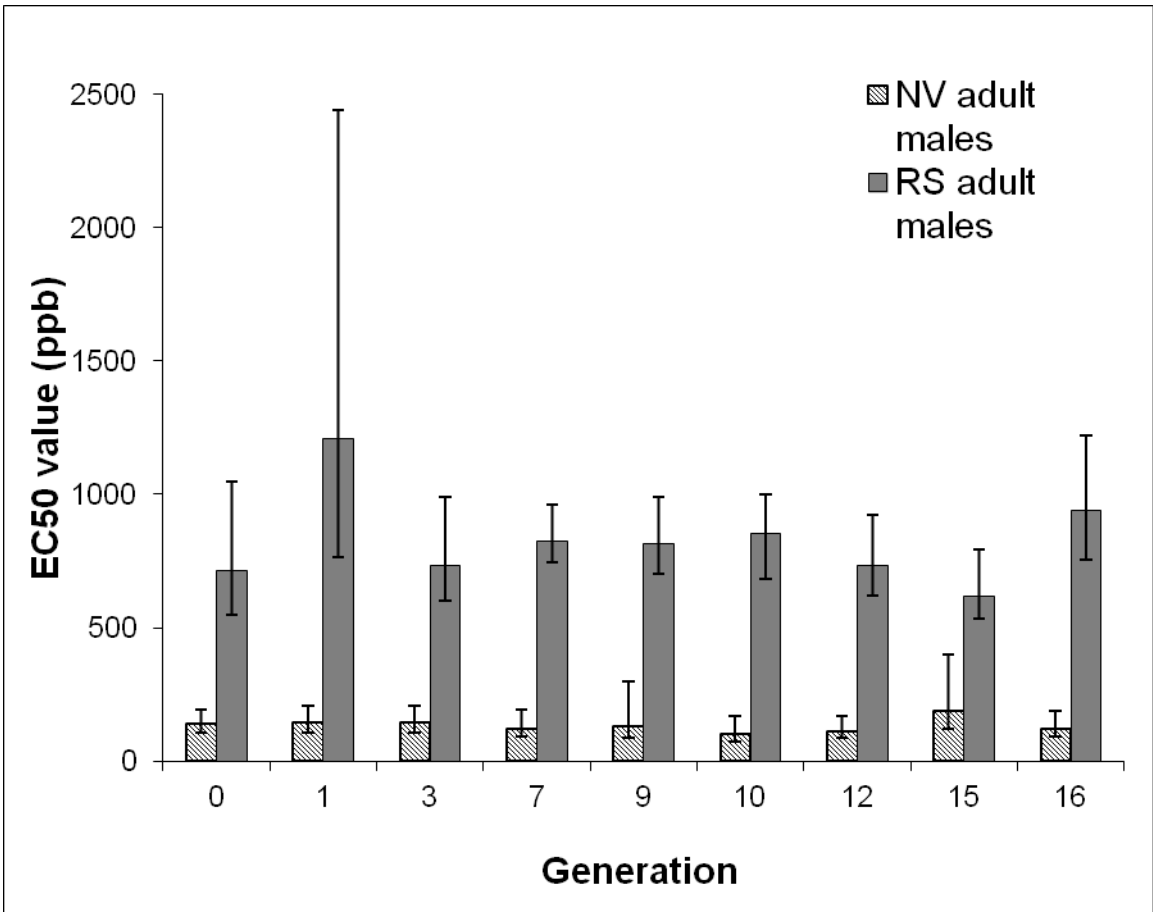

Figure 1: EC_{50} values of emamectin benzoate for NV and RS strains of lice. Lice were cultured in laboratory conditions in the absence of any louse treatments. Error bars denote 95% confidence intervals.

In all assays, the adult male lice from the resistant RS strain were less sensitive to EB than those from the naïve strain. The level of sensitivity in both strains was fairly consistent over the 16 generations with approximately six-fold less sensitivity in the RS strain. There was an anomalous result for the RS strain in Generation 1 where confidence intervals were very high. This may be explained as there were fewer adult males available for testing in this assay (n=10 per dose) compared to other assays (n≥20 per dose).

3.3 Fecundity and egg hatching

Relative fecundity and egg hatchability were not evaluated for the reasons described in section 2.3 and discussed in section 4.

3.4 Determination of sensitivity to EB of F1 hybrids between resistant and naïve lice strains

3.4.1 Bioassay results for two strains of hybrid lice (F1a and F1b)

Bioassay results for the two strains of hybrid lice (F1a and F1b) are presented in Table 4 and graphically presented in Figure 2. EC_{50} values were between those seen for NV and RS lice. In particular, the EC_{50} of F1 hybrid males were 348 and 435. These lay between the NV EC_{50} (114) and RS EC_{50} (732) of males in generation 12 (parents of the hybrids). The EC_{50} value did not differ greatly according to the sex of the resistant parent. The two types of F1 hybrid produced were therefore mixed for further use in tank trials.

Table 4. EC_{50} values for adult male and adult female (pre-gravid) lice exposed to emamectin benzoate at 12 ºC for 24 hours in dark conditions. All values are ppb, with 95% confidence intervals reported. Lw 95% = lower 95% confidence interval, Up 95% = upper 95% confidence interval.

Hybrid strain	Parents	Test Subject	EC_{50}	Lw 95%	Up 95%
F1a	NV female + RS male	Adult male	435	335	573
		Adult female	250	196	318
F1b	NV male + RS female	Adult male	348	231	525
		Adult female	174	144	300

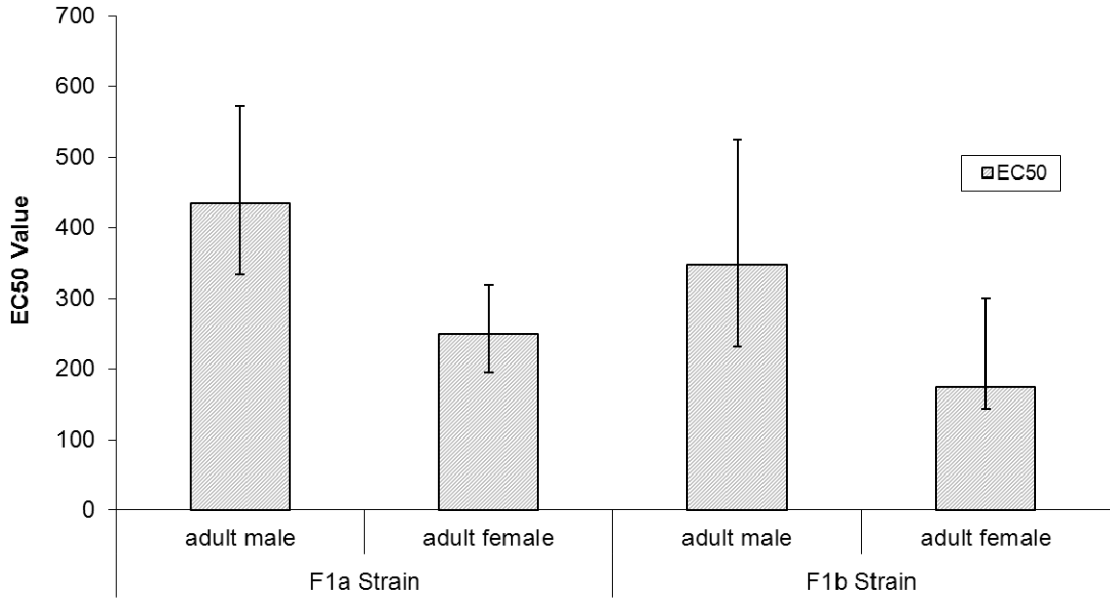

Figure 2: EC_{50} values for EB for two hybrid strains of lice: F1a and F1b. Strains have different parentage. Results are presented for male and female lice separately. Error bars denote 95% confidence intervals.

3.4.2 In vivo results for hybrid lice

3.4.2.1 Time-point 1: Days -8 and -7

The mean lice counts for the three strains of lice when examined at chalimus III stage prior to treatment are presented in Table 5. A non-parametric Kruskal-Wallis test showed that there were no significant differences in the number of lice per fish between tanks ($p = 0.24$, $KW=6.70$).

Table 5. Summary data of lice counts at Time-point 1 (pre- treatment). The table presents the mean, standard deviation (SD) and median number of lice per fish for each group (n= 25 fish/group). The majority of lice were at the chalimus III stage.

Group	Mean no. lice per fish	SD	Median no. lice per fish
NV Control	25.8	21.4	22
NV Treated	22.6	12.9	19
HB Control	26.6	10.7	25
HB Treated	26.8	11.8	26
RS Control	23.8	7.3	22
RS Treated	21.9	7.3	21

3.4.2.2 In-feed Dose

Slightly above target dosing was achieved for all treatment groups. Mean dose delivery for each treatment group was as follows:

NV treated group – 53.1 µg/kg/day

HB treated group – 53.2 µg/kg/day

RS treated group – 52.7 µg/kg/day

3.4.2.3 Time-point 2: Day 8 (27/01/11)

The summary data for lice counts for the three strains of lice when examined on Day 8 are presented in Table 6. At this time, Slice was shown to be 80% effective against the naïve strain of lice, ineffective (0%) against the hybrid strain and only 11.5% effective against the RS strain. A Mann-Whitney U test revealed significantly fewer lice per fish in treated than control groups of the NV strain (U =25 , p =<0.01). Tests did not reveal a significant difference between the control and treated groups for the hybrid strain (U =18, p =0.31) or the RS strain (U =14.5, p =0.75).

Table 6. Summary data of lice counts at Time-point 2 (immediately following treatment). The table presents the mean, standard deviation (SD) and median number of lice per fish for each group (n=5 fish/group). The majority of female lice were at the pre-adult II stage. The majority of male lice were at the adult stage. Efficacy of treatment is also given.

Group	Mean no. lice per fish	SD	Median	Efficacy (%)
NV Control	20.2	3.4	21.0	
NV Treated	4.0	1.9	4.0	80.0
HB Control	19.0	2.4	19.0	
HB Treated	15.8	6.8	17.0	0
RS Control	18.6	7.2	21.0	
RS Treated	17.8	8.2	21.0	11.5

3.4.2.4 Time-point 3: Day 28 (16/02/11)

The summary data for lice counts for the three strains of lice when examined on Day 28 are presented in Table 7 and illustrated in Figure 3. Slice was shown to be 98% effective against the naïve strain of lice, 3.2% against the Hybrid strain and 0% against the RS strain. A Mann-Whitney U test revealed significantly fewer lice per fish in treated than control groups of the NV strain (U =330.5 , p =<0.01). Tests did not reveal a significant difference between the control and treated groups for the hybrid strain (U =201.5, p =0.11) or the RS strain (U =231, p =0.41).

Table 7. Summary data of adult lice counts at Time-point 3 (Day 28). The table presents the number of fish sampled and the mean, standard deviation (SD) and median number of adult lice per fish for each group. All lice were adults. Efficacy of treatment is also given.

Group	Number of fish	Mean no. lice per fish	SD	Median	Efficacy (%)
NV Control	18	5.3	4.4	4.0	
NV Treated	19	0.1	0.3	0.0	98
HB Control	17	8.1	3.4	9.0	
HB Treated	18	10.4	3.6	10.0	3.2
RS Control	20	7.7	3.3	7.0	
RS Treated	20	6.9	3.1	6.5	0

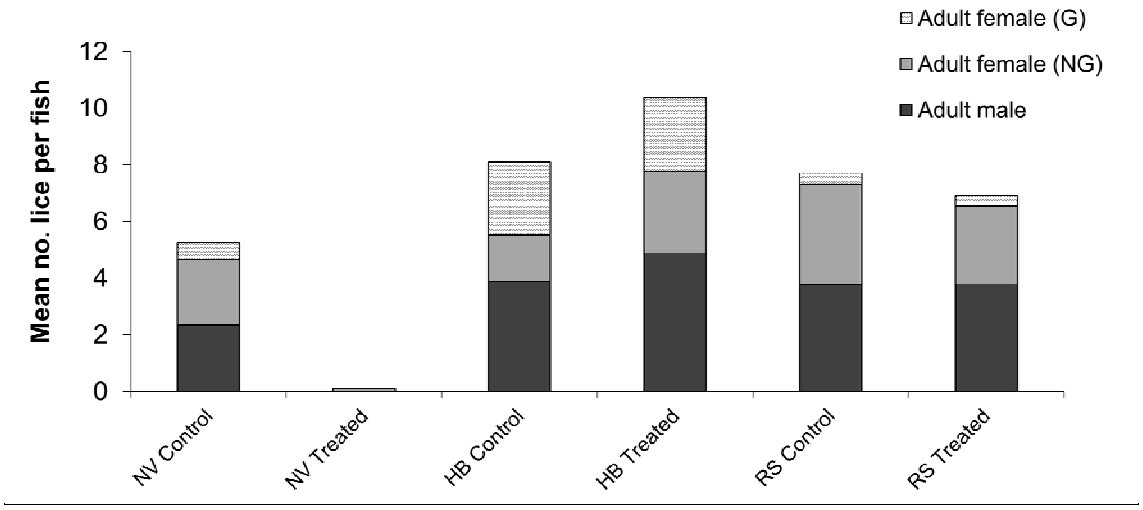

Figure 3: Mean number of lice per fish for the control and treated groups within the NV, HB and RS lice strains. Data is presented for Sample 3 (3 weeks post Slice treatment). Bars are divided into the mean number of adult males and adult females NG (non-gravid) and G (gravid) per fish (see Table 6 for the number of fish sampled).

3.4.3 Bioassay results for hybrid lice following the in vivo trial

Lice were removed from the three control tanks (NV control, Hybrid control, RS control) from the *in vivo* study and tested in a bioassay. The lice were tested for sensitivity to emamectin. The results from the bioassay are summarised in Table 8 and graphically presented in Figure 4. Estimated EC_{50} values for F1 Hybrids (HB) were between those seen for the pure NV and RS strain for both male and female lice.

Table 8. Estimated EC_{50} values for adult male and adult female *L. salmonis* exposed to emamectin benzoate at 12 ºC for 24 hours in dark conditions. All values are ppb, with 95% confidence intervals reported. Lw 95% = lower 95% confidence interval, Up 95% = upper 95% confidence interval.

	Adult males			Adult females		
Group	**EC_{50}**	**Lw 95%**	**up 95%**	**EC_{50}**	**Lw 95%**	**up 95%**
NV control	151.8	66.9	208.3	117.6	24.8	144.1
HB control	739.2	570.5	957.5	314.0	213.9	404.2
RS control	944.1	774.5	1150.4	445.1	370.5	543.6

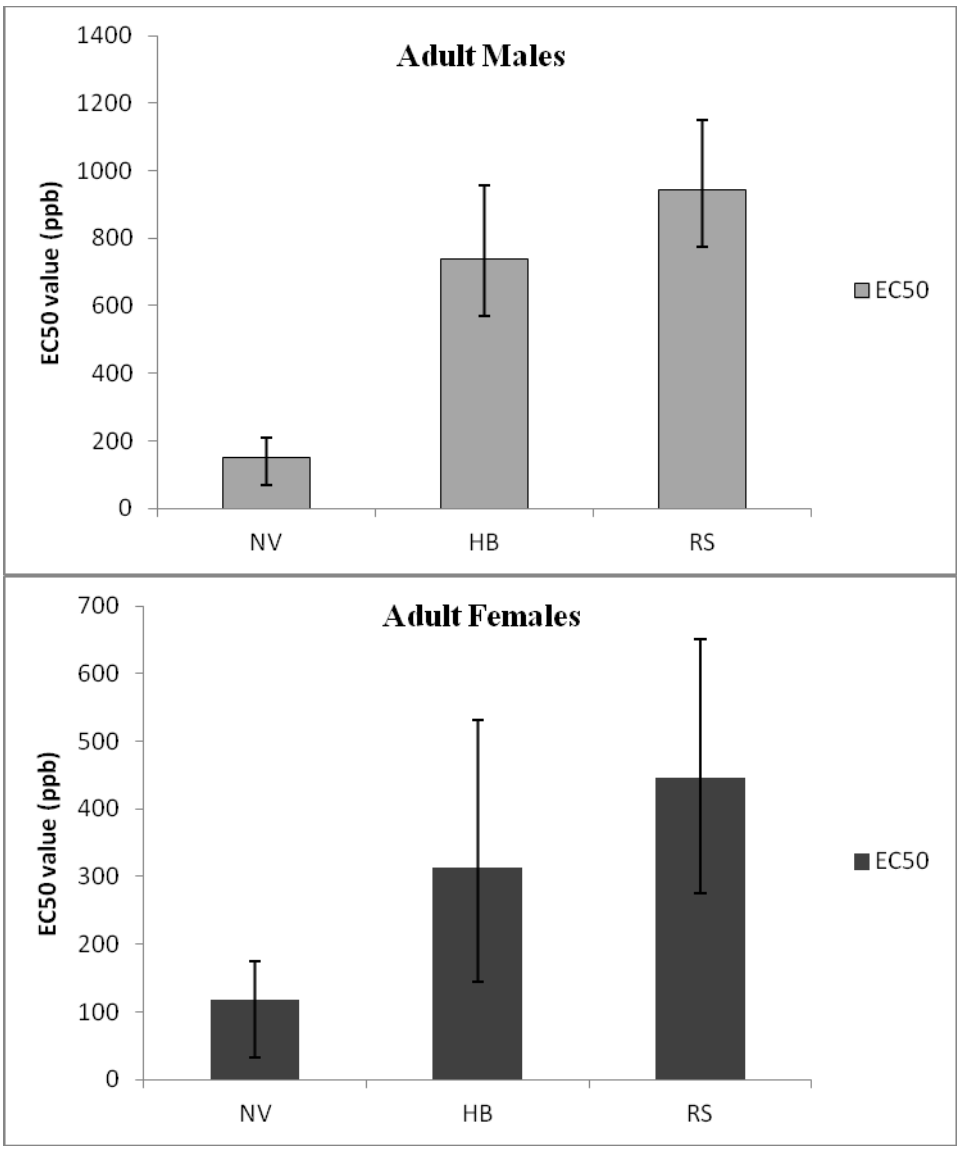

Figure 4: EC_{50} values of EB for NV, HB and RS strains of lice. Results for male and female lice are presented separately. Lice were removed from fish from the control tanks from the *in vivo* study. Error bars denote 95% confidence intervals.

4. DISCUSSION

This study has demonstrated that lice of the two strains, naïve (NV) and resistant (RS), can be successfully cultured through multiple generations in laboratory conditions.

Sensitivity measured by EC_{50} to emamectin benzoate within the two strains did not change considerably during 16 generations in the absence of either selection by drugs or gene flow. This implies that there is a heritable genetic basis to sensitivity in the NV strain and to reduced sensitivity in the RS strain. The stability of the generations gives confidence in the value of these strains as reference standards for the determination of chemotherapeutant efficacy.

F1 Hybrid lice between the NV and RS strains showed an estimated EC_{50} value between those of parent strains when tested using bioassays. This was found to be independent of the strain of the father or mother. This supports the idea that reduced sensitivity has a genetic basis and is heritable from either the mother or father in a semi-dominant fashion.

In the tank study using Slice at the recommended dose (50 µg EB/kg/day), there was no clear sensitivity in either the RS strain or F1 hybrid strain whereas 98% of NV strain were affected. Thus a potential threshold dose which might start to affect hybrid lice but not RS lice had not been reached. It is difficult to compare the dosage in the two approaches taken (bioassay *versus* tank). It seems likely that in the tank study, hybrid lice were being exposed to concentrations below the EC_{50} level identified by bioassay. Indeed the routes tested in the bioassays and tanks trials prevent comparisons - lice in the bioassays were topically exposed to EB for 24 hours whereas lice in the tank study were exposed to EB by feeding on the fish over a number of days.

This study has focussed on sea lice resistance in experimental culture conditions and provided information as to the mechanism of resistance in sea lice. It has confirmed that reduced sensitivity to EB is a heritable trait in sea lice. It has also shown that in the absence of selection pressure (in favour of EB resistance) or gene flow, sensitivity within an isolated lice population was not restored in our study over 16 generations. Gene flow

through breeding with more sensitive lice appears to, however, reduce or dilute genetic *in vitro* resistance in the absence of selection pressure. This effect is likely to be present in any farm setting, albeit to unknown levels. This suggests that forgoing drugs to which genetic or cross-resistance resistance has appeared and allowing seeding of the population with more sensitive lice, could over time lead to a restoration of sensitivity.

Further work is necessary to address whether genetic variants conferring resistance incur a 'fitness cost' to the lice. In drug-resistant *Drosophila melanogaster*, Kane *et al*. (2000) identified reduced brood size and a reduction in locomotion and bang sensitivity. Espedal *et al*. (2010) investigated the fitness cost of emamectin resistance in *L. salmonis* in terms of fecundity and developmental success. They compared the length of egg strings, the hatching success and the survival of copepodids for a naïve strain of lice versus a strain with reduced EB sensitivity. They reported not to have uncovered any fitness costs related to reduced EB sensitivity. It is possible that resistance may incur fitness costs linked to environmental parameters. For example, do resistant lice show lower tolerance to more extreme salinities versus naïve lice?

This study has demonstrated that where consistency of response over time is required, the naïve and resistant lice held at MERL are available as predictable reference strains. Their stability over time supports the idea that they are valid reference points for evaluation of the efficacy of potential new chemotherapeutants and feed additives, new combination treatments, and to investigate cross-resistance towards different pharmaceutical products.

5. REFERENCES

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