

# Optimization and field use of a bioassay to monitor sea lice *Lepeophtheirus salmonis* sensitivity to emamectin benzoate

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**ABSTRACT:** A bioassay for sea lice *Lepeophtheirus salmonis* sensitivity towards emamectin benzoate (EMB) was validated for field use. A probit regression model with natural responsiveness was used for the number of affected (moribund or dead) sea lice in bioassays involving different concentrations of EMB. Bioassay optimization included an evaluation of the inter-rater reliability of sea lice responsiveness to EMB and an evaluation of gender-related differences in susceptibility. Adoption of a set of bioassay response criteria improved the concordance (evaluated using the concordance correlation coefficient) between raters' assessments and the model estimation of EC<sub>50</sub> values (the 'effective concentration' leading to a response of 50% of the lice not prone to natural response). An evaluation of gender-related differences in EMB susceptibility indicated that preadult stage female sea lice exhibited a significantly larger sensitivity towards EMB in 12 of 19 bioassays compared to preadult males. In order to evaluate sea lice sensitivity to EMB in eastern Canada, the intensive salmon farming area in the Bay of Fundy in southwestern New Brunswick was divided into 4 distinct regions based on industry health management practices and hydrographics. A total of 38 bioassays were completed from 2002 to 2005 using populations of preadult stage sea lice collected from Atlantic salmon *Salmo salar* farms within the 4 described regions. There was no significant overall effect of region or year on EC<sub>50</sub> values; however, analysis of variance indicated a significant effect of time of year on EC<sub>50</sub> values in 2002 and a potential effect in 2004 to 2005. Although the range of EC<sub>50</sub> values obtained in this 3 yr study did not appear sufficient to affect current clinical success in the control of sea lice, the results suggest a seasonal- or temperature-associated variation in sensitivity to EMB. This will need to be considered if changes in EMB efficacy occur in the future.

**KEY WORDS:** Sea lice · *Lepeophtheirus salmonis* · Emamectin benzoate · SLICE® · Bioassay · Gender-difference · Inter-rater reliability · Field monitoring

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## INTRODUCTION

Sea lice *Lepeophtheirus salmonis* are ectoparasitic, copepodid crustaceans capable of inflicting serious physical damage upon their salmonid hosts if not managed carefully at commercial salmon culture sites (Ramstad et al. 2002). The requirement for continual monitoring and control of sea lice is a costly economic burden for Atlantic salmon *Salmo salar* producers throughout North America and northern Europe (Costello 1993, Treasurer & Pope 2000). Although

there are a number of biological and chemical means of sea lice management and control, reduced sensitivity and resistance development of sea lice towards several chemotherapeutants have been reported (Jones et al. 1992, Treasurer et al. 2000, Tully & McFadden 2000, Sevatdal & Horsberg 2003, Fallang et al. 2004).

An important principle of preventing or minimizing the development of resistance is the avoidance of reliance on single products, or on those treatments most likely to select for the same mechanism of resistance (Denholm et al. 2002). This is often difficult when

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a limited range of chemotherapies is available. The only drugs currently available for sea lice control in Canada are the chitin-synthesis inhibitor teflubenzuron (Calicide®) and the avermectin, emamectin benzoate (EMB, active chemical in SLICE®), both of which are administered in-feed. A recent survey indicated that >90% of sites in the Bay of Fundy are using EMB for sea lice control (Westcott et al. 2004). With such a limited range of medicines registered for use, and widespread reliance on a single chemotherapeutant, the potential for resistance development is an ongoing concern. Although there have been no known documented cases of treatment failures with EMB or of sea lice resistance to the drug, early detection of changes in the sensitivity of sea lice towards EMB should be a key component of a successful resistance management strategy.

The conventional means of detecting insecticide resistance has been by bioassay, which quantifies the response of a test subject to increasing concentrations or doses of an agent (Brogdon 1989). The agent is typically a drug and the subject response a change in a particular characteristic, morbidity and/or death (Hubert 1980, Robertson & Preisler 1992). Bioassays are valuable tools in the detection of individuals or cases with a decreased sensitivity towards a chemotherapeutant, especially when the mechanism of resistance is unknown (Denholm et al. 2002). Bioassays represent the best method for the standardization of variables that may influence sea lice sensitivity to EMB, as non-responsive treatments in the field may be related to factors that cannot be detected through simple clinical observations. Using bioassays to aid in the early detection of changes in the sensitivity of a population of individuals before the onset of resistance development will allow for the adoption of alternative control measures and possible prevention or delay of resistance development. Bioassays for several chemicals administered as bath treatments to combat sea lice infections have been developed (Sevatdal & Horsberg 2003, Sevatdal et al. 2005a). However, the present study is a report of a bioassay developed for a drug administered in-feed to farmed salmon.

The dependence of bioassays for sea lice on subjective assessments of sea lice responsiveness (i.e. vigour/mortality) to varying concentrations of a chemotherapeutant may influence the reproducibility of evaluations between 2 independent raters. The need to quantify agreement arises when 2 raters independently

but simultaneously assess a response (King & Chinchilli 2001). The inter-rater reliability, or inter-observer agreement, is used to assess the consistency of results for the same outcome using the same subject/individual at the same time provided by independent raters. Lack of inter-rater reliability may arise from deviations between rater evaluations or instability over time of the attribute being measured.

There is currently an absence of standardized methods and techniques for detecting and monitoring resistance of sea lice to EMB. The objectives of this study were to: (1) develop and optimize a bioassay protocol for EMB using field-collected sea lice; (2) validate the bioassay protocol; (3) evaluate the inter-rater reliability of the subjective assessment of the bioassay protocol; (4) evaluate gender-related differences in the susceptibility of sea lice to EMB; and (5) use the developed bioassay protocol with field-collected sea lice from Atlantic salmon farms in the Bay of Fundy region of southwestern New Brunswick, Canada, to test for differences in the susceptibility to EMB over a 3 yr period.

## MATERIALS AND METHODS

**Sea lice collections.** Due to the fact that an element of subjectivity exists in this type of bioassay evaluation, specific criteria for lice *Lepeophtheirus salmonis* condition were adopted (Table 1). An evaluation of inter-rater agreement prior to and following the adoption of the bioassay response criteria was conducted on sea lice collected in 2002 and 2003 from fish originating at 4 different Atlantic salmon *Salmo salar* sea cage sites located in the Bay of Fundy. Prior to the adoption of the bioassay response criteria, sea lice were collected on 4 separate days over a 1 wk period from 1 site using market-sized fish (3 to 4 kg) that were part of a routine har-

Table 1. *Lepeophtheirus salmonis*. Bioassay response criteria for sea lice (adapted from Sevatdal & Horsberg 2003)

Response	Criteria
Live	(1) Normal swimming behavior (ability to swim in a straight line) (2) Securely adheres to Petri dish (3) Normal movement of extremities
Moribund	(1) Disabled swimming but capable of weak uncoordinated movement (loop to loop swimming) (2) Inability to firmly adhere to Petri dish (adherence to dish for a period before dropping off) (3) Minimal movement of extremities
Dead	(1) Inability to swim (2) Floating in Petri dish (3) No movement of extremities

vest (i.e. not anesthetized). Following the adoption of the refined bioassay response criteria, sea lice were collected from 3 different sites, on separate days, during routine sea lice counting on site. On those days sea lice were collected from fish that were immobilized using tricaine methanesulfonate at a dose of approximately  $100 \text{ mg l}^{-1}$  (TMS, Syndel Laboratories).

Sea lice used for the evaluation of gender-related differences in EMB susceptibility and those used in field bioassays were collected during the years 2002 to 2005 from 16 Atlantic salmon marine farm sites. Based on hydrographics and industry health management policies, the Bay of Fundy salmon farming area was divided into 4 distinct regions: (1) Campobello Island (C) and Deer Island (D); (2) Grand Manan Island (GM); (3) Lime Kiln Bay (LK); and (4) Passamaquoddy Bay (PB) (Fig. 1). Bioassays were conducted on sea lice collected from 4 sites in CD, 2 in GM, 9 in LK, and 1 site in PB. Sampling was dictated by the sporadic availability of sea lice and the cooperation and participation of sea cage sites. Sea lice were collected at sea cage sites from Atlantic salmon smolts, pre-market fish, or brood-stock, during routine sea lice counting or harvest(s). Sea lice collected during routine sea lice counting were removed from fish anesthetized with TMS, and those collected during the harvest were collected from cage-side fish lightly sedated with carbon dioxide or those immobilized in ice water or by percussive stunning.

For all bioassays only healthy preadult stages of both male and female *Lepeophtheirus salmonis* were used to minimize potential variation in sensitivity that may be related to age or size of the test subjects (Sevatdal 2005). Sea lice were gently removed from Atlantic salmon using forceps and placed into a sealed container of seawater collected from the site. Except for initial collections, battery-operated air pumps were added to collection containers. An additional container was filled with 7 to 10 l of seawater collected from the

site for use in later bioassay EMB dilutions. Ice packs were placed in a cooler with the collection containers to ensure that the lice were kept cool during transport to the laboratory.

Bioassays involving field-collected sea lice were set up either at the Atlantic Veterinary College (AVC), University of Prince Edward Island in Charlottetown, PE, or the New Brunswick Department of Agriculture, Fisheries and Aquaculture Diagnostic Laboratory in Black's Harbour, NB. All bioassays were initiated within a maximum of 6 h of collection. Seawater obtained from each sea lice collection site was used to prepare the bioassay dilutions. A stock solution was prepared for each bioassay by dissolving 5 mg EMB (Emamectin benzoate PESTANAL<sup>®</sup>, Sigma-Aldrich) in 50 ml of methanol. A working solution was prepared by diluting 10 ml of the stock solution with 990 ml of seawater. EMB concentrations were prepared by diluting the working solution with seawater in order to create 7 concentrations of EMB (0, 1, 3, 10, 30, 100, 300 ppb).

The number of sea lice used in each bioassay dish and the number of replicates at each treatment dilution was dependent upon the availability of sea lice on collection days. Due to the greater number of sea lice available from one site that was being harvested (prior to the adoption of the bioassay response criteria), an average of 15 sea lice (equal proportions of males and females were used, where possible) were carefully transferred from the collection containers into each of 28 or 35 (4 or 5 replicates of each of the 7 treatment dilutions) Petri dishes containing seawater from the collection site. In 2003 and 2004, sea lice collections resulted in fewer total sea lice due to low availability at sites in those years. Therefore, for these collections, an average of 10 to 15 live and healthy sea lice were transferred to each of 7 Petri dishes. Placement of sea lice in Petri dishes was conducted in such a manner as to ensure that dishes were filled simulta-

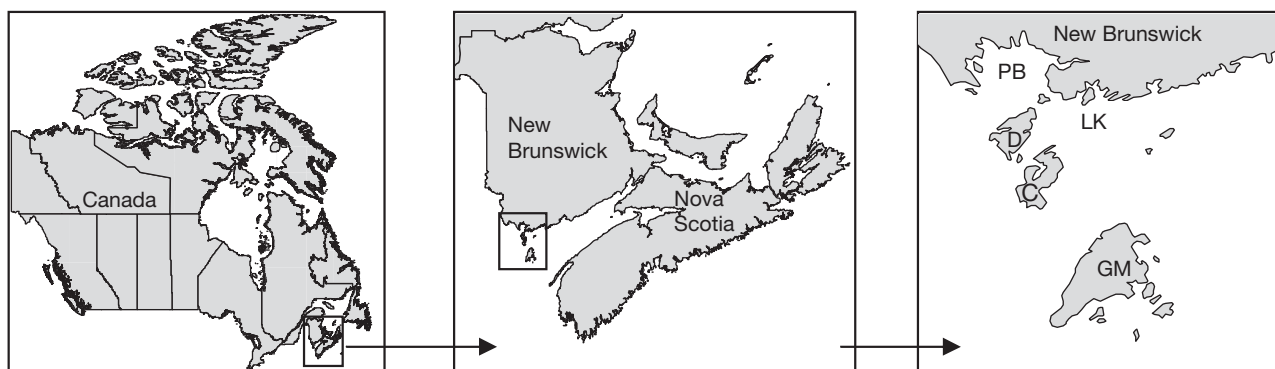


Fig. 1. Bay of Fundy fish farm sea lice *Lepeophtheirus salmonis* sampling regions: Campobello Island (C) and Deer Island (D), Lime Kiln Bay (LK), Grand Manan Island (GM), and Passamaquoddy Bay (PB)

neously. Petri dishes were maintained on a chilled surface, and shaded from exposure to fluorescent lighting. Allocation of Petri dishes to treatment dilutions was blinded to prevent bias during the bioassay outcome assessment. Seawater was replaced with approximately 50 ml volumes of 7 different EMB dilutions allotted to 1 of 7 Petri dishes. Petri dishes were incubated in a temperature-controlled chamber at 10 to 12°C for an exposure period of 24 h. The conditions of the sea lice were evaluated after 24 h according to an adopted set of bioassay response criteria (Sevatdal & Horsberg 2003).

**Sea lice cultivation.** Laboratory-cultivated sea lice used in this study were the first generation progeny of *Lepeophtheirus salmonis* adult females collected in July 2005 during a cage harvest on an Atlantic salmon sea cage site. Bioassays involving laboratory-cultivated sea lice were set up at the AVC. Gravid females were collected and transported to the laboratory as described above for preadult sea lice collections. In the laboratory, egg-strings were carefully removed from females and incubated in flow-through chambers containing seawater collected from the Bay of Fundy at a temperature of approximately 10°C. Following the development of the majority of sea lice stages to copepodids, they were transferred to a fish-holding module to challenge Atlantic salmon. A total of 250 Atlantic salmon, age 30 to 42 mo with an average weight of  $417 \pm 114$  g, were held in a re-circulation system containing synthetic saltwater (Instant Ocean, Aquarium Systems) at a salinity of  $30 \pm 2\%$  and a temperature of  $12 \pm 1^\circ\text{C}$ . Infestation of Atlantic salmon with copepodids occurred during 3 separate exposure periods. In each case, saltwater flow to tanks was stopped, and the water level in the tanks was lowered to accommodate saltwater containing copepodids. Static exposure of fish to copepodids occurred for 1 h, after which time water flow was restored to tanks. Fish were maintained in the saltwater recirculation system to allow time for the sea lice to develop on the host. Following the progression of sea lice to the preadult stages, fish were anaesthetized using TMS ( $100 \text{ mg l}^{-1}$ ), and sea lice were removed and placed into Petri dishes containing synthetic saltwater. Two collection days were employed 2 wk apart. For laboratory-cultivated sea lice, the proportion of males and females was approximately equal. Thus, subsequent bioassays contained 50% males and 50% females. Two bioassays were set up on each collection day; one with synthetic saltwater and the other using Bay of Fundy seawater. Ten sea lice were transferred to each of 42 Petri dishes (3 replicates at each treatment dilution using natural seawater and 3 replicates at each treatment dilution using synthetic saltwater as the solvent). Bioassays were set up as described previously.

**Bioassay analysis.** For each bioassay, the number of affected (moribund or dead) sea lice at different concentrations of EMB was analyzed by a probit regression model with natural responsiveness (Finney 1971). Specifically, the modeling equation for the probability ( $p$ ) of sea lice subjected to a certain dose of EMB being affected was  $p = p_0 + (1 - p_0) \times \text{probit}^{-1} [\alpha + \beta \times \log(\text{dose})]$ , where  $p_0$  is the probability of response in controls,  $\log$  is the natural logarithm, and probit is the cumulative distribution function of a standard normal distribution. The parameter of primary interest was  $\text{EC}_{50} = -\alpha/\beta$ , the 'effective concentration' leading to a response of 50% of the lice not prone to natural response. For example, if the natural responsiveness was 40%, the  $\text{EC}_{50}$  was the dose level corresponding to 70% (40% + 50% of 60%) mortality. Following Williams (1986), in order to improve identifiability of the  $\text{EC}_{50}$  parameter the equation was rewritten using  $\alpha + \beta \times \log(\text{dose}) = \beta \times [\log(\text{dose}) - \log(\text{EC}_{50})]$ ; parameter estimates were obtained by maximum-likelihood estimation, and confidence intervals were computed by the profile-likelihood method. The analyses were implemented in SAS 8.2 software (SAS Institute) using probit and nlmixed procedures (Stryhn & Christensen 2003).

**Inter-rater agreement.** The inter-rater agreement (2 raters) of the number of affected (moribund or dead) sea lice in the Petri dishes used for each bioassay was assessed prior to and following the adoption of the bioassay response criteria. Prior to the adoption of the bioassay response criteria, rater agreement was compared 3 times for each of the bioassays conducted on 6, 9, 10, and 11 September 2002. The first comparison involved rater agreement for proportions of affected lice computed for each of the replicate dishes at the 7 bioassay concentrations (i.e. on 6 September 2002, Dishes 1 to 4 at each EMB concentration). The second and third comparisons involved an assessment of only the first and second dish read by each rater at each EMB concentration (i.e. Dish 1 and 2 at each EMB concentration; Table 2). In each case, the raters' proportions of affected lice were compared using the concordance correlation coefficient (CCC) (Lin 1989). The CCC evaluates the agreement between 2 readings from the same sample by measuring the variation from the 45° line through the origin (line of perfect agreement). CCC values of 0 and 1 represent no and complete agreement, respectively. The analysis included approximate 95% confidence intervals (CI) based on a z-transformation and was carried out using the concord command (Steichen & Cox 2002) for Stata 8 software (Stata Corp.). The agreement between raters was also assessed for  $\text{EC}_{50}$  values computed separately for each rater.

Table 2. *Lepeophtheirus salmonis*. Agreement (concordance correlation coefficient) of emamectin benzoate (EMB) bioassay evaluations of individual or multiple dishes of sea lice between 2 independent raters prior to and following the adoption of the bioassay response criteria. EC<sub>50</sub>: effective concentration of EMB, leading to a response of 50% of sea lice not prone to natural mortality; Y: yes; N: no

Date	Dish(es) evaluated by each rater at each EMB concentration	Bioassay response criteria adopted (Y/N)	EC <sub>50</sub> (ppb) EMB		Concordance correlation coefficient (95% CI)
			Rater 1	Rater 2	
6 Sep 2002	1–4	N	35	27	0.93 (0.86–0.97)
9 Sep 2002	1–4	N	51	36	0.85 (0.69–0.93)
10 Sep 2002	1–5	N	– <sup>a</sup>	– <sup>a</sup>	0.57 (0.31–0.75)
11 Sep 2002	1–5	N	28	32	0.78 (0.62–0.88)
6 Sep 2002	1	N	48	20	0.72 (0.08–0.94)
6 Sep 2002	2	N	– <sup>a</sup>	– <sup>a</sup>	0.98 (0.91–1.00)
9 Sep 2002	1	N	71	33	0.93 (0.64–0.99)
9 Sep 2002	2	N	71	– <sup>a</sup>	0.47 (–0.07–0.80)
10 Sep 2002	1	N	– <sup>a</sup>	– <sup>a</sup>	0.67 (–0.06–0.93)
10 Sep 2002	2	N	– <sup>a</sup>	– <sup>a</sup>	0.45 (–0.33–0.86)
11 Sep 2002	1	N	21	– <sup>a</sup>	0.90 (0.55–0.98)
11 Sep 2002	2	N	29	42	0.88 (0.48–0.98)
2 Oct 2002	1	Y	39	43	0.99 (0.96–1.00)
12 Aug 2003	1	Y	31	31	1.00 (0.98–1.00)
11 Sep 2003	1	Y	83	76	0.95 (0.74–1.00)

<sup>a</sup>EC<sub>50</sub> estimation impossible because data did not exhibit a dose–response relationship

#### Gender-related differences in EMB susceptibility.

Differences in EMB susceptibility related to sea lice gender were evaluated by estimating separate-sex probit regression models with natural responsiveness. First, the previously described model was used separately for male and female sea lice. However, this approach required substantial lice counts of both sexes and was therefore limited to the subset of samples in which sufficient representation of both male and female sea lice occurred. Second, the model for data of both sexes was extended to allow for different EC<sub>50</sub> values for males and females. To ensure identifiability, common values of the natural responsiveness ( $p_0$ ) and the dose-response regression coefficient ( $\beta$ ) were retained. Fig. 2 shows results for the laboratory-cultivated sea lice bioassay with the overlaid dose-response curves estimated from the model. In the latter model, a statistically significant difference in EC<sub>50</sub> values between sexes was assessed by a likelihood-ratio test, a p-value < 0.05 being indicative of a statistically significant gender-related difference in sea lice susceptibility to EMB.

**Analysis of EC<sub>50</sub> values for field data.** An analysis of variance (ANOVA) for a linear model was used to determine whether EC<sub>50</sub> values showed any significant differences between years, regions, and time of year (modeled by a linear effect of the number of days elapsed within a year since 24 July, the earliest sampling time in the 3 years). All first-order interactions were initially included and later removed if judged

unimportant. The residuals were used to validate model assumptions and determine any influential observations. The significance level was set at  $p < 0.05$ .

**Analysis of clustering in time and space for field data.** The temporal correlation derived from repeated measures on the same sites was assessed in a linear mixed model with the correlation,  $\rho$ , between 2 mea-

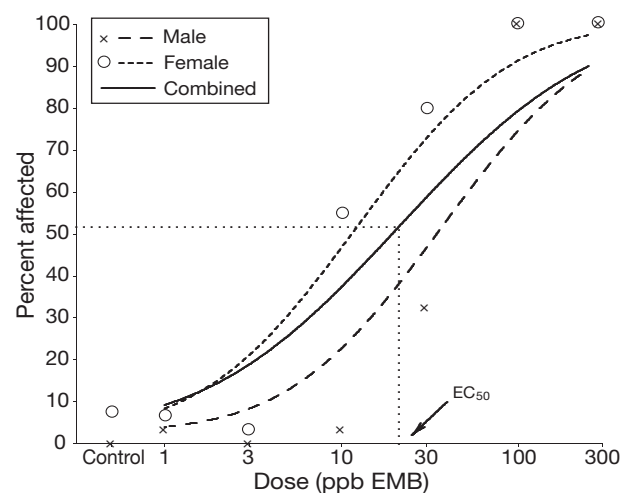


Fig. 2. *Lepeophtheirus salmonis*. Dose-response curves for laboratory-cultivated sea lice showing the combined and gender-specific curves and the determination of the combined EC<sub>50</sub>: effective concentration of emamectin benzoate (EMB) leading to a response of 50% of sea lice not prone to natural response

surements at the same site being  $\rho^d$ , where  $d$  is the number of days between them. The presence of spatial correlation in the residuals of the linear model was assessed by computing Moran's  $I$ . This analysis was based on digitized site coordinates and was carried out using R 2.10 software (Ihaka & Gentleman 1996) with the `spdep` library.

## RESULTS

### Evaluation of inter-rater agreement

CCCs for bioassays with replicates ranged from 0.57 to 0.93, representing moderate to almost perfect agreement between raters. CCCs for bioassays in which the first plate read by each rater was evaluated for agreement ranged from 0.67 to 0.93, and CCCs for those in which the second plate read by each rater was evaluated for agreement ranged from 0.45 to 0.98. Following the adoption of the bioassay response criteria, due to the limited availability of sea lice, the 3 bioassays conducted did not have replicates. CCCs ranged from 0.95 to 1.00, representing almost perfect agreement between raters. The 95% CIs for these coefficients were much narrower than earlier assessments (Table 2). Also, the  $EC_{50}$  values were less variable between raters following adoption of the criteria.

### Assessment of gender-related differences in EMB susceptibility

The number of preadult stage male and female sea lice *Lepeophtheirus salmonis* collected for each bioassay was variable. There were higher numbers of preadult stage males collected in the field than preadult stage females; on average, bioassays consisted of 15% females and 85% males. Preadult female sea lice were significantly more sensitive to EMB (i.e. lower  $EC_{50}$  values) compared to preadult stage males in 12 of the 19 bioassays assessed for gender-related differences in EMB susceptibility, with values ranging from 39 to 116 and 1 to 59 ppb EMB for males and females, respectively. Seven of the remaining 19 bioassays did not demonstrate significant gender-related differences in EMB susceptibility, although all estimated  $EC_{50}$  values were higher for males than females (Table 3). Out of 39, 20 bioassays were not included in the gender-difference analysis, either because there were too few preadult stage female sea lice collected for the bioassay or the information regarding the sex of susceptible sea lice in the bioassay was not recorded (early protocols did not include this assess-

ment). Preadult female sea lice cultivated in the laboratory also exhibited the same increased EMB sensitivity as seen in field samples when compared to preadult males. The ratios of male-to-female  $EC_{50}$  values for significant and non-significant gender-specific analyses ranged from a minimum of 1.17 to a maximum of 39.00, with a median value of 3.04. This suggests that on the average the 'effective concentration' leading to a response of 50% of preadult female sea lice not prone to natural response was 3 times lower than that of preadult stage males.

### Bioassay results for field collected and laboratory cultivated sea lice

A total of 38 field bioassays were completed during the years 2002 to 2005; the  $EC_{50}$  values are shown in Fig. 3 and listed in Table 3. Sea lice collected from October 2004 to March 2005 were grouped as 2004 lice, since the fish populations were the same and new sea lice infestations do not normally occur until after this point in time under culture situations in the Bay of Fundy. The most bioassays were completed for the LK region, due to a higher concentration and accessibility of sites within this area. Relatively few bioassays were completed in 2003, due to lower preadult lice burdens on the sampled sites during that year, making it difficult to consistently obtain the minimum 70 preadult sea lice required for each bioassay. On average, bioassays using field-collected sea lice consisted of 15% females and 85% males. However, bioassays using sea lice derived from a laboratory-grown generation contained 50% males and 50% females.  $EC_{50}$  values for 2002 ranged from 25 to 118 ppb EMB; 31 to 104 for 2003; 26 to 89 for 2004; and 49 to 116 for 2005. The  $EC_{50}$  value for laboratory-cultivated sea lice was 21 ppb EMB.

### Region, year, season effects

Region, year, days, and the interaction term between days and year were included in the final ANOVA model. The ANOVA indicated no overall significant effect of region ( $p = 0.68$ ) or year ( $p = 0.36$ ) on  $EC_{50}$  values. There was a significant effect of time ( $p = 0.007$ ) (expressed in days) on  $EC_{50}$  values, indicating a seasonal- and/or temperature-associated variation in efficacy of EMB. Although the interaction term between year and time was not significant overall ( $p = 0.10$ ), there was a significantly positive slope ( $\beta = 0.62$ ,  $p = 0.002$ ) of the 2002 data, indicating an increase in  $EC_{50}$  values towards late fall and early winter (Table 4). The 2003 data showed no increase in  $EC_{50}$  values later in the sampling season ( $\beta = 0.03$ ,  $p =$

Table 3. *Lepeophtheirus salmonis*. Combined (n = 39) and gender-specific (n = 19) EC<sub>50</sub> (effective concentration leading to a response of 50% of sea lice not prone to natural mortality) estimations used to determine gender-related differences in sea lice susceptibility to emamectin benzoate. ns: not significant (p > 0.05)

Date	No. of sea lice used in bioassay(s)			Combined EC <sub>50</sub> estimation		Gender-specific EC <sub>50</sub> estimation		
	Total	Male	Female	EC <sub>50</sub> (95% CI)	Natural mortality	Male	Female	p(gender)
Jul 2002	130	— <sup>a</sup>	— <sup>a</sup>	25 (17, 35)	0.132	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Jul 2002	254	— <sup>a</sup>	— <sup>a</sup>	41 (34, 76)	0.156	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Aug 2002	90	— <sup>a</sup>	— <sup>a</sup>	75 (42, 115)	0.249	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Aug 2002	95	— <sup>a</sup>	— <sup>a</sup>	73 (38, 142)	0.228	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Sep 2002	167	— <sup>a</sup>	— <sup>a</sup>	35 (26, 48)	0.072	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Sep 2002	411	— <sup>a</sup>	— <sup>a</sup>	35 (28, 45)	0.290	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Sep 2002	497	— <sup>a</sup>	— <sup>a</sup>	48 (35, 78)	0.607	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Sep 2002	493	— <sup>a</sup>	— <sup>a</sup>	— <sup>b</sup>	— <sup>b</sup>	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Sep 2002	500	— <sup>a</sup>	— <sup>a</sup>	28 (23, 33)	0.444	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Sep 2002	74	— <sup>a</sup>	— <sup>a</sup>	44 (30, 88)	0.262	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Oct 2002	150	— <sup>a</sup>	— <sup>a</sup>	102 (80, 134)	0.095	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Oct 2002	85	— <sup>a</sup>	— <sup>a</sup>	39 (31, 82)	0.057	— <sup>a</sup>	— <sup>a</sup>	— <sup>a</sup>
Oct 2002	99	75	24	103 (80, 148)	0.056	114	34	<0.001
Oct 2002	323	203	120	48 (37, 62)	0.029	95	20	<0.001
Oct 2002	178	128	50	107 (96, 151)	0.162	116	31	<0.001
Oct 2002	43	31	12	103 (55, 300)	0.038	110	18	<0.001
Oct 2002	141	85	56	98 (71, 123)	0.093	104	59	<0.05
Nov 2002	168	147	21	118 (106, 292)	0.158	— <sup>c</sup>	— <sup>c</sup>	— <sup>c</sup>
Nov 2002	105	93	12	107 (95, 165)	0.078	— <sup>c</sup>	— <sup>c</sup>	— <sup>c</sup>
Nov 2002	134	121	13	90 (54, 104)	0.184	94	17	<0.05
Nov 2002	117	93	24	95 (55, 116)	0.175	99	29	<0.05
Aug 2003	81	73	8	31 (23, 43)	0.049	39	1	<0.001
Aug 2003	75	56	19	65 (41, 104)	0.048	83	30	ns <sup>d</sup>
Sep 2003	125	112	13	87 (48, 97)	0.135	86	31	ns
Nov 2003	75	60	15	60 (39, 96)	0.068	85	30	ns
Nov 2003	77	52	25	104 (56, 152)	0.037	115	61	ns
Nov 2003	70	64	6	40 (32, 85)	0.077	55	17	<0.05
Nov 2003	70	59	11	37 (30, 57)	0	35	30	ns
Oct 2004	74	54	20	26 (17, 42)	0	55	5	<0.001
Oct 2004	82	63	19	38 (31, 56)	0	55	28	0.01
Oct 2004	74	59	15	29 (18, 48)	0	55	30	ns
Nov 2004	77	76	1	89 (53, 103)	0.054	— <sup>c</sup>	— <sup>c</sup>	— <sup>c</sup>
Dec 2004	86	74	12	42 (27, 69)	0.138	50	20	ns
Dec 2004	87	81	6	79 (48, 92)	0.048	— <sup>c</sup>	— <sup>c</sup>	— <sup>c</sup>
Feb 2005	71	69	2	116 (86, 163)	0.020	— <sup>c</sup>	— <sup>c</sup>	— <sup>c</sup>
Feb 2005	69	69	0	100 (71, 138)	0	— <sup>c</sup>	— <sup>c</sup>	— <sup>c</sup>
Feb 2005	70	69	1	104 (79, 152)	0	— <sup>c</sup>	— <sup>c</sup>	— <sup>c</sup>
Feb 2005	70	64	6	113 (100, 182)	0.020	— <sup>c</sup>	— <sup>c</sup>	— <sup>c</sup>
Mar 2005	70	70	0	49 (31, 80)	0	— <sup>c</sup>	— <sup>c</sup>	— <sup>c</sup>
Lab lice <sup>e</sup>	420	208	212	21 (17, 26)	0.035	37	12	<0.001

<sup>a</sup>Gender data not recorded on these dates of the bioassay protocol  
<sup>b</sup>EC<sub>50</sub> estimation impossible because data did not exhibit a dose–response relationship  
<sup>c</sup>Too few females to obtain reliable gender-specific estimates  
<sup>d</sup>Actual p-value = 0.058  
<sup>e</sup>Sea lice from 1 laboratory-cultivated population of sea lice (bioassay set up using Bay of Fundy seawater as solvent) in July 2005

0.90). In the 2004 to 2005 data, estimation of the slope was strongly affected by the EC<sub>50</sub> value for 30 March 2005 ( $\beta = 0.29$  and  $p = 0.09$ , with the value included;  $\beta = 0.59$  and  $p = 0.03$ , without this value). The data offer no conclusive evidence as to whether the 30 March 2005 value should be considered an outlier. We conclude cautiously that there was some indication of an increase in EC<sub>50</sub> values towards fall and winter.

### Spatial clustering

There was a marginally significant ( $p = 0.09$ ) and moderate correlation between measurements taken at the same site close in time ( $\rho = 0.50$ ,  $SE = 0.25$ , for measurements taken 7 d apart). Only minor effects were seen in the results of the linear model when accounting for the repeated measures. Spatial clustering of the

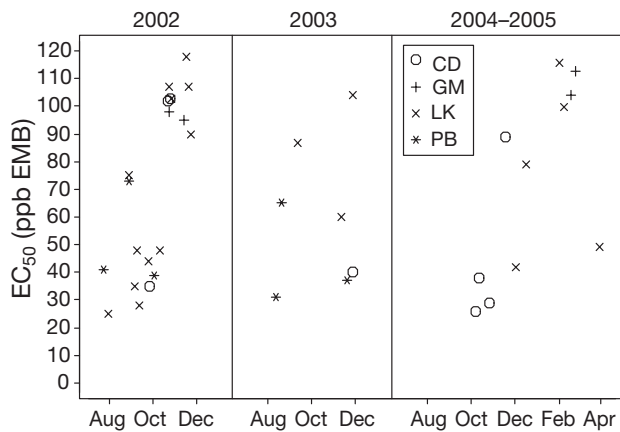


Fig. 3. *Lepeophtheirus salmonis*. Emamectin benzoate (EMB) bioassay  $EC_{50}$  values (ppb EMB) for sea lice collected from Campobello Island and Deer Island (CD), Grand Manan Island (GM), Lime Kiln Bay (LK), and Passamaquoddy Bay (PB) for the years 2002 to 2005.  $EC_{50}$ : effective concentration of EMB leading to a response of 50% of sea lice not prone to natural mortality

Table 4. Slopes and descriptive statistics of  $EC_{50}$  data for the years 2002 to 2004 (days, year, and region were included in the ANOVA model; 2004 and 2005 data grouped together as 2004 for ANOVA).  $EC_{50}$ : effective concentration of emamectin benzoate leading to a response of 50% of sea lice not prone to natural mortality. CD: Campobello Island and Deer Island; GM: Grand Manan Island; LK: Lime Kiln Bay; PB: Passamaquoddy Bay

Predictor	Predictor level	Coefficient	SE	p-value
Region	CD	21.20	18.53	0.682
	GM	40.88	21.61	
	LK	27.79	14.57	
	PB	18.18	15.02	
Year	2002	0		0.360
	2003	35.58	24.79	
	2004	-1.71	31.66	
Days 2002		0.62	0.18	0.002
Days 2003		0.03	0.23	0.907
Days 2004		0.29	0.17	0.090

bioassay data from the years 2002 to 2005 was determined to be of no statistical significance for this data set (Moran's  $I = -0.31$ ,  $p = 0.93$ ).

## DISCUSSION

Sea lice *Lepeophtheirus salmonis* bioassay development to date has focused on chemotherapeutants used as bath treatments. The development of bioassays using chemotherapeutants applied in-feed is a new appli-

cation of the bioassay assessment. As a result, an appropriate gold standard or reference assay against which to compare the current EMB bioassay results is lacking. However, bioassays are commonly employed in the field of agriculture to determine sensitivity values for macrocyclic lactones against ectoparasites such as the cattle tick *Boophilus microplus*. Exposure of the cattle tick to pour-on and injectable avermectin compounds (e.g. Ivermectin or Ivomec®, Abamectin or Avomec®) is through feeding on the host's blood. Sensitivity toward the compound is measured using the larval immersion test (LIT) or adult immersion test (AIT), in which larval or adult-stage ticks are bathed in the test compound for a pre-determined exposure period, after which survival of the larvae or egg production in the adults is assessed to determine sensitivity toward the test compound (Sabatini et al. 2001, Klafke et al. 2006).

Under field conditions, sea lice become exposed to EMB by ingestion while feeding on the mucus, epidermis, and blood of treated fish. Following a week-long medication period ( $50 \mu\text{g kg}^{-1} \text{ feed d}^{-1}$ ), the average concentrations of EMB in Atlantic salmon *Salmo salar* were shown to be 128 ppb in plasma, 105 ppb in mucus, and 68 ppb in muscle (Sevatdal et al. 2005b). The EMB concentrations chosen for inclusion in the bioassay cover the range of concentrations of EMB that sea lice would be naturally exposed to under field conditions while feeding on the mucus and blood of Atlantic salmon treated with SLICE®. However, the EMB bioassay relies on direct penetration and absorption of EMB through the cuticle of the sea louse so that it reaches its target site, the glutamate-gated chloride channels. Differences in the efficiency of the absorption of EMB through the cuticle in this experiment compared to exposure of a sea louse to EMB through feeding on the mucus and blood of an EMB-treated salmon in the field, are unknown. The bioassay provides no information on the amount of EMB penetrating each sea louse. EMB may be inadvertently lost or its absorption limited due to adherence of EMB to the glass Petri dishes or non-target tissues in the sea louse, or the use of seawater as a solvent. Seawater collected from each site was used when setting up the bioassays and, although EMB degradation is relatively slow in distilled water (<10% over 30 d) (Roberts & Hutson 1999), its degradation in seawater is unknown. Seawater also contains organic particles that may bind EMB (Sevatdal 2005), limiting the amount of active EMB available to sea lice in the bioassay.

## Rater agreement

Reproducibility is important to ensure uniformity among investigators testing different test subjects in dif-



ferent laboratories (Robertson & Preisler 1992). Reproducibility of a method can be determined by test/retest of the assessments (intra-rater repeatability) or by measuring the reliability between independent raters (inter-rater reliability). Discordance between the assessments of independent raters is not uncommon when subjective measures are evaluated. Inconsistencies between the outcomes of diagnostic tests run by different laboratories on the same sample using the same test have been shown to be partly due to subjectivity of the diagnostic test evaluations experienced by independent raters (see for example McClure et al. 2005). Training and experience would likely improve inter-rater reliability (Seidman et al. 2003, Sevatdal 2005).

The inter-rater reliability of the current bioassay protocol was evaluated by comparing 2 independent rater's evaluations of sea lice responsiveness to EMB using the CCC. Subjectivity of initial bioassay evaluations experienced by 2 independent raters led to the adoption of a set of bioassay response criteria. Although the CCCs prior to the adoption of the bioassay response criteria were reasonably high, our results suggest improvements in several aspects of rater agreement following adoption of the bioassay response criteria. These include a substantial improvement in rater agreement as well as an improvement in  $EC_{50}$  estimation. Detailed descriptions of inter-rater agreement involving sea lice bioassays have not been published.

### **Gender-related differences in EMB susceptibility**

A number of human and animal studies show that males and females may differ in their biological response to drugs, and the safety and effectiveness of many drugs exhibit some degree of sex dependence in both vertebrate and invertebrate species (Beierle et al. 1999, Miller et al. 2003, Pica-Mattocchia & Cioli 2004, Simon & Resnick 2004).

The reason for differences in male and female sea lice susceptibility towards EMB under bioassay conditions in this study are unknown. A gender difference in EMB efficacy following treatment was reported in a laboratory efficacy study involving sea lice grown on Atlantic salmon fed EMB in freshwater (Stone et al. 2002). The results of their study suggested that overall efficacy of EMB against Preadult II sea lice following transfer to saltwater was higher in female sea lice than in males on treated fish, although efficacy against adult males was lower compared to adult females. Bioassay studies conducted by Sevatdal et al. (2005a) suggested that adult females were 5 times less susceptible to pyrethroids than were adult males. Although the

study did not specifically look at gender-related differences in susceptibility, the results suggest that adult males and Preadult I sea lice of both sexes displayed approximately the same sensitivity as mixed-sex populations of Preadult II sea lice towards pyrethroids. It is evident that further studies will be required to quantify the effects of age or stage of sea lice on chemotherapeutant susceptibility under bioassay and field conditions.

The stages of sea lice that could be used in this bioassay were limited by the availability of sea lice in the field, the higher proportion of males collected in field samples, and the variation in size and weight between male and female sea lice at different stages of the life cycle. In an effort to minimize potential variation in sensitivity that may have been related to age/stage and/or size of the test subjects, size matching of preadult-stage sea lice was conducted where possible. However, this can be challenging given the natural variation in the size of preadult males and females (Johnson & Albright 1991). There may have been adult males included in bioassays with preadult sea lice as it can be challenging to distinguish between Preadult Stage I and adult males. Immature males are distinguished from mature males by the surfaces of the second antennae and the presence of a rough-surfaced pad (post-oral adhesions pad) located near the base of the first maxilla (Johnson & Albright 1991). Recent pyrethroid bioassay studies conducted by Sevatdal et al. (2005a) suggested that adult and Preadult I and II male sea lice displayed similar responses. Further studies are required to understand the impacts of the effects of gender and sea lice stage-related sensitivity towards EMB for use in this bioassay.

### **EMB susceptibility of laboratory cultivated and field collected sea lice**

In this study, laboratory-cultivated sea lice had an  $EC_{50}$  value of 21 ppb, which was lower than the values recorded for field samples. Unlike the field-collected sea lice used in all other bioassays in this study, laboratory-cultivated sea lice were first generation progeny of sea lice collected from the field with no possibility of previous direct exposure to EMB. These sea lice were hatched in Bay of Fundy saltwater then developed to preadult stages on fish held in a system containing synthetic saltwater. Although sea lice have been cultured successfully in systems containing synthetic saltwater (Bowers et al. 2000, Nolan et al. 2000), there is no information available on potential effects of synthetic saltwater on sea lice susceptibility to EMB. However, in the current study, bioassays involving laboratory-

reared sea lice were conducted using both synthetic saltwater and seawater, and no differences were found in sea lice sensitivity to EMB in the bioassays.

Day-to-day variations in bioassay results using field-collected sea lice were substantial, as was evident from the wide range of  $EC_{50}$  values for field samples of lice (e.g. 25 to 118 ppb) and the repeated measures analysis where only a marginally significant correlation between bioassay  $EC_{50}$  values taken at the same site close in time was found. A feature of all biological assays, even under carefully controlled experimental conditions, is the variability in the reaction of test subjects and the difficulty in reproducing the same result in successive trials (Finney 1971).

It was difficult to collect sea lice from responsive fish using forceps without the possibility of damaging the sea lice. Therefore, sea lice were collected from salmon that were anaesthetized or immobilized. It is possible that the method used to immobilize the salmon (i.e. carbon dioxide, ice bath, TMS) in order to collect sea lice had an effect on sea lice health; although there was a delay between the time of collection and set-up in the bioassay, which may have allowed potentially affected sea lice to recover, and only apparently healthy sea lice were chosen for inclusion in the bioassays. There is no published literature available on the effects of carbon dioxide or TMS on sea lice. Furthermore, methanol was used in the stock solution of each bioassay, and there was no methanol control tested in this study; therefore, its potential effect on sea lice health is unknown. However, methanol is a common solvent used in bioassays and in no case did the methanol concentration exceed 0.3%.

### EMB susceptibility factors

The results of this 3 yr study suggest that there is a seasonal- or temperature-associated variation in efficacy of EMB in field-collected sea lice. Increased efficacy with increasing water temperature, as indicated by lower  $EC_{50}$  values earlier in the sampling season, was found for sea lice collected and assayed in 2002, and there was some indication of this increase in 2004 to 2005. It is possible that variations in the field, transportation, and laboratory temperatures have affected the bioassay results (Schouest & Miller 1988). For example, lack of acclimation from the collection water temperature to the bioassay temperature may have altered the observed response.

Temperature is one of the most important factors affecting biological processes, and the effects of temperature on pesticide toxicity have been well documented in mammals, birds, insects, and other invertebrates (Scott 1995). Alterations in penetration, metabolism, and distribution within the animal and/or

altered effectiveness at the target site have been suggested as possible factors contributing to temperature-toxicity effects (Scott 1995). Although seasonal variations in the metabolism of other copepods have previously been documented (Siefken & Armitage 1968), the metabolic rate response of *Lepeophtheirus salmonis* to acclimation periods or to small differences in field and test temperatures are unknown (Tully & McFadden 2000) and there is a lack of information on temperature compensation in *L. salmonis*. However, previous arthropod research has shown that acclimation prior to insecticide treatment had no effect on toxicity in insecticide-susceptible and -resistant German cockroaches (Valles et al. 1988). Thus, the effects of temperature on EMB toxicity of *L. salmonis* are evident, but mechanisms of the effect require further investigation.

Sea lice control at Atlantic salmon aquaculture sites is critical during warmer periods (i.e. late summer), and little or no treatment is required during the winter months in Atlantic Canada. The results of this study suggest that sea lice collected during warmer field temperatures, when control is critical (i.e. in the spring and summer months), are susceptible to EMB. This seasonal- and/or temperature-associated variation in EMB efficacy suggests that sea lice may be less sensitive to EMB at colder water temperatures or as they approach the colder seasons of fall and winter. Temperature may be an important variable when assessing sea lice sensitivity towards EMB in the field.

The lack of a significant effect of region on  $EC_{50}$  values may be attributed to factors influencing spatial variation in *Lepeophtheirus salmonis* sensitivity to EMB. It is well established that the genes conferring resistance towards a chemotherapeutant are passed from one generation to the next as individuals within a population survive treatment, and the development of resistance depends on limited gene exchange between populations. Patterns of *L. salmonis* dispersal, recruitment, and treatment may influence population dynamics and the spread of genes conferring resistance to EMB. As many as 5 or 6 successive generations of *L. salmonis* are possible in 1 yr (Tully 1992), and the infective planktonic copepodid stage can be transported many kilometers from a point source (Murray & Gillibrand 2006); therefore, it is unlikely that a single population or generation of sea lice is present in any one region of the Bay of Fundy at a given time. Unlike laboratory populations of sea lice, where individuals of the same generation can be chosen for inclusion in a bioassay, under field conditions it is likely that sea lice collected for the bioassays are of mixed genetic backgrounds. Thus, their susceptibility to EMB may vary naturally. The frequency of treatments at a site may be sporadic or regular, depending on sea lice abundance

in a given region. Thus, field populations of sea lice are likely comprised of a mixture of susceptible and less susceptible individuals. Tully & McFadden (2000) recorded short-term variability in sensitivity of *L. salmonis* to dichlorvos based on bioassays conducted on adult female lice collected from salmon farms in Ireland during 2 periods of sampling.

It was not possible to fully validate the bioassay because there were no proven EMB-resistant sea lice populations to use for comparison. As a result of the inherent variability between bioassays, this protocol applied to field samples of sea lice should be used to describe population trends of  $EC_{50}$  values only. Individual bioassay values should not be interpreted in isolation as indicative of resistance development, and reduced treatment response should not automatically be equated with resistance development without eliminating possible extenuating factors more related to host or environmental factors.

### Recommendations for optimizing bioassays

In this section we review a number of factors considered or experienced to be of importance for optimization of the EMB bioassay protocol. Only live and apparently healthy sea lice were used in the bioassay to reduce pre-exposure factors affecting the reliability of the bioassay results (Sevatdal 2005). Careful removal of sea lice from fish using forceps reduced physical damage of the sea lice and minimized handling. Since the distance between farms and the nearest laboratory caused variable transport times, battery-operated air pumps were used in collection containers to provide continual aeration during transport to the laboratory and maximize survival of the sea lice.

Glass Petri dishes, rather than polystyrene plastic, minimized changes in EMB concentration. In a pilot assay using EMB in plastic containers, the concentration of EMB following an incubation of 24 h was 50 to 60% of the initial concentration (observations made in our laboratory and by Sevatdal 2005), likely due to adherence of the EMB to the plastic dishes. Sea lice of equal size were used to ensure comparable adherence and absorption of EMB by all sea lice in each dish. As it is unknown whether or not there is a stage-related difference in sea lice sensitivity to EMB, sea lice should be staged following the completion of the bioassay to indicate the sea lice stages tested. Where possible, increasing the number of replicates within each bioassay is desirable as is the standardization of the number of sea lice used in each Petri dish. Although as many as 120 test subjects are required for a reliable full-scale, dose-response experiment, as few as 60 test subjects can be used successfully as

long as the doses are carefully selected (Robertson & Preisler 1992). In the current study, all bioassays were performed on a minimum of 70 healthy preadult sea lice exposed to 7 concentrations of EMB. However, a sufficient number of test subjects are often difficult to consistently obtain under field conditions (Brogdon 1989). Although it may not be possible to collect equal numbers of males and females from field samples for use in the bioassay, it is important to ensure adequate sample sizes are used and the sex of each louse is recorded. The use of 2 independent evaluators for each bioassay was advantageous to ensure consistency of bioassay evaluations, and blinding of evaluators to test concentrations was essential to reduce any pre-conceived expectations of treatment effects during bioassay evaluations.

### CONCLUSION

The high level of agreement achieved between the bioassay evaluations of 2 independent raters provides confidence that the adopted bioassay response criteria were clearly defined, the raters understood consistent definitions of live, moribund, and dead sea lice and were able to consistently apply those definitions to their evaluations. This study indicated a gender-related difference in the sensitivity of preadult stage sea lice towards EMB such that females were more susceptible to EMB than males. Thus, sex will influence bioassay results such that the use of unequal proportions of each sex in a bioassay may contribute to unwanted variability in bioassay results. Further studies are required to understand the impacts of the effects of gender- and sea lice stage-related sensitivity towards EMB for use in this bioassay.

Although the bioassay protocol shows promise as a method to verify clinical resistance, it lacks rapidity and simplicity for use as a routine test. The time requirement for sea lice collection, bioassay set-up, and the 24 h incubation period, as well as the difficulty in consistently obtaining sufficient numbers of viable sea lice in the field make this bioassay impractical in many situations. As a result, the bioassay protocol is currently useful as a research tool at the descriptive level, but has limited use as a field resistance monitoring tool. However, it may be possible in a setting where resistance is relatively common, to establish bioassays as indicators of susceptibility (i.e. restrict bioassay only to a concentration known to reflect susceptibility). The occurrence of an apparent lack of response to an EMB treatment on a farm does not automatically indicate a change in sea lice sensitivity to EMB, because there may be other extenuating circumstances resulting in treatment failure.

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#### LITERATURE CITED

- Beierle I, Meibohm B, Derendorf H (1999) Gender differences in pharmacokinetics and pharmacodynamics. *Int J Clin Pharmacol Ther* 37:529–547
- Bowers JM, Burka JF, Mustafa A, Speare DJ, Conboy GA, Sims DE, Brimacombe M (2000) The effects of a single experimental challenge of sea lice, *Lepeophtheirus salmonis*, on the stress response of Atlantic salmon, *Salmo salar*. *J Fish Dis* 23:165–172
- Brogdon WG (1989) Biochemical resistance detection: an alternative to bioassay. *Parasitol Today* 5:56–60
- Costello MJ (1993) Review of methods to control sea lice (Caligidae: Crustacea) infestations in salmon (*Salmo salar*) farms. In: Boxshall GA, Defaye D (eds) Pathogens of wild and farmed fish: sea lice. Ellis Horwood, Chichester, p 219–252
- Denholm I, Devine GJ, Horsberg TE, Sevatdal S, Fallang A, Nolan DV, Powell R (2002) Analysis and management of resistance to chemotherapeutants in salmon lice *Lepeophtheirus salmonis* (Krøyer) (Copepoda: Caligidae). *Pest Manag Sci* 58:528–536
- Fallang A, Ramsay JM, Sevatdal S, Burka JF, Jewess P, Hammell KL, Horsberg TE (2004) Evidence for occurrence of an organophosphate-resistant type of acetylcholinesterase in strains of sea lice (*Lepeophtheirus salmonis* Krøyer). *Pest Manag Sci* 60:1163–1170
- Finney DJ (1971) Probit analysis. Cambridge University Press, Cambridge
- Hubert JJ (1980) Bioassay. Kendall/Hunt, Dubuque, IA
- Ihaka R, Gentleman R (1996) R: a language for data analysis and graphics. *J Comput Graph Statist* 5:299–314
- Johnson SC, Albright LJ (1991) The developmental stages of *Lepeophtheirus salmonis* (Krøyer, 1837) (Copepoda: Caligidae). *Can J Zool* 69:929–950
- Jones MW, Sommerville C, Wooten R (1992) Reduced sensitivity of the salmon louse, *Lepeophtheirus salmonis*, to the organophosphate dichlorvos. *J Fish Dis* 15:197–202
- King TS, Chinchilli VM (2001) A generalized concordance correlation coefficient for continuous and categorical data. *Stat Med* 20:2131–2147
- Klafke GM, Sabatini GA, de Albuquerque TA, Martins JR, Kemp DH, Miller RJ, Schumaker TTS (2006) Larval immersion tests with ivermectin in populations of the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) from State of Sao Paulo, Brazil. *Vet Parasitol* 142:386–390
- Lin LI (1989) A concordance correlation coefficient to evaluate reproducibility. *Biometrics* 45:255–268
- McClure CA, Hammell KL, Stryhn H, Dohoo IR, Hawkins LJ (2005) Application of surveillance data in evaluation of diagnostic tests for infectious salmon anemia. *Dis Aquat Org* 63:119–127
- Miller JA, Davey RB, Oehler DD, Pound JM, George JE (2003) Efficacy of the Ivomec SR Bolus for control of horn flies (Diptera: Muscidae) on cattle in South Texas. *J Econ Entomol* 96:1608–1610
- Murray AG, Gillibrand PA (2006) Modelling salmon lice dispersal in Loch Torridon, Scotland. *Mar Pollut Bull* 53:128–135
- Nolan DT, Ruane NM, van der Heijden Y, Quabius ES, Costelloe J, Wendelaar Bonga SE (2000) Juvenile *Lepeophtheirus salmonis* (Krøyer) affect the skin and gills of rainbow trout *Oncorhynchus mykiss* (Walbaum) and the host response to a handling procedure. *Aquacult Res* 31:823–833
- Pica-Mattocchia L, Cioli D (2004) Sex- and stage-related sensitivity of *Schistosoma mansoni* to *in vivo* and *in vitro* praziquantel treatment. *Int J Parasitol* 34:527–533
- Ramstad A, Colquhoun DJ, Nordmo R, Sutherland IH, Simons R (2002) Field trials in Norway with SLICE® (0.2% emamectin benzoate) for the oral treatment of sea lice infestation in farmed Atlantic salmon *Salmo salar*. *Dis Aquat Org* 50:29–33
- Roberts T, Hutson DH (1999) Metabolic pathways of agrochemicals. Royal Society of Chemistry, Cambridge
- Robertson JL, Preisler HK (1992) Pesticide bioassays with arthropods. CRC Press, Boca Raton, FL, p 1–15
- Sabatini GA, Kemp DH, Hughes S, Nari A, Hansen J (2001) Tests to determine LC<sub>50</sub> and discriminating doses for macrocyclic lactones against the cattle tick, *Boophilus microplus*. *Vet Parasitol* 95:53–62
- Schouest LP, Miller TA (1988) Factors influencing pyrethroid toxicity in pink bollworm (Lepidoptera: Gelechiidae): implications for resistance management. *J Econ Entomol* 81:431–436
- Scott JG (1995) Effects of temperature on insecticide toxicity. In: Roe RM, Kuhr RJ (eds) Reviews in pesticide toxicology. Toxicology Communications, Raleigh, NC, p 111–135
- Seidman JJ, Steinwachs D, Rubin HR (2003) Design and testing of a tool for evaluating the quality of diabetes consumer-information web sites. *J Med Internet Res* 5:e30. Available at: [www.jmir.org/2003/4/e30/HTML](http://www.jmir.org/2003/4/e30/HTML)
- Sevatdal S (2005) Sea lice resistance to chemotherapeutants: bioassays as diagnostic tools for determination of sensitivity patterns in sea lice (*Lepeophtheirus salmonis* Krøyer). PhD dissertation, Norwegian School of Veterinary Science, Oslo
- Sevatdal S, Horsberg TE (2003) Determination of reduced sensitivity in sea lice (*Lepeophtheirus salmonis* Krøyer) against the pyrethroid deltamethrin using bioassays and probit modeling. *Aquaculture* 218:21–31
- Sevatdal S, Copley L, Wallace C, Jackson D, Horsberg TE (2005a) Monitoring of the sensitivity of sea lice (*Lepeophtheirus salmonis*) to pyrethroids in Norway, Ireland and Scotland using bioassays and probit modelling. *Aquaculture* 244:19–27
- Sevatdal S, Magnusson Å, Ingebrigtsen K, Haldorsen R, Horsberg TE (2005b) Distribution of emamectin benzoate in Atlantic salmon (*Salmo salar* L.). *J Vet Pharmacol Ther* 28: 101–107
- Siefken M, Armitage KB (1968) Seasonal variation in metabolism and organic nutrients in three *Diaptomus* (Crustacea: Copepoda). *Comp Biochem Physiol* 24:591–609
- Simon V, Resnick E (2004) Drug therapy and gender. *US Pharm* 9:37–38
- Steichen TJ, Cox NJ (2002) A note on the concordance correlation coefficient. *Stata J* 2:183–189. Available at: [www.stata-journal.com](http://www.stata-journal.com)

- Stone J, Roy WJ, Sutherland IH, Ferguson HW, Sommerville C, Endris R (2002) Safety and efficacy of emamectin benzoate administered in-feed to Atlantic salmon, *Salmo salar* L., smolts in freshwater, as a preventative treatment against infestations of sea lice, *Lepeophtheirus salmonis* (Krøyer). *Aquaculture* 210:21–34
- Stryhn H, Christensen J (2003) Confidence intervals by the profile likelihood methods, with applications in veterinary epidemiology. In: Proceedings of 10th Symposium of the International Society for Veterinary Epidemiology and Economics. Vina Del Mar, November 17–21, 2003
- Treasurer JW, Pope JA (2000) Selection of host sample number and design of a monitoring program for ectoparasitic sea lice (Copepoda: Caligidae) on farmed Atlantic salmon, *Salmo salar*. *Aquaculture* 187:247–260
- Treasurer JW, Wadsworth S, Grant A (2000) Resistance of sea lice, *Lepeophtheirus salmonis* (Krøyer), to hydrogen peroxide on farmed Atlantic salmon, *Salmo salar* L. *Aquacult Res* 31:855–860
- Tully O (1992) Predicting infestation parameters and impacts of caligid copepods in wild and cultured fish populations. *Invertebr Reprod Dev* 22:91–102
- Tully O, McFadden Y (2000) Variation in sensitivity of sea lice [*Lepeophtheirus salmonis* (Krøyer)] to dichlorvos on Irish salmon farms in 1991–92. *Aquacult Res* 31:849–854
- Valles SM, Sánchez-Arroyo H, Brenner RJ, Koehler PG (1988) Temperature effects on  $\lambda$ -cyhalothrin toxicity in insecticide-susceptible and -resistant German cockroaches (Diptera: Blattellidae). *Fla Entomol* 81: 193–201
- Westcott JD, Hammell KL, Burka JF (2004) Sea lice treatments, management practices and sea lice sampling methods on Atlantic salmon farms in the Bay of Fundy, New Brunswick, Canada. *Aquacult Res* 35:784–792
- Williams DA (1986) Interval estimation of the median lethal dose. *Biometrics* 42:641–645

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