A Fixed-Dose Approach to Conducting Emamectin Benzoate Tolerance Assessments on Field-Collected Sea Lice (*Lepeophtheirus salmonis*)

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ABSTRACT

In New Brunswick (NB), Canada, the sea louse, *Lepeophtheirus salmonis*, poses an on-going management challenge to the health and productivity of commercially cultured Atlantic salmon, *Salmo salar*. While the in-feed medication, emamectin benzoate (SLICE® Merck, Canada) has been highly effective for many years, evidence of increased tolerance has been observed in the field since late 2008. Although bioassays on motile stages are a common tool to monitor sea lice sensitivity to emamectin benzoate in field collected sea lice, they require the collection of large numbers of sea lice due to inherent natural variability in the gender and stage response to chemotherapeutants. In addition, sensitive instruments such as EC_{50} analysis may be unnecessarily complex to characterise susceptibility subsequent to a significant observed decline in efficacy. This study proposes an adaptation of the traditional, dose-response format bioassay to a fixed-dose method. Analysis of 657 bioassays on pre-adult and adult stages of sea lice over the period 2008-2011, indicated a population of sea lice in NB with varying degrees of susceptibility to emamectin benzoate. A seasonal and spatial effect was observed in the robustness of genders and stages of sea lice, which suggest that mixing different genders and stages of lice within a single bioassay may result in pertinent information being overlooked. Poor survival of adult female lice in bioassays, particularly during May/June, indicates it may be prudent to consider excluding this stage from bioassays conducted at certain times of the year. This work demonstrates that fixed-dose bioassays can be a valuable technique in detecting reduced sensitivity in sea lice populations with varying degrees of susceptibility to emamectin benzoate treatments.

Introduction

Emamectin benzoate (SLICE®) is an avermectin chemotherapeutant that is used as an in-feed treatment of sea lice, *Lepeophtheirus salmonis* on Atlantic salmon, *Salmo salar*, and has been used extensively in New Brunswick since the late 1990s initially through the Emergency Drug Release programme of Health Canada's Veterinary Drugs Directorate, before receiving full approval for use in Canada in 2009. The effectiveness of emamectin benzoate resulted in it becoming the preferred chemotherapeutant for control of sea lice in coastal finfish aquaculture operations in Atlantic Canada, and in other parts of the world over the last decade, often to the exclusion of other products. It has been demonstrated that reliance on therapies with a single mode of action has proven to be a factor in the selection for resistance (Denholm, Devine, Horsberg, Sevetdal, Fallang, Nolan, & Powell, 2002) and indeed, decreasing sea lice sensitivity to emamectin benzoate has since been reported in Chile (Bravo, Sevatdal & Horsberg, 2008), Ireland (Hamish Rodgers, pers comm), Scotland (Lees, Baillie, Gettinby & Revie, 2008a), Norway (Pettersen, 2009; Horsberg, 2012), and Canada (Jones, Hammell, Dohoo & Revie, 2012).

Although published reports are limited, anecdotal evidence of a reduction in sensitivity of sea lice to emamectin benzoate in New Brunswick exists (Mark Moore, pers comm.; Chang, Page, Beattie & Hill, 2011) based on a reduction in the efficacy of this once highly effective chemotherapeutant in certain sea lice populations at marine aquaculture sites in the Bay of Fundy. In Canada, therapeutants for control of sea lice and similar ectoparasites on cultured fish which are administered externally by direct or indirect application are subject to the Pest Control Products and Food and Drug Acts, administered by the Pest Management Regulatory Authority (PMRA) and the Veterinary Drugs Directorate, respectively. Both these agencies suggest using therapeutants with different modes of action in rotation as a means of decreasing resistance pressure. Caution should, however, be exercised when attributing clinical treatment failures with emamectin benzoate to resistance development without considering other extenuating circumstances which can influence treatment outcome (Lees, Baillie, Gettinby & Revie, 2008b). Misdiagnosis of tolerance will negatively influence determination of the optimum dose sufficient to kill the maximum number of lice present but minimize toxicity to fish and the environment (Denholm *et al*., 2002). For this reason, it is important to use laboratory-based assessment methods (e.g. bioassays) to identify changes in sea lice susceptibility to therapeutants.

Bioassays are the conventional means by which insecticide resistance is detected. Over the past decade, bioassay protocols have been adapted for use as a common tool to monitor sea lice resistance to chemotherapeutants globally, including sensitivity to emamectin benzoate in laboratory-reared (Sevatdal & Horsberg, 2003; SEARCH Consortium 2006) and field collected

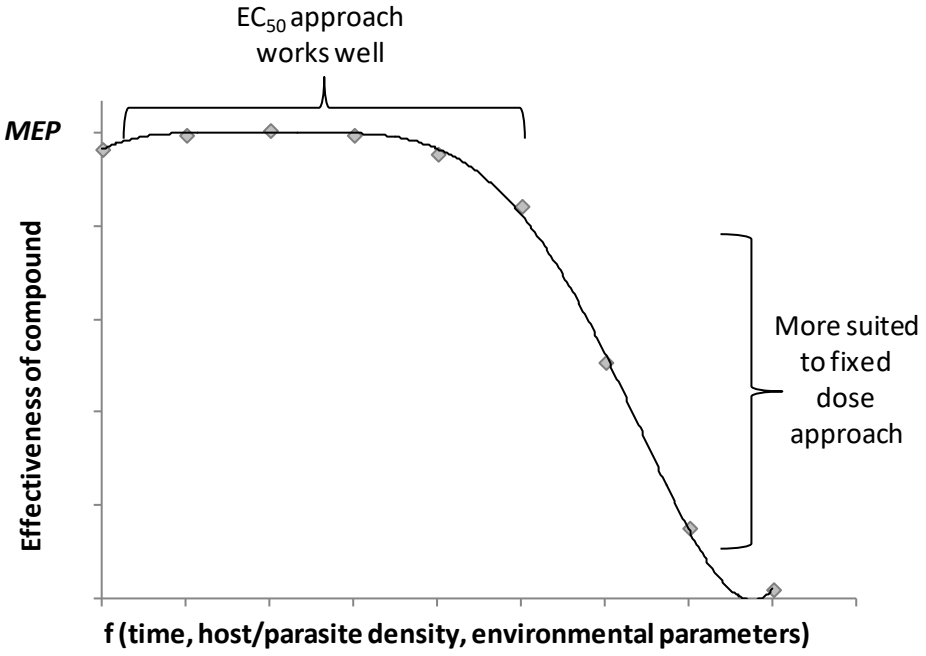
(Westcott, Stryhn, Burka & Hammell, 2008) sea lice. As expected, variations in bioassay methods exist between research groups (e.g. solvent used, doses tested, separation of sea lice by gender and stage for testing, statistical approach used to estimate effective concentration) (Sevatdal, Copley, Wallace, Jackson, & Horsberg, 2005; Westcott *et al*., 2008; Bravo *et al*., 2008). While these protocols are appropriate and effective, they require the collection of large numbers of sea lice due to inherent natural variability in response to chemotherapeutants and differences in gender and stage response to treatment. In addition to the fact that not all stages of lice are readily available at all periods of the year, their collection is labour-intensive and thus costly. The adaptation of the traditional, dose-response format bioassay to one that significantly reduced the number of sea lice required to obtain reliable efficacy assessments is the focus of this paper.

In addition to the costs and difficulties of collecting lice from the field, there is evidence that as tolerance develops within a sea lice population it appears to reach a "tipping point", after which it spreads rapidly through the remaining population of lice. This was seen in the results of clinical evaluations of tolerance to emamectin benzoate as measured by post-treatment efficacy in populations of lice on Scottish salmon farms (Lees *et al*, 2008a; 2008b). In these studies a gradual reduction in efficacy was observed between 2002 and 2005, before a sudden and dramatic drop in 2006, at which time approximately two-thirds of all treatments were deemed to be ineffective. Of particular interest in the context of the present study is the fact that a very similar situation appears to have prevailed in the New Brunswick salmon industry, albeit at a slightly later time period (Jones *et al*., 2012).

The conceptual diagram shown in Figure 1 provides a representation of the situation that may arise with respect to sea lice tolerance to a given mode of action. During the initial use of a given product it can be expected that the compound will be maximally effective. Indeed, the curve shown accepts the argument that the maximum effectiveness will not be achieved until some point after initial introduction, as a consequence of the learning period associated with developing best practice on the part of the user. This situation can remain reasonably unchanged for some time, reflected in the graph by the maximum effectiveness plateau (MEP). Following this phase, there is likely to be a period of gradually reducing effectiveness until a 'tipping point' is reached after which, effectiveness declines rapidly. The length of time (i.e. the scale on the x-axis) over which reasonable effectiveness can be maintained will, of course, vary by treatment compound and across situations. Factors including host density, mode of action, environmental conditions, e.g. area-wide flushing, and the relative density of farmed and wild salmonid hosts, may all have an impact on the trajectory of the effectiveness profile over time.

The purpose of introducing this concept in the current context is not to engage in speculation as to the relative importance of these various factors, but rather to illustrate where the various types of bioassay are likely to be most appropriate. In the initial stages of a given product's use, changes in effectiveness are likely to be modest and relatively sensitive tests are required to detect any change. During this phase it is recommended that traditional bioassays using the dose-response format be used as they are more suited to picking up subtle changes in parasite response to chemical use – as measured, for example, by changes in the value of EC_{50} estimates. Given the 'noise' associated with measurement in any natural/biological system, even these tests may not be sensitive enough to detect small changes in efficacy, but they should be able to demonstrate whether the chemotherapeutant is still relatively effective. However, once the tipping point has been reached, effectiveness begins to reduce so rapidly that less sensitive instruments can be used to detect such declines. This paper explores the utility of a fixed-dose emamectin benzoate bioassay in the context of the declining effectiveness of SLICE® to control sea lice populations across multiple aquaculture sites in New Brunswick.

Figure 1 Schematic view of the typical scenario that might be observed as tolerance to a compound develops within an ectoparasitic population following multiple exposures to the compound



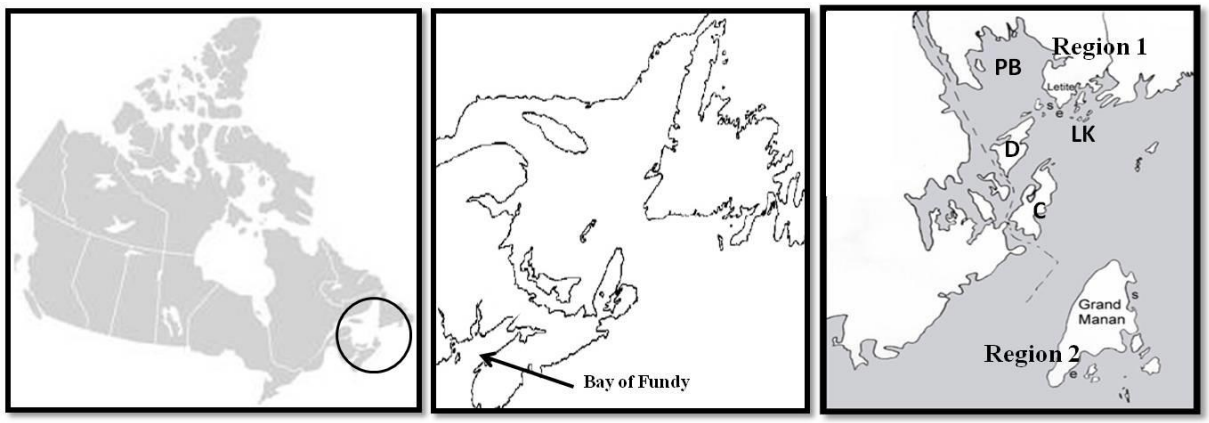
Materials and Methods

Sea Lice Collections

Motile stages of sea lice - adult females (AF), pre-adult males and adult males (PAM-AM) and pre-adult females (PAF) - were collected during 2008 to 2011 from 38 Atlantic salmon *Salmo salar* marine farm sites within the Bay of Fundy, New Brunswick, Canada, during routine harvests (i.e. market-sized fish that were not anaesthetized) or while routine sea lice counting was taking place on site (i.e. pre-market sized fish that were anaesthetized using tricaine methanesulfonate at a dose of approximately 100 mg l^{-1}) (TMS, Syndel Laboratories). Collections occurred throughout the year. For the purposes of this study two seasons were considered, according to mean sea water temperatures. In the analysis, winter-spring is defined as January to June and summer-fall is defined as July to December. Mean (± sd) water temperatures were 4.6 (± 2.0) °C in winter-spring and 11.8 (± 2.3) °C in summer-fall. Based on distinctly separate hydrographics and geographical locations, the bay management areas (BMAs) were categorised into two main regions for analytical purposes: Region 1 - Campobello Island, Deer Island, Lime Kiln Bay and Passamaquoddy Bay; and Region 2 – Grand Manan Island (Figure 2).

Sea lice were gently removed from the salmon using forceps and placed into a sealed container containing seawater collected from the sea cage site. Battery operated air pumps were added to collection containers for aeration during transport and these were transported back to the laboratory in coolers containing ice packs. Sea lice were held overnight at 10-12°C in an incubator to allow them to acclimate prior to bioassay set-up the following morning.

Figure 2 Farm sites in the Bay of Fundy, New Brunswick where Sea Lice Counts and Collections were performed: Campobello Island (C), Deer Island (D), Passamaquoddy Bay (PB), Lime Kiln Bay (LK) and Grand Manan Island



Bioassay Procedure

A total of 657 bioassays were performed between 2008 and 2011 comprising 3,532 individual dishes (10 lice per dish), including three life stages (i.e. AF, PAM-AM and PAF). All bioassays were performed at the Atlantic Veterinary College, University of Prince Edward Island, using a standardized protocol. The same personnel conducted all trials. All bioassays were initiated within 24h of collection; sea lice have been found to be more robust after storage at 10°C with air pumps for 12 hours or more to recover from handling and transport (Westcott *et al*., 2008). A stock solution of emamectin benzoate was prepared for each bioassay by dissolving 5 mg of emamectin benzoate (PESTANAL[7], Sigma-Aldrich, St. Louis, MO) in 50 ml methanol (or 25 ml of methanol + 25 ml de-ionized water when dilution was necessary). This working solution was used to prepare experimental solutions of 400 ppb and 800 ppb emamectin benzoate. The concentrations of emamectin benzoate chosen for inclusion in the bioassays were based on preliminary bioassay results showing that a majority of lice died in this range when collected from a susceptible population (Westcott *et al*., 2008). Control dishes (seawater only) were included in each trial. In all cases the experimental solutions used seawater taken from the same site from which the sea lice were collected. The exposure protocol involved exposing ten apparently healthy sea lice in glass Petri dishes to the treatment or control solutions; triplicate dishes of treatment and control were used. The Petri dishes were incubated in a temperature-controlled chamber at 10°C for an exposure period of 24 hours, as specified by Westcott *et al*.

(2008). All dishes were blinded as to treatment allocation. Following exposure, the condition (live, weak, moribund, or dead) of each sea louse was evaluated, according to an adopted set of bioassay response criteria (Westcott *et al*., 2008). For the purposes of this study, evaluations of sea lice were aggregated into two groups: live (live, weak) and dead (moribund, dead).

Statistical Analysis

Descriptive statistics of the proportion of dead sea lice by trial, lice stage, and dose were tabulated. A random effects Poisson model was used to investigate the association between the expected count of dead lice and each of the predictors of interest after adding random effects for site and trial. The outcome variable was the number of dead lice per dish and the Poisson exposure was defined as the total number of lice per dish (n=10). The predictor variables were: region, year, stage of lice, dose of emamectin benzoate and season. The unconditional association between the outcome and each of the independent variables was examined at $P<0.15$. Significant variables were further included in a multivariable random effects Poisson regression model keeping only those variables which have a significant association with the outcome at $P<0.05$ (Dohoo, Martin & Stryhn, 2009). Two-way interactions between all significant predictors in the final main effects model were evaluated. The impact of the interaction terms on the proportion of dead lice were presented graphically. Mortality in the control group was modeled separately using the same predictors. All analyses were performed using Stata version 12 (Stata Corp., College Station, TX).

Results

In the bioassays performed over the period 2008 to 2011, there was considerable difference in the numbers of the different stages collected opportunistically in the field. Of the total number of bioassays, 14.5 % were performed using pre-adult females, compared with 35.0 % using pre-adult and adult males and 50.5 % using adult females. For each bioassay conducted and each sea lice stage tested in the bioassay (i.e. adult females, pre-adult/adult males and pre-adult females) the proportion of dead sea lice was calculated (Table 1). Over the period 2008-2011, a total of 657 bioassays were conducted on adult females, adult males, pre-adult males and pre-adult females.

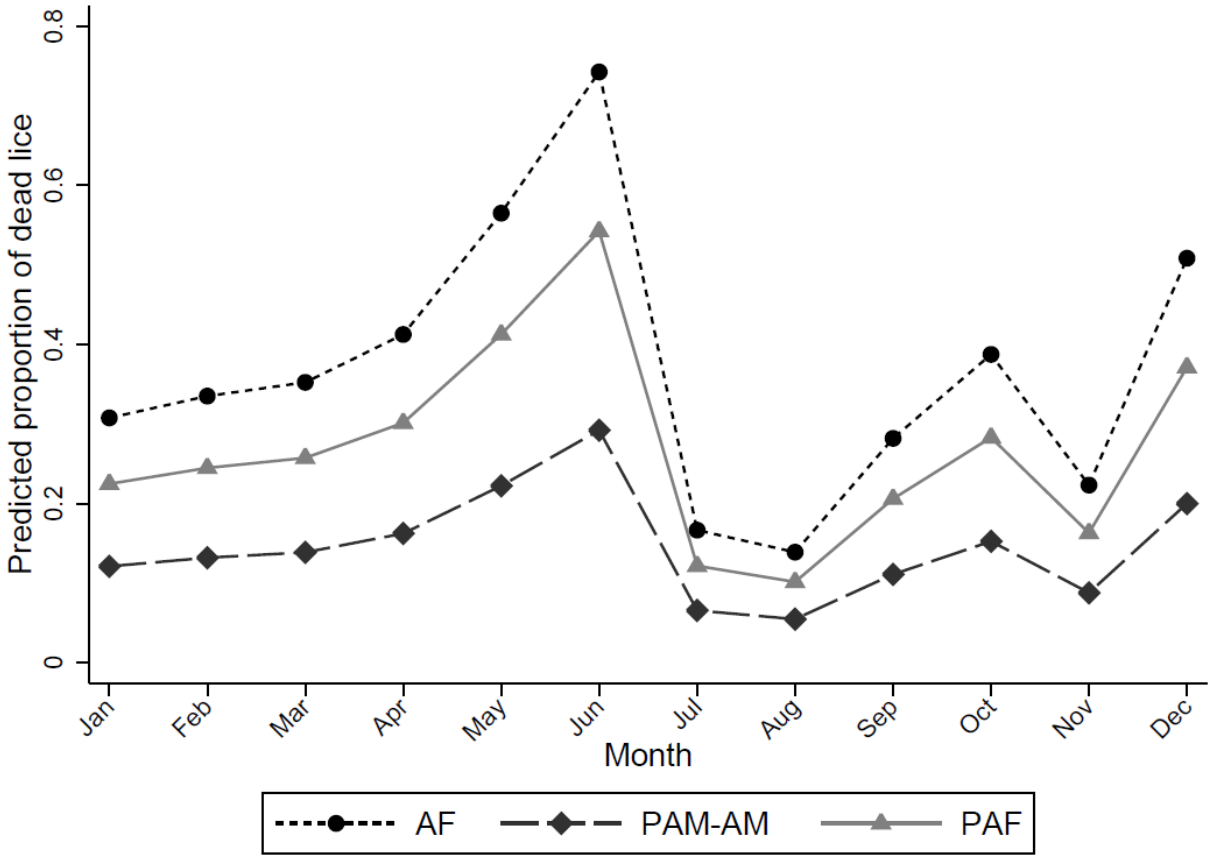
Table 1 Frequency distribution of average trial mortality by dose & stage

Dose	N	Percentile					Mean	Range
		10%	25%	50%	75%	90%		
Adult Female								
0	115	0.05	0.15	0.30	0.61	0.85	0.39	0-1
400	110	0.29	0.45	0.70	0.90	0.99	0.66	0.03-1
800	107	0.55	0.72	0.88	0.97	1	0.82	0.15-1
Pre-Adult Male/Adult Male								
0	79	0	0	0.06	0.20	0.40	0.13	0-1
400	76	0.08	0.14	0.27	0.54	0.80	0.36	0.05-1
800	75	0.30	0.40	0.60	0.94	1	0.62	0.1-1
Pre-Adult Female								
0	34	0	0.03	0.10	0.2	0.53	0.17	0-0.70
400	31	0.29	0.33	0.44	0.70	0.95	0.52	0-1
800	30	0.58	0.77	0.89	1	1	0.84	0.23-1

The mean control mortality for adult female lice was 39%, which was much higher than either pre-adult males and adult males (13%) or pre-adult females (17%). All trials were included in the analysis. Excluding trials from the analysis based on a specific threshold for control mortality, for example > 20%, would add bias to the model by excluding trials which have a greater proportion of adult female lice. At the 400 ppb dose of emamectin benzoate, the proportion of dead lice was higher for pre-adult female and adult female lice compared to pre-adult male and adult male lice. Similar results were also found at the 800 ppb dose. As expected, there was an increase in the proportion of dead lice, for all stages, as the dose of emamectin benzoate increased.

There was no difference in mortality of control lice between Regions 1 and 2, although overall, control mortality was higher in 2009-2011 compared to 2008. Figure 3 illustrates the predicted proportion of dead lice in the control group as a function of lice stage and time of year.

Figure 3 Predicted proportion of dead lice in the control group, by lice stage and month



A higher proportion of dead lice were observed in control adult female lice compared to pre-adult female, pre-adult male and adult male lice. An increase in control mortality was observed during May and June for all stages. Separate analyses (not shown) considering each lice stage in each of the four years (2008-2011) produced a similar trend.

Table 2 describes the random effects Poisson model for the association between the number of dead lice exposed to 400 and 800 ppb emamectin benzoate and the different predictors.

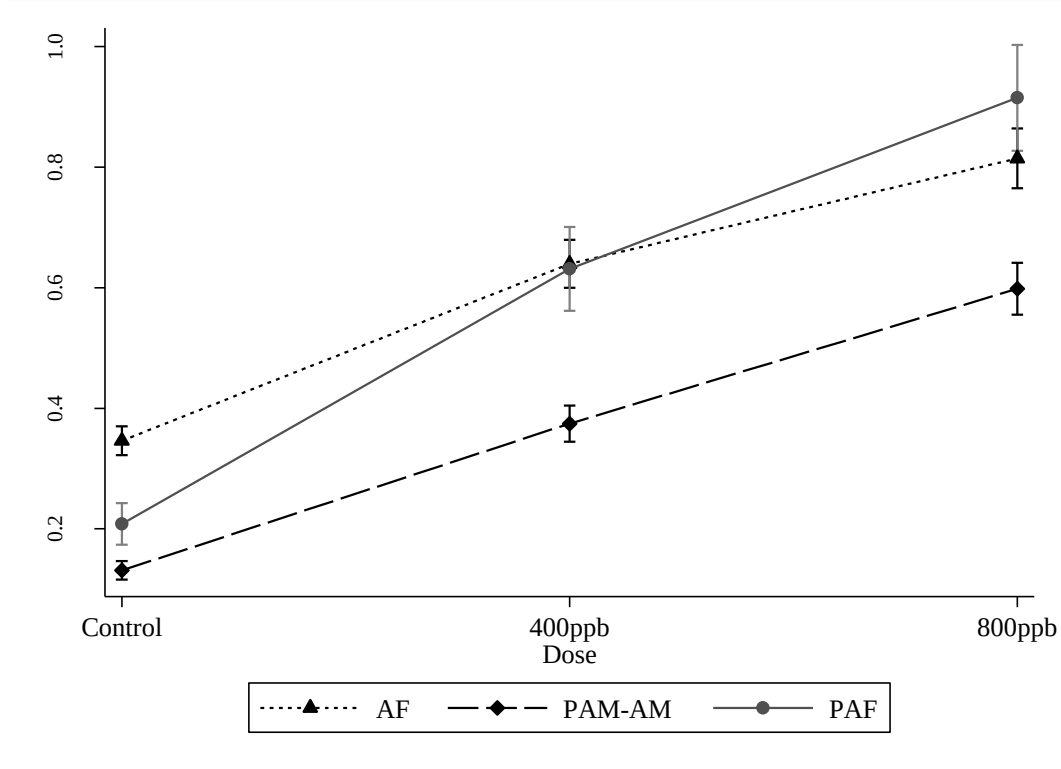
Table 2 Random effects Poisson model for the association between the number of dead lice exposed to 400 and 800 ppb emamectin benzoate and different predictors.

Covariate	Coefficient	Standard Error	P - value	Lower 95% Confidence Interval	Upper 95 % Confidence Interval
Region (reference = Region 1)					
Region 2	0.305	0.092	0.001	0.126	0.485
Year (ref = 2008)					
2009	0.049	0.105	0.636	-0.155	0.254
2010	0.153	0.102	0.135	-0.048	0.353
2011	0.566	0.108	0.000	0.355	0.777
Dose (reference = Control 0 ppb)					
400 ppb	1.317	0.064	0.000	1.190	1.443
800 ppb	1.955	0.061	0.000	1.835	2.075
Stage (reference = Pre-adult males-adult males)					
Adult females	0.972	0.057	0.000	0.861	1.083
Pre-adult females	0.462	0.093	0.000	0.280	0.643
Season‡ (reference = Summer-Fall)					
Winter-Spring	0.524	0.079	0.000	0.369	0.680
Intercept	-2.695	0.110	0.000	-2.910	-2.479
Random part					
Site	0.013	0.009		0.003	0.051
Trial	0.087	0.014		0.063	0.119

‡ Summer-Fall (July –December); Winter-Spring (January – June)

A higher number of dead lice were evident for Region 2 compared to Region 1. The expected number of dead lice was also higher for 2009-2011 compared to 2008, however, this effect was only significant for 2011. Dose was highly significant, with a higher dose being associated with an increase in mortality. Both pre-adult female and adult female lice stages exhibited higher mortality compared to pre-adult male and adult male lice. The expected number of dead lice was higher in the winter-spring period compared to the summer-fall period. Significant interactions between dose and stage of lice (Figure 4) and between dose and season (Figure 5) were observed.

Figure 4 Effect of interaction between dose and stage on the proportion of dead lice (95% confidence intervals shown)

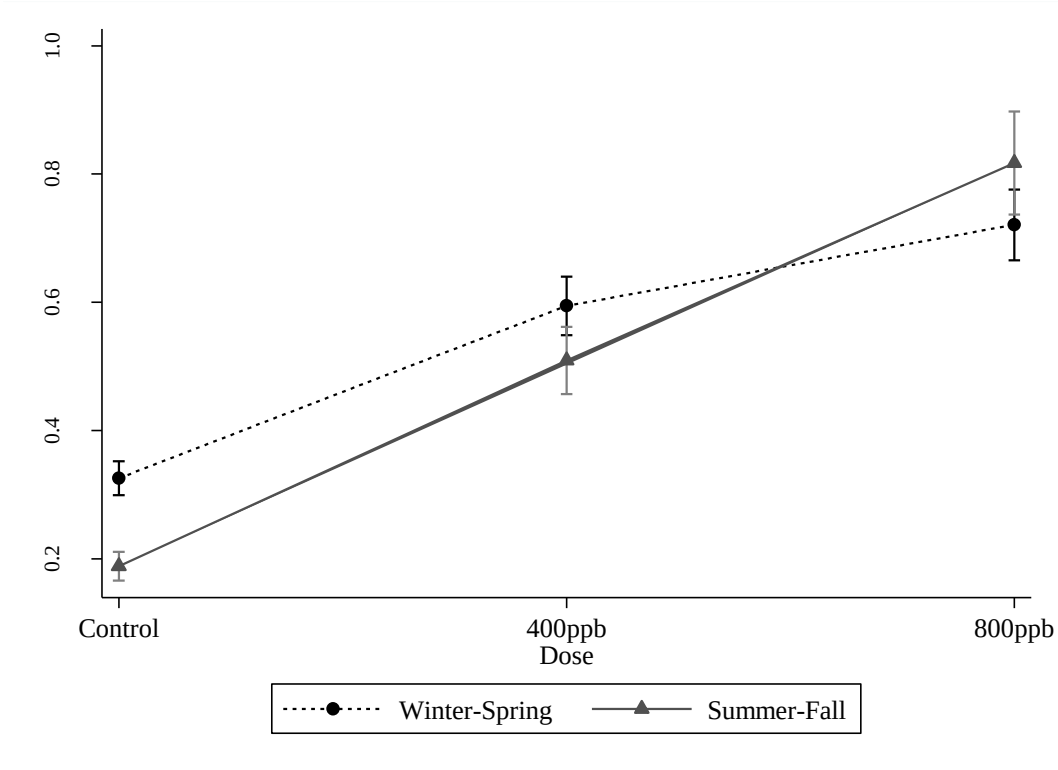


Mortality in pre-adult male and adult male lice was consistently lower than mortality in adult female or pre-adult female lice regardless of the dose used (Figure 4). Mortality in pre-adult female lice was significantly lower than adult female lice for the control dose only, whereas at the 400 and 800 ppb dose, mortality in female lice tended to be similar.

Figure 5 illustrates a higher proportion of dead lice in winter-spring compared to summer-fall in the absence of emamectin benzoate exposure (control). At the 400 ppb dose, season is no longer significant. Furthermore, at the 800 ppb dose, a higher, but not significant, proportion of dead lice were observed in the summer-fall season. In the winter-spring season, distinctions between mortality at the 400 ppb and 800 ppb doses were less obvious. However, during the summer-fall season, the differences in mortality between the two doses were more distinguishable. The

distribution of variance in the final model indicates that most of the variation was attributed to between-trial variance, whereas site to site variation was very small.

Figure 5 Effect of interaction between dose and season on the proportion of dead lice (95% confidence intervals shown)



Discussion

Regular bioassay monitoring using farm-collected lice supports field-based observations, which are typically subject to high levels of ecological 'noise' (Westcott *et al*., 2008). Several authors have reported increased tolerance to chemotherapeutants based on bioassay evidence (Roth, Richards, Dobson & Rae, 1996; Tully & McFadden, 2000; Sevatdal & Horsberg, 2003; Sevatdal *et al*., 2005). Increased tolerance to emamectin benzoate has been described in Chile for *Caligus rogercresseyi* (Bravo *et al*., 2008) which the authors attributed to the extensive use of this compound in the local industry. They also noted that the product is often used at higher than recommended doses and using prolonged treatment regimes, and was preceded by the use of ivermectin for approximately 10 years. While Westcott *et al*.

(2008) observed no bioassay evidence of increased tolerance to emamectin benzoate in sea lice in the Bay of Fundy, Canada, for collections done prior to 2005, seasonal or temperature associated trends were noted. Lees *et al*. (2008a) also reported spatial variation in efficacy to emamectin benzoate and a decline over time using epidemiological modeling of pre- and post-treatment farm data in Scotland. In a subsequent study based on the same data set (Lees *et al*., 2008b), the authors indicated that emamectin benzoate treatments administered on Scottish salmon farms were approximately 11 times more likely to be ineffective in 2006 compared to 2003 (their reference year).

The classic EC_{50} approach to bioassay data analysis requires a minimum of six therapeutant doses and a large number of sea lice in order to generate reliable EC_{50} estimates (10 sea lice per Petri dish with at least 3 replicates per dose, for a total of 180 viable sea lice of a given stage per bioassay). There are a number of challenges in estimating EC_{50} values from bioassays involving field collected sea lice using the classic approach. These include, but are not limited to, inherent individual variability in response to test compounds making EC_{50} estimates unreliable, varying levels of sea lice exposure to chemotherapeutants used in aquaculture operations which may influence sea lice health and response in bioassays, different ages of sea lice collected, and the influence of season/temperature on sea lice viability. In addition, the varying availability of sea lice from season to season will limit the number of individuals available for use in bioassays at any given time. These factors have the potential to influence estimates of EC_{50} values such that they cannot be made with any confidence as a result of constraining the fit of the data with the assumptions of the probit model, resulting in unacceptably wide 95% confidence intervals or inestimable EC_{50} values. This is a noted deficiency of the maximum likelihood or minimum chi-square method for estimating ED_{50} values based on the probit or logit models (Hamilton, Russo, & Thurston, 1977; Sanathanan, Gade & Shipkowitz, 1987). Similar influences have been observed in bioassays on the cattle tick, *Boophilus microplus* (Jonsson, Miller, Finn, & George 2003; Jonsson, Miller, & Robertson, 2007).

In contrast, the fixed-dose method affords a number of advantages over the traditional EC_{50} approach. For example, fewer sea lice are needed; two doses (control and chosen fixed-dose) with the same number of lice per dish and replicates might require, for example, only 60 viable sea lice per bioassay. A reduction in the number of doses tested also significantly simplifies the set-up of each bioassay experiment compared to traditional bioassays with multiple doses. In addition, statistical analysis of results can be simplified to a straightforward comparison of mortality for 2 groups. Finally, the use of a single emamectin benzoate concentration/dose should be sufficient to allow for discrimination between susceptible and resistant populations of sea lice in the field, thus allowing decisions around the use of emamectin benzoate on sites to be informed in a more efficient and practical manner.

There is evidence that the efficacy of emamectin benzoate in New Brunswick declined significantly in 2008 when compared to the previous three years (Jones *et al*., 2012). Anecdotal reports would suggest that this represented the 'tipping point' for many sea lice populations in the region and we have contended that sensitive instruments such as EC_{50} analysis are not well-suited for characterising susceptibility subsequent to such a decline. This challenge has also been observed in other parasite populations where drug-resistance management practices have a better chance of succeeding if resistance is detected when the allele conferring resistance is still at a low frequency in the population. Sensitive molecular assays which identify the operation of selection for drug resistance before treatment failure becomes clinically observable are valuable at this point in time. However, once the resistance allele reaches a sufficiently high frequency in the population, the rate of spread of drug resistance, and treatment failure, over time is highly nonlinear (Churcher & Basanez, 2009).

Many pharmacokinetic studies in animals have identified gender-specific biological responses to therapeutic exposure (Czerniak, 2001;Umeh & Currier, 2006; McKellar & Gokbulut, 2011) and sea lice are no exception in this regard (Stone, Roy, Sutherland, Ferguson, Sommerville & Endris 2002; Sevatdal & Horsberg, 2003; Sevatdal *et al*., 2005; Westcott, Revie, Giffin & Hammell, 2010; Igboeli, Fast, Heumann & Burka, 2012). Female sea lice collected in this study demonstrate an increased susceptibility to emamectin benzoate under bioassay conditions compared with male sea lice. The reasons for this are unclear. Susceptibility was also observed to increase over time from 2008 to 2011, with lice becoming significantly more susceptible to emamectin benzoate in 2011. The reasons for this are also unclear but, in general, the robustness of the lice, particularly adult female lice stages, appeared to decline from 2008 to 2011, as illustrated by increasing numbers of dead adult female lice in the control dose. This could be speculated as being due to the treatment pressure of emamectin benzoate use at increased doses as well as the use of other chemotherapeutants with possible temporal and environmental impacts on the overall robustness of the lice. Use of emamectin benzoate in eastern Canada was almost exclusive until 2009, at which time bath treatments were introduced due to observed treatment failures with emamectin benzoate. Azamethiphos (Salmosan®), hydrogen peroxide (Interox® Paramove®50) and Ivermectin, and for a short time, deltamethrin (AlphaMax™), have subsequently been included in sea lice management practices.

An apparent seasonal and spatial effect was observed in the robustness of genders and stages of sea lice included in emamectin benzoate field bioassays. Survival in all control groups was significantly lower in winter-spring, compared to survival in summer-fall. This was again, particularly noticeable for the adult females. Variation in water temperature has been identified as an influencing factor in treatment efficacy of emamectin benzoate (Westcott *et al*., 2008; Lees

et al., 2008a; 2008b). It has been suggested that adult female lice developing in summer-fall have a shorter generation time in warmer temperatures and, as such, the numbers are more likely to increase exponentially (Costello, 2006). Whether a more rapid development implies a more robust, emamectin benzoate tolerant louse is unknown, although it has been shown that parasites that have a high reproduction rate per individual female and a high rate of natural parasite turnover, can rapidly transmit resistance (Wolstenholme, Fairweather, Prichard, Samson-Himmelstjerna & Sangster, 2004; Prichard, 2005). As noted by Sevatdal & Horsberg (2003), age and gender also influence sensitivity to pesticides. The poor survival of adult females in the bioassays performed in this study could also be due to age of females, since it is likely that the majority present in the early months of the year were overwintering females (Jacobsen & Gaard, 1997; Heuch, Knutsen, Knutsen & Schram, 2002; Boxaspen, 2006). Body size of lice may influence the exposure and uptake of emamectin benzoate in an immersion bioassay and it is possible that the overwintered adult females were larger than summer generations (Costello, 2006) although considerable variation in size has been reported depending on the source of the specimens (Pike & Wadsworth, 1999). No determination of age (young vs old) was made between the two halves of each year; all adult females in this study were simply categorized to this stage according to Johnson & Albright (1991). Given the poor survival of adult female lice during the first half of the year, particularly May/June, it may be prudent to consider not using this stage in bioassays conducted during that time of the year. Some authors have suggested using only pre-adult II and adult male sea lice stages, or 50% of each gender, as the standard method for conducting field bioassays or similar controlled sensitivity tests (Sevatdal & Horsberg, 2003; SEARCH Consortium, 2006). However, the observations reported here raise questions as to the advisability of mixing different genders and stages of lice within a single bioassay as pertinent information may be overlooked.

It is known that sea lice abundance and susceptibility in other regions of the world vary by geopraphic location (Lees *et al*., 2008b). Similarly, in this study, sea lice collected from Region 2 (Grand Manan Island) appeared to be significantly more susceptible to emamectin benzoate than sea lice collected from Region 1 (Campobello and Deer Islands, Passamaquoddy Bay and Lime Kiln Bay). Recently it has been demonstrated that densities of farmed salmonids surrounding individual farms have a strong effect on sea lice infection pressure as well as increasing efforts of chemotherapeutic control thereby enhancing the risk of the development and spread of treatment resistance (Jansen, Kristoffersen, Viljugreun, Jimenez, Aldrin & Stein, 2012). The density of aquaculture sites in Region 1 over the period 2008-2011 was significantly higher than in Region 2 and, in terms of emamectin benzoate treatments, records for between 2009 and 2011 indicate that significantly more treatments occurred in Region 1 compared with Region 2. It is possible that the increased density and treatment pressure in Region 1 contributed to the decrease in susceptibility of lice to emamectin benzoate during the period studied.

The value of the single fixed dose biossay lies in the ability to categorise results into meaningful interpretations regarding developing resistance for different stages of lice within the population. The population of sea lice in the Bay of Fundy comprises lice with varying degrees of susceptibility to emamectin benzoate. The development of a statistical model to assess survival at a single fixed dose permits health professionals and producers to identify significant changes in susceptibility and compare these results across the industry. This information is important in the management strategies implemented to control sea lice infestations on aquaculture sites as optimal strategies to control resistance situations will differ according to the situation.

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