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Role of P-glycoprotein in emamectin benzoate (SLICE®) resistance in sea lice, *Lepeophtheirus salmonis*

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ABSTRACT

Emamectin benzoate (EMB; SLICE®) has been the drug of choice for the control of sea lice in salmon aquaculture within the past decade due to its ease of administration as well as efficacy on all parasitic stages of sea lice. This over-reliance has led to increased tolerance to the drug and a consequent decline in its use. ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp) are known to be involved in drug resistance. The present study investigated 1) the interaction of EMB with P-gp, 2) the effect of increasing EMB concentrations on P-gp mRNA expression in male and female sea lice, *Lepeophtheirus salmonis*, from Atlantic salmon (*Salmo salar*) farms in the Bay of Fundy, NB, as well as 3) changes in the mRNA expression of the transporter in archived adult female *L. salmonis*. Analysis of bioassay results indicated a 4 to 26 fold higher EMB EC_{50} for samples collected in 2011 compared to a similar study carried out between 2002 and 2004 suggesting loss of EMB efficacy in the parasite. An assay for ATPase activity as well as a competitive inhibition test showed that EMB interacts with the transporter. Emamectin benzoate had a significant concentration-dependent effect on P-gp mRNA expression in the parasite. There was a temporal increase in levels of P-gp mRNA in sea lice samples collected from 2002 to 2011. Our results indicate that EMB is a substrate for P-gp and that the transporter could be involved in the loss of efficacy of the parasiticide in *L. salmonis*.

1. Introduction

Lepeophtheirus salmonis is one of the major species of sea louse that infest both wild and farmed Atlantic salmon, *Salmo salar*, (Burka et al., 2012) and is currently the greatest challenge to profitable commercial salmon aquaculture in North America and Europe (Costello, 2009). The adult parasite will normally feed on mucus, but may also ingest blood following damage of superficial epidermal capillaries exposed by extensive abrasion of the skin (Bron et al., 1993). The attachment and feeding activities of the parasite cause stress to the host (Fast et al., 2005) as well as osmoregulatory problems and secondary bacterial infections (Pike and Wadsworth, 1999). These will lead to high treatment costs, reduced growth, reduced food conversion rate and, consequently, reduced profit margin for the salmon farmer (Costello, 2009).

Drugs used for the control of sea lice are either administered orally in the feed, e.g. emamectin benzoate (EMB), or topically as a bath treatment, e.g. deltamethrin and azamethiphos (Burridge et al., 2010). Emamectin benzoate, a macrocyclic lactone (ML), has been one of the most effective drugs (Stone et al., 1999) for combatting *L. salmonis* infestation in the past decade. It is administered in salmon feed as the chemotherapeutant SLICE® at a dose of 50 μg kg^{-1} fish biomass for 7 days (Stone et al., 1999). When fed to fish, it is absorbed from the gut and distributed throughout the fish with the least and highest concentrations in the muscle and mucus, respectively (Sevatdal et al., 2005). *L. salmonis* ingests EMB while feeding on the host mucus and the drug acts to block nerve transmission leading to flaccid paralysis and death of the parasite (Burka et al., 2012). Unlike bath treatments, which are more labor intensive and can be stressful to the fish, EMB is easily administered even during unfavorable weather conditions (Stone et al., 2000). It has high efficacy against all parasitic stages of the louse, disrupting the life cycle at multiple points (Stone et al., 1999). Its effectiveness and advantages over other sea lice parasiticides made it the preferred drug for the control of the parasite in salmon farms in the Bay of Fundy, NB (Westcott et al., 2004), and elsewhere, leading to concerns over resistance development (Westcott et al., 2008, 2010).

Drug resistance is an evolutionary adaptative process whereby susceptible parasites are eliminated causing resistant survivors to multiply and become the dominant population. This has been proposed for resistance development by sea lice to anti-parasitic drugs (Denholm et al., 2002). The speed at which resistance develops and its extent depend on such factors as the nature of parasite-induced damage to the host, the mechanism of resistance, frequency of drug use or selection pressure, and the parasite's biology. In a study by Bravo et al. (2008), over reliance on EMB was the major cause of the loss of sensitivity to

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another species of sea louse, *Caligus rogercresseyi*, in Chilean salmon aquaculture. Several years of use of another ML, ivermectin, prior to the introduction of EMB, also favored resistance development towards this class of drugs, suggesting lack of selectivity among the MLs.

P-glycoprotein (P-gp), also known as ABCB1, a member of the ATP-binding cassette (ABC) transporter protein superfamily has been linked to ivermectin resistance in nematodes (Eng and Prichard, 2005; Xu et al., 1998). These transporters function by causing the efflux of chemically diverse substances from within the cell to the outside. Extrusion of lipophilic chemicals from the cell by P-gp is powered by ATP hydrolysis, and this can be employed to determine drugs that interact with the transporter using the ATPase activity assay (Lespine et al., 2007; Schwab et al., 2003). It is hypothesized that over-expression of P-gp, most-likely in the gut epithelium, will impede the absorption of various compounds, including EMB, ingested by the salmon louse (Tribble et al., 2007). Furthermore, a novel *L. salmonis* P-gp SL-PGY1 (GenBank accession no. HQ684737) was recently cloned by Heumann et al. (2012).

There have been reports of sea lice tolerance to EMB on fish farms in New Brunswick, Canada (Westcott et al., 2010), but whether P-gp is involved in reduced EMB efficacy in the parasite is largely unknown. Early detection of changes in the sensitivity of sea lice towards EMB using drug resistance monitoring techniques should be a key component of a successful parasiticide resistance management plan (Westcott et al., 2008). Drug resistance monitoring should be precise, easy to perform, simple and repeatable. Sea lice bioassays are commonly used in diagnosing clinical resistance, but cannot be routinely performed due to their lack of rapidity and simplicity. Since the bioassay must be performed shortly after detaching the parasite from the host, to avoid biased endpoints from stressed sea lice, endpoints can be unclear especially between weak and moribund parasites following exposure in this system (Denholm et al., 2002; Westcott et al., 2008). Results of bioassays can differ widely based on time of year and site of the parasite collection. Also, no single bioassay technique will be suitable for all therapeutants used for sea lice control due to differences in the characteristics, stage specificity and speed/duration of action of the drugs (Denholm et al., 2002). These factors create the need for alternative methods of monitoring resistance development in the parasite, for example, using such molecular tools as reverse transcription quantitative PCR (RT-qPCR). Identification and monitoring expression of resistance-associated genes can assist to detect the development of resistance and to modify treatment strategies (Eng and Prichard, 2005). MLs can induce over-expression of P-gp in parasites (Lespine et al., 2012) and evidence for the involvement of such a gene in resistance development to a drug can be obtained by examining differences in expression of the gene between sensitive and resistant individuals (Williamson and Wolstenholme, 2011), where available.

The objectives of the present study were 1) to investigate possible links between P-gp and EMB by identifying whether EMB interacts with the transporter using an assay for ATPase activity and through competitive inhibition of the efflux pump, 2) to explore the use of RT-qPCR as a tool for monitoring resistance development to EMB in *L. salmonis* through temporal and dose response analysis of P-gp gene expression, and 3) identify whether P-gp expression analysis tracks resistance development in archived *L. salmonis* samples.

2. Materials and methods

2.1. Materials

Emamectin benzoate (PESTANAL®) and chemicals used for this study were of analytical grade and purchased from Sigma-Aldrich, St. Louis, MO. Membrane preparation (SB-MDR1-PREDEASY™-ATPase, SOLVO Biotechnology) from *Sf*9 (*Spodoptera frugiperda*) was obtained from Xenotech, Lenexa, KS.

2.2. Parasite collection

Sample collections for sea lice bioassays were done in March (winter collection) and July (summer collection) 2011. These months were selected to identify seasonal influence on EMB efficacy (Lees et al., 2008; Westcott et al., 2008). Adult male and female *L. salmonis* were carefully detached from host Atlantic salmon from fish farms in the Bay of Fundy, NB, and brought back alive to the laboratory in cold seawater collected from the sampling site and maintained at 10 °C overnight with aeration. Archived adult female *L. salmonis* (November 2002, February 2008, and February 2010) were collected in a similar manner, but were immediately flash frozen on arrival at the laboratory and stored at −80 °C prior to further processing. Samples were also collected in December 2011 from fish farms in the Bay of Fundy (adult male and female sea lice) and from the laboratory (Westcott et al., 2008) for P-gp competitive inhibition tests. Similarly, the sea lice samples were maintained at 10 °C overnight with aeration prior to use in the P-gp inhibition experiments.

2.3. Bioassay

Adult *L. salmonis* (240 male and 240 female) were selected within 12 h of the 10 °C overnight storage and randomly distributed into Petri dishes at 10 sea lice per dish; each Petri dish contained either male or female *L. salmonis*. The lice were exposed to EMB at 0, 10, 100, 300, 1000 and 3000 ppb in 4 replicates per EMB concentration in a 24 h bioassay at 10 °C. The EMB concentrations were coded to avoid biased analyses of the bioassay endpoint. Following the overnight incubation, the number of live, weak, moribund and dead lice per dish was determined according to Westcott et al. (2008) with slight modifications. 'Weak' refers to parasites that displayed poor and irregular swimming and were unable to attach to the Petri dish while 'moribund' refers to immotile parasites with twitching appendages. Percentage mortality was calculated following the 24 h bioassay and the current half-maximal effective concentration (EC_{50}) for EMB was derived from Trimmed Spearman–Kaber analysis (TSK) (Hamilton et al., 1977). Half-maximal effective concentration (EC_{50}) is the concentration of EMB that will cause 50% mortality (moribund and dead) of the parasite. To ascertain whether 24 h 10 °C incubation had any effect on P-gp mRNA expression, 40 live adult female *L. salmonis* were flash frozen shortly after collection while another 40 lice were incubated (10 lice per dish) for 24 h at 10 °C without any treatment; the pre-incubated and post-incubated samples were compared for changes in P-gp mRNA expression. All the bioassay survivors (none from the 3000 ppb exposure group) were stored in RNAlater® (Sigma-Aldrich) at 4 °C for 24 h and then at −80 °C prior to RNA extraction.

2.4. ATPase assay

Membrane preparations (SB-MDR1-*Sf*9, SOLVO Biotechnology) were incubated with EMB and ivermectin in separate assays, according to the manufacturer's protocol (Sarkadi et al., 1992). Briefly, the test compounds were dissolved in ethanol (2% maximum solvent concentration) to achieve 5 mM initial concentration of each test drug followed by a 4-fold serial dilution. The basic and verapamil-activated membrane suspensions as well as the KH_2PO_4 controls [0, 4 and 8 nmol inorganic phosphate (P_i) final assay concentration] were dispensed into a 96 well plate according to the manufacturer's setup such that each well contained 4 μg membrane (total) protein. Membrane suspensions were not added to the wells for phosphate calibration. The test drugs were added to the respective duplicate wells to achieve a concentration range of 0.01 to 100 μM final assay concentration. Prior to the initiation of ATPase reaction, the plate and MgATP solution were preincubated at 37 °C for 10 min, MgATP was then added to each well with the exception of the wells for phosphate calibration and incubated at 37 °C for 10 min. The ATPase reaction was terminated by addition of 100 μl of

the supplied developer solution at room temperature. Two minutes later, 100 µl of the supplied blocker solution was added to the wells at room temperature. The plate was then incubated for 10 min at 37 °C and the optical density read immediately at 620 nm using a BioTek® Synergy HT microplate reader (BioTek Instruments, Winooski, VT). The amount of P_i liberated in the presence and absence of 1.5 mM orthovanadate and relative to the KH_2PO_4 calibration curve were determined for both EMB and ivermectin and reported as the vanadate-sensitive ATPase activity (nmol P_i/mg protein/min). This is the activation assay and indicates whether the test compounds will stimulate baseline vanadate sensitive ATPase activity. The inhibition study, performed concurrently with the activation assay, was used to determine if the test compounds will decrease maximum vanadate-sensitive ATPase activity following stimulation by verapamil, a strong activator of P-gp ATPase activity. P-gp substrates will stimulate baseline vanadate-sensitive ATPase activity while inhibitors of the efflux pump will decrease verapamil-stimulated maximum vanadate-sensitive ATPase activity.

2.5. P-glycoprotein inhibition test

In a preliminary study, adult male and female *L. salmonis* from host Atlantic salmon from fish farms were exposed in duplicates at 10 lice per Petri dish to increasing verapamil concentrations (0, 10, 30, 100, and 300 µM), and also to increasing verapamil concentrations with 100 or 300 ppb EMB concurrently in separate bioassays. This was done to verify any toxicity of the inhibitor on the parasite as well as to ascertain the concentration of EMB at which inhibition of the transporter will be evident. 100 and 300 ppb EMB concentrations were chosen based on results of previous bioassays. In the subsequent experiment, adult male and female *L. salmonis* from the same sampling location were exposed to increasing verapamil concentrations (0, 1, 3, 10, and 30 µM), and also to the same range of increasing verapamil concentrations with 100 ppb EMB (adult female *L. salmonis*) or 300 ppb EMB (adult male *L. salmonis*) in concurrent but separate bioassays. Also, first generation adult male *L. salmonis* grown in the laboratory (Westcott et al., 2008) were exposed to 300 ppb EMB with or without 10 µM verapamil; 10 µM of the P-gp inhibitor was chosen based on results of initial experiments (Table 1). The bioassays were done according to previously described procedure and the criteria for determining the endpoints were the same.

2.6. RNA extraction

Total RNA was extracted from bioassay survivors and archived samples using the RNeasy® Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with minor modifications. One or two *L. salmonis* were placed in a 5 ml plastic tube containing 1 ml Trizol and homogenized using a hand held electrical tissue homogenizer. The quality of all isolated RNA samples was verified with Experion™ RNA StdSens Chips (Bio-Rad Laboratories, Hercules, CA). The RNA concentration and the 260/280 nm ratio were confirmed using the Nanodrop 1000 Spectrophotometer (NanoDrop Products, Wilmington, DE). The samples were then stored at −80 °C prior to further use.

Table 1
Mortality analysis of adult male and female *Lepeophtheirus salmonis* following exposure to emamectin benzoate (EMB) in a 24 h bioassay with or without increasing verapamil concentrations: December 2011 data.

Concentration of verapamil (µM)	% Mortality			
	Male[a]		Female[a]	
	Verapamil alone	Verapamil with 300 ppb EMB	Verapamil alone	Verapamil with 100 ppb EMB
0	0	5	20	10
1	0	0	15	5
3	0	0	30	25
10	15	35	10	60
30	55	100	65	100

[a] *L. salmonis* collected from Atlantic salmon farms in the Bay of Fundy, NB.

2.7. Reverse transcription quantitative PCR

All the RT-qPCR steps were done according to standard procedures following the manufacturer's protocol. Briefly, 1 µg RNA of each sample was treated with DNase I (Invitrogen, Carlsbad, CA) and then reverse transcribed using SuperScript® III (Invitrogen). The PCR reactions were carried out using SYBR® GreenER™ qPCR SuperMix (Invitrogen) on a Rotor-Gene 3000 (Corbett Life Science, Concorde, NSW, AU). Primers for the RT-qPCR were designed based on the SL-PGY1 sequence (Heumann et al., 2012) using the Primer 3 software. Expression of P-gp mRNA in the different samples were normalized to 4 reference genes — glyceraldehyde-3-phosphate dehydrogenase, 18S rRNA, translation eukaryotic elongation factor 1α and structural ribosomal protein S20 using the geNorm software (Vandesompele et al., 2002). P-glycoprotein mRNA expression was derived from $2^{-\Delta\Delta Cq}$ analysis followed by comparison of each sample to the calibrator.

2.8. Statistical analysis

Relative P-gp mRNA expression was determined using one way ANOVA with the Minitab 15 statistical software (Minitab Inc., State College, PA). Statistical significance was set at $p<0.05$. Graphical representation of the ATPase inhibition and activation assay result for EMB and ivermectin were created using the GraFit Version 7 software (Erithacus Software Ltd., Horley, UK). The rest of the graphs were plotted using SigmaPlot 10.0 (Systat Software Inc., IL). The ATPase activity EC_{50} (concentration of test drug that will cause half-maximal stimulation of basal vanadate-sensitive ATPase activity) and IC_{50} (concentration of the test drug that will cause half maximal inhibition of maximum vanadate-sensitive ATPase activity) for the test compounds were determined using SigmaPlot 10.0 software.

3. Results

3.1. Bioassay

The March bioassay showed a dose-dependent effect of EMB on the survival of both the male and female *L. salmonis* (Fig. 1). The female *L. salmonis* control group recorded a higher % mortality compared to the male control group. The % mortality for the male *L. salmonis* 0, 10, 100 and 300 ppb treatment groups were generally lower than those of the female counterparts (Fig. 1) except for the male 1000 ppb treatment group which had a higher % mortality compared to the female counterpart.

There was also a dose-dependent EMB effect on the parasite for the July bioassay (Fig. 1). Unlike the female control group, no mortality was recorded for the male control group. Again, the % mortality for the male *L. salmonis* treatment groups were generally lower than those of the female *L. salmonis* treatment groups with the exception of the 1000 ppb treatment groups which were 100% and 96% for the male and female parasite respectively.

The March 2011 EMB EC_{50} values (Fig. 1) derived from TSK analysis of the bioassay results were 457.20 ± 55.30 and 399.50 ± 93.70 ppb for the adult male and female *L. salmonis* respectively. These values are 1.4–1.5 fold higher than the July 2011 EC_{50} values which were 315.30 ± 56.80 ppb for the male parasite and 279.30 ± 57.20 ppb for the female.

3.2. ATPase assay

EMB and ivermectin decreased baseline vanadate sensitive ATPase activity with 50% inhibition of 26.35 µM (Fig. 2A) and 0.14 µM (Fig. 2B) respectively. Both compounds inhibited maximum vanadate-sensitive

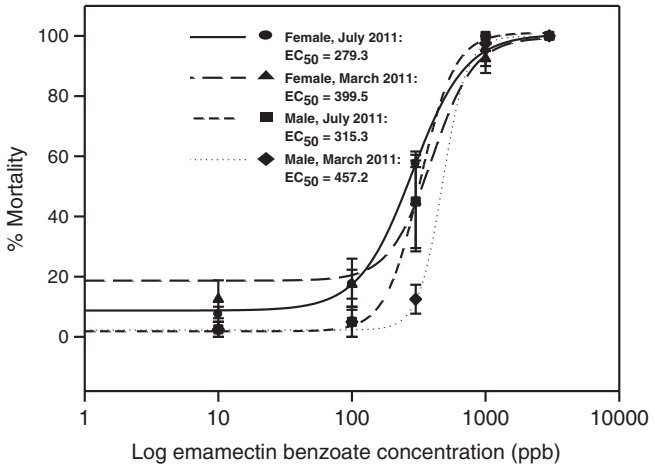

Fig. 1. Concentration–% mortality relationship for adult male and female *Lepeophtheirus salmonis* exposed to emamectin benzoate in a 24 h bioassay (mean ± SEM derived from 4 replicates per EMB concentration). *L. salmonis* samples collected in March and July 2011.

ATPase activity with IC_{50} of 7.82 μM for EMB (Fig. 2C) and 4.98 μM for ivermactin (Fig. 2D).

3.3. P-glycoprotein inhibition test

In the P-gp inhibition study, the male parasite recorded less than 5% mortality for the 0, 1 and 3 μM verapamil (with or without 300 ppb EMB) treatment groups (Table 1). Exposure of the male parasite to 10 μM verapamil with 300 ppb EMB caused 35% mortality while exposure to 300 ppb EMB alone resulted in 15% mortality. Also in the male parasite, concurrent exposure to 30 μM verapamil and 300 ppb EMB caused 100% mortality while exposure to the same concentration of the parasiticide without verapamil caused 55% mortality. Percentage mortality in the female parasite following exposure to increasing concentration of verapamil was irregular (Table 1). While 10 μM verapamil caused 10% mortality, simultaneous exposure to 10 μM verapamil and 100 ppb EMB caused 60% mortality for the female parasite.

For the laboratory-grown adult male sea lice, exposure to 300 ppb EMB without verapamil caused 6% mortality while exposure to the same concentration of EMB with 10 μM verapamil caused 93.75% mortality (Fig. 3).

3.4. Reverse transcription quantitative PCR

For the March 2011 bioassay survivors, EMB induced higher P-gp relative mRNA expression in the male adult *L. salmonis* (Fig. 4A) compared to the adult female parasite (Fig. 4B). For the male *L. salmonis*, the 1000 ppb treatment group had significantly higher relative P-gp mRNA expression compared to the other treatment groups and control with the exception of the 300 ppb group which also did not significantly differ from the mRNA expression levels of the transporter in the 0, 10 and 100 ppb treatment groups. For the female *L. salmonis*, the highest treatment group (1000 ppb) had significantly higher relative P-gp mRNA expression compared to the other treatment groups and control; the mRNA expression of the transporter in the control, 10, 100 and 300 ppb treatment groups did not significantly differ. No significant differences in P-gp relative mRNA expression were observed for the July 2011 bioassay survivors (data not shown). Overnight incubation had no effect on P-gp mRNA expression in the parasite (data not shown).

Analysis of the relative P-gp mRNA expression for the archived adult female *L. salmonis* (Fig. 5) revealed that the 2011 samples had a

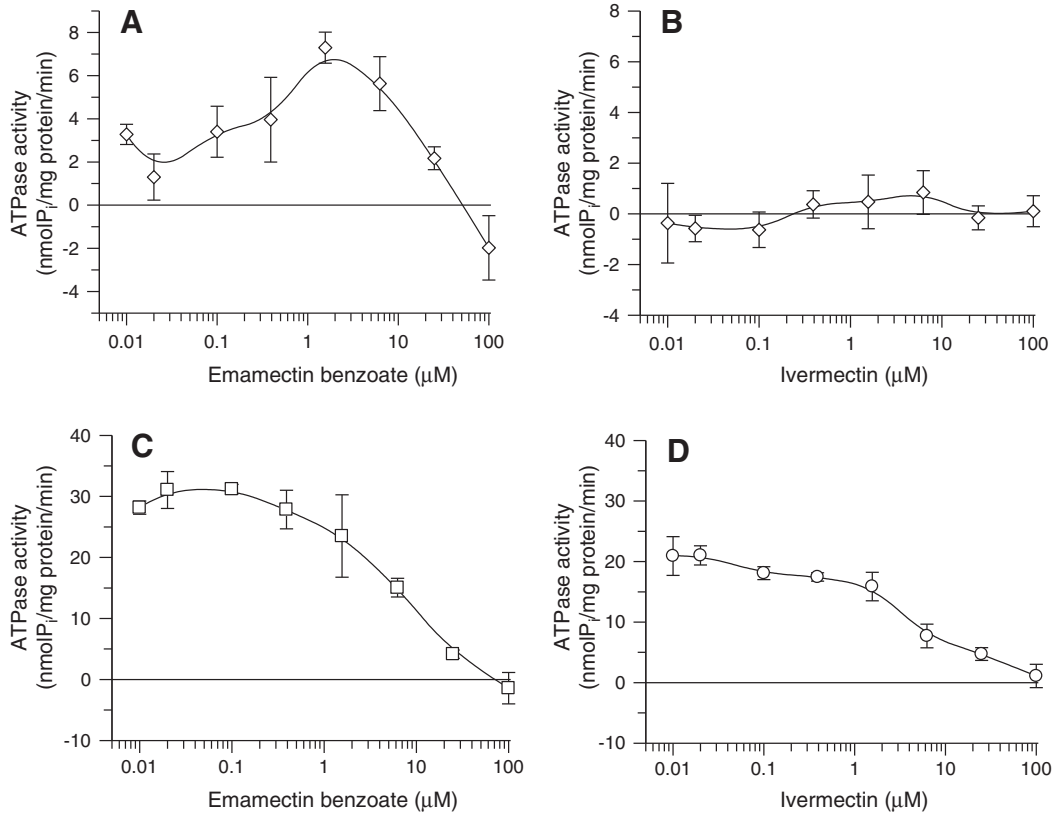

Fig. 2. Activation (A and B) and inhibition (C and D) of ATPase activity (nmol P_i/mg protein/min) by emamectin benzoate and ivermectin using membrane preparation from *Sf*9 overexpressing P-glycoprotein.

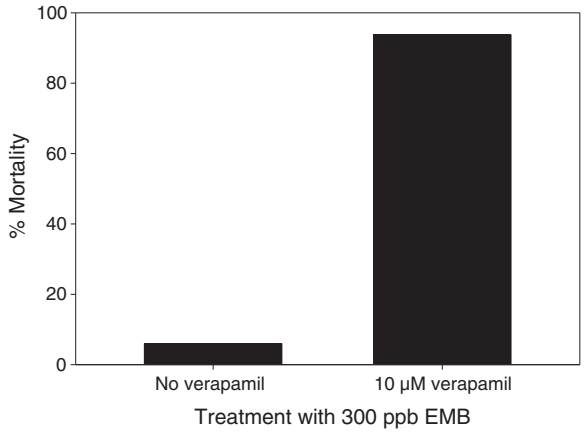

Fig. 3. Differences in % mortality of laboratory-grown adult male *Lepeophtheirus salmonis* exposed to 300 ppb emamectin benzoate (EMB) in a 24 h bioassay with and without 10 μM verapamil.

significantly higher (over 3-fold) levels compared to the samples from previous years (November 2002, February 2008 and February 2010). Although there was a general upward trend in the expression of P-gp mRNA from 2002 to 2010 (Fig. 5), the levels did not significantly differ.

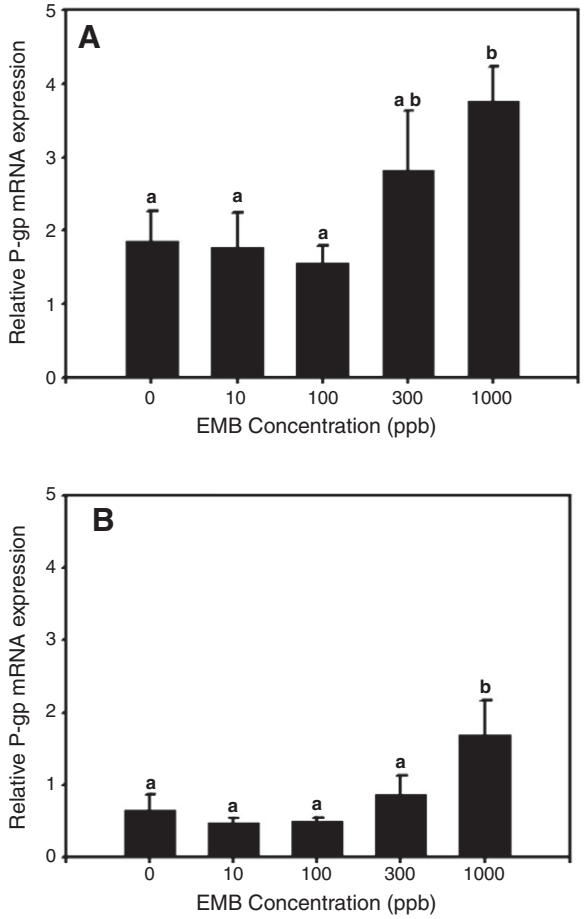

Fig. 4. Relative P-glycoprotein mRNA expression in adult male (A) and female (B) *Lepeophtheirus salmonis* emamectin benzoate bioassay (EMB) survivors (mean relative expression ± SEM). *L. salmonis* samples collected in March 2011. Different superscripts (a, b) denote significant ($p<0.05$) difference between two means.

4. Discussion

Macrocyclic lactones have been widely used for the control of human and veterinary parasites since 1981 (reviewed by Shoop and Soll, 2002), but unfortunately resistance has been developing to this family of parasiticides (reviewed by Prichard and Roulet, 2007). Mechanisms of resistance include changes in the target sites or decreased concentration of the drug at the receptor due to increased metabolism of the drugs or decreased uptake/increased excretion in the parasite. It is currently unknown whether EMB undergoes any significant enzymatic breakdown within the salmon louse. However, EMB undergoes only limited metabolism in the host (Kim-Kang et al., 2004) and, if this can be extrapolated to the parasite, would suggest that changes in metabolism would not significantly contribute to resistance. Although genetic changes in the glutamate-gated chloride channel have been associated with ML resistance (Njue et al., 2004), increased expression of the ABC transporter, P-gp, is widely believed to be the primary mechanism responsible for loss of parasite sensitivity to MLs (reviewed by Prichard and Roulet, 2007).

Kerboeuf and Guégnard (2011) and several other studies demonstrated that nematode P-gp interacts with EMB as well as other MLs, hence our interest in verifying such involvement in the salmon ectoparasite, *L. salmonis*. Resistance to parasiticides has been attributed to drug selective pressure on parasite populations whereby susceptible strains are eliminated, allowing individuals that survive therapeutic concentrations of the drug to multiply and become the dominant strain, as proposed for resistance development in sea lice (Denholm et al., 2002). For an anti-infective drug to be clinically useful, resistance needs to be monitored during treatment, and concomitant use of closely related drugs should be avoided to prevent selection for drug resistance. This has been reported for *L. salmonis* in New Brunswick (Westcott et al., 2010) and *C. rogercresseyi* in Chilean salmon aquaculture where several years of use of ivermectin followed by over-reliance on EMB led to the emergence of resistant populations of the parasite (Bravo et al., 2008). It is therefore important to identify genes and mechanisms associated with parasiticide resistance followed by monitoring the expression level of the identified genes as well as changes to the efficacy of the parasiticide. Such a combined monitoring approach will help prevent development of drug resistance in economically important parasites such as *L. salmonis*. Studying parasites at the gene expression level also yields information such as stage and gender-dependent differences in drug sensitivity that could be exploited in parasite control strategies.

Bioassays can be useful in detecting clinical resistance and Tribble et al. (2007) have previously investigated potential effects of EMB on gene

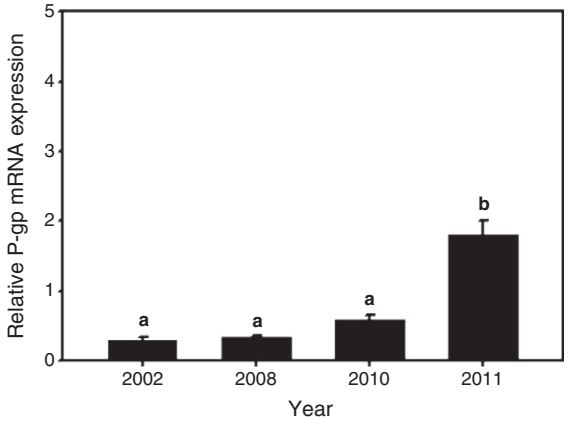

Fig. 5. Relative P-glycoprotein mRNA expression in adult female *Lepeophtheirus salmonis* collected in November 2002, February 2008, February 2010 and March 2011 from salmon farms (mean relative expression ± SEM). Different superscripts (a, b) denote significant ($p<0.05$) difference between two means.

expression in sea lice following a 24 h bioassay. For the March (winter) bioassay in this study, the female *L. salmonis* control group recorded slightly higher % mortality than the male *L. salmonis* counterpart but this trend was less obvious for the July (summer) bioassay (Fig. 1). Poor fitness is generally observed in samples collected during the colder winter period compared to the warmer summer months [pers. comm. Dr. J.D. Westscott, Centre for Aquatic Health Sciences (CAHS), AVC, Charlottetown, PE], but it is not clear as to why this was more evident in the female parasite compared to the male in the present investigation. Also, it has been reported that female *L. salmonis* are more sensitive to EMB than the males (Westcott et al., 2008); this was confirmed in the current study where the EC_{50} values for the female treatment groups were lower than the values for the male treatment groups. Although the reason for the gender discrepancy in EMB sensitivity in the parasite is yet to be elucidated, the reproductive burden imposed on the female *L. salmonis* could be a predisposing factor. Whether the intrinsically higher expression of P-gp mRNA in the male *L. salmonis* (Fig. 4A) compared to the female *L. salmonis* (Fig. 4B) is also a contributing factor to the gender differences in EMB sensitivity is unknown. Bioavailability of MLs in dogs and rats has been shown to be higher in females than males and was suggested to be likely due to differences in P-gp and/or other MDR transporter activity or expression levels (reviewed by Lespine et al., 2009). The 1.4 to 1.5 fold higher EMB EC_{50} for the March bioassay compared to the July bioassay could be attributed to differences in water temperature in the Bay of Fundy between the two seasons; the mean water temperature in the Bay in March and July 2011 were 2.6 and 11.2 °C, respectively (pers. comm. Dr. S.K. Whyte, CAHS, AVC, Charlottetown). Variations in seasonal temperature have been associated with seasonal differences in EMB efficacy in sea lice (Westcott et al., 2008), and Lees et al. (2008) have previously shown that seasonal temperature could be a risk factor for the outcome of EMB treatment episodes. Since the bioassays in the present study were done at the same temperature, the differences in EMB EC_{50} between the March and July bioassays could be traced to differing temperature preconditioning at the sampling site. Although the mechanism of such temperature effects on the parasiticide is not yet understood, knowledge of such seasonal differences in the efficacy of EMB can be useful in the timing of treatment episodes for sea lice on salmon farms. The differences in EMB EC_{50} between the two sampling periods could also be due to variations in EMB sensitivity in sea lice population between different salmon farms in the Bay of Fundy. Sea lice samples used in the two bioassays were collected from different sites in the Bay of Fundy and Westcott et al. (2010) have previously shown that sea lice sensitivity to the parasiticide can vary between different salmon farms within the Bay of Fundy. When compared to the EC_{50} in the present study, the EC_{50} values derived from a similar study by Westcott et al. (2008) between 2002 and 2004 using preadult stages of the parasite were 4 to 26 fold lower and ranged from 50.00 ± 0.00 to 107.80 ± 3.80 ppb for the male parasite and 15.00 ± 14.50 to 34.50 ± 9.35 ppb for the female parasite, and is indicative of reduced EMB potency in the bioassays. The 2002–2004 bioassays were carried out using preadult stages of the parasite, whereas the present study utilized adult stages. Whether the difference in the stage of *L. salmonis* used in the bioassays accounts for the differences in EMB EC_{50} values between both studies and the magnitude of such differences are yet to be ascertained. Notwithstanding the foregoing, we suspect that a rightward shift in the EC_{50} of the parasiticide in *L. salmonis* found on Atlantic salmon in fish farms in the Bay of Fundy has occurred. This normally occurs through drug selective pressure (Lespine et al., 2012) and has been previously demonstrated for sea lice parasitosis in salmon farms elsewhere (Bravo et al., 2008; Lees et al., 2008).

Typical of MLs, EMB and ivermectin inhibited basal ATPase activity; Schwab et al. (2003) previously showed that ivermectin is an inhibitor of basal P-gp ATPase activity. In a study by Lespine et al. (2007), the following MLs: abamectin, eprinomectin, doramectin, ivermectin, selamectin and moxidectin, inhibited the basal P-gp ATPase activity with 50% inhibition at 0.2, 0.3, 0.5, 2, 3 and 10 μM, respectively. Slowly transported substrates have been shown to inhibit basal P-gp ATPase activity, while substrates that are more readily transported activate the ATPase activity (Lespine et al., 2007). In this study, EMB recorded an EC_{50} of 26.35 μM (Fig. 2A), suggesting that it is probably a more readily transported substrate than ivermectin (EC_{50}: 0.14 μM, Fig. 2B) as well as the previously mentioned MLs. The difference in ivermectin EC_{50} values between the study by Lespine et al. (2007) and our study may be due to the type of membrane used in the different experiments. Whereas Lespine et al. (2007) used membranes derived from DC-3F/ADX cells from Chinese hamster lung fibroblasts overexpressing P-gp, the current study utilized SB-MDR1-*Sf*9 membranes overexpressing the transporter. It has been shown that variations in basal ATPase activity can exist between different membrane preparations overexpressing P-gp (Lespine et al., 2007). More studies to determine the rate of EMB transportation by the efflux pump using a cell-based model (Brayden and Griffin, 2008) should be done to clarify our current observations. The ATPase activity inhibition study (Fig. 2C and D) showed that EMB similar to ivermectin inhibited maximal vanadate-sensitive ATPase activity with IC_{50} of 7.82 and 4.98 μM, respectively. Emamectin benzoate and ivermectin are lipophilic compounds and are slowly transported *in vivo* and such compounds have been shown to inhibit maximal vanadate-sensitive ATPase activity. Competitive inhibitors of P-gp could also be substrates of the transporter (Garrigos et al., 1997) and further studies are necessary to confirm whether EMB, similar to ivermectin, is a substrate of the efflux pump. Ivermectin has previously been shown to be a competitive inhibitor as well as substrate of P-gp, hence initial attempts to use the parasiticide as a multidrug-reversing agent in drug resistant parasites (Mottier et al., 2006) and cells (Pouliot et al., 1997).

P-gp competitive inhibition test using MDR-reversing agents such as verapamil (Lespine et al., 2012) is a well established means of determining compounds that are substrates of the efflux transporter during drug development and the concept is the basis for the reversal of P-gp mediated drug resistance in anthelmintic and anticancer chemotherapy. Verapamil, a well known calcium channel blocker, caused ≥10% mortality in *L. salmonis* at 10 and 30 μM (Table 1) and 100% mortality at 100 μM (data not shown), possibly due to blockade of calcium channels in the parasite. However, concurrent exposure of *L. salmonis* to EMB and verapamil caused significantly higher mortality compared to exposing the parasite to the parasiticide alone (Table 1, Fig. 3). This data provides further evidence that EMB interacts with P-gp in the parasite and also suggests that verapamil may be interacting at the same site on the efflux pump as EMB. The increased sensitivity of *L. salmonis* to EMB following concomitant verapamil exposure is most likely a consequence of competitive inhibition of P-gp by the MDR-reversal agent. Inhibition of the transporter limits the efflux of the parasiticide, thereby causing an increase in the concentration of EMB in the parasite. Also, the sum of mortalities due to separate exposure of the parasite to EMB and verapamil (Table 1) is several fold lower than the mortality recorded following combined exposure to both drugs at the 10 and 30 μM verapamil concentrations. The gender differences in the concentrations of verapamil and EMB combined exposure that caused over 50% mortality in the parasite further reinforces the idea that the gender differences in EMB sensitivity observed in the parasite could be due to differences in P-gp expression between male and female *L. salmonis*. Again, the female sea lice treatment groups displayed suboptimal fitness compared to the male parasite groups, but that notwithstanding, the effect of concomitant verapamil and EMB exposure on % mortality on both parasite genders is very evident at the 10 and 30 μM verapamil exposures. This observation is consistent with previous studies on competitive inhibition of P-gp to ivermectin by verapamil in rat (Alvinerie et al., 1999) and *Haemonchus contortus* (Molento and Prichard, 1999), and further confirms our hypothesis that P-gp could be involved in reduced sensitivity of *L. salmonis* to EMB. This implies that concurrent administration

of MDR-reversing agents (such as verapamil) and EMB could increase the sensitivity of sea lice to the parasiticide and bring about significant reduction in EMB-resistant parasite population to lower acceptable numbers in salmon farms where reduced EMB efficacy have been reported. More studies are necessary to confirm the possibility of using MDR-reversing agents in the control of sea lice in Atlantic salmon farms plagued with EMB-resistant strains of the parasite. Verapamil was used in this study as a pharmacological tool to ascertain the role of P-gp in resistance development *in vitro*, but would probably not be an ideal agent to use therapeutically as it is not likely to accumulate in the skin, limiting availability to the parasite. It would also have the potential of inducing cardiac toxicity to the host salmon due to Ca^{++} channel inhibition. Other ABC-transporter inhibitors with appropriate pharmacokinetics and host and human safety parameters need to be developed.

Emamectin benzoate induced overexpression of P-gp mRNA in a concentration-dependent manner for the March, but not July 2011 sampling period. The approximately 2-fold P-gp mRNA expression (March 2011 sampling) in the 1000 ppb male treatment group compared to the 0, 10 and 100 ppb treatment groups (Fig. 4A), as well as the dose-dependent upward trend in P-gp mRNA expression (Fig. 4A and B) suggest that there is a positive correlation between EMB concentration and P-gp mRNA expression. Previous studies in nematodes have linked ivermectin resistance to over-expression of P-gp and the transporter has been reported to be responsible for multidrug resistance to structurally diverse drugs and chemicals used in agriculture, medicine and veterinary medicine. The upward trend in the relative P-gp mRNA expression in archived samples (Fig. 5) and the fact that P-gp mRNA expression for the March 2011 samples was significantly (>2-fold) higher than the expression for 2002, 2008 and 2010 samples suggest that P-gp mRNA expression levels was increasing over the years. Although lipophilic xenobiotics in the sampling site can induce increased expression of the transporter gene in the parasite, presence of such contaminants was not confirmed and the genetic effect of continuous use of EMB on salmon farms since 2000 could be the prevailing factor (Bravo et al., 2008; Westcott et al., 2010). This pattern is similar to what was observed for the March 2011 bioassay survivors (Fig. 4A and B) whereby higher concentrations of EMB induced higher P-gp mRNA expression and vice versa. Such pattern of expression was not observed for the July bioassay, suggesting possible correlation between EMB EC_{50} values and P-gp mRNA expression in *L. salmonis*. The EC_{50} value was higher in the March 2011 sampling compared to the July 2011 sampling and there were concentration-dependent differences in P-gp mRNA expression for the earlier sampling period but not for the latter. Unfortunately, only adult female parasites were archived and analyzed, otherwise, it would have been interesting to investigate the changes in the expression of the transporter in the male parasite over the same period. This is because male *L. salmonis* are less sensitive to EMB and has a higher level of P-gp expression (Fig. 4A and B), hence will likely be a better model for tracking development of EMB resistance compared to the female parasite.

5. Conclusion

Our results, especially the P-gp competitive inhibition test results, strongly indicate that the efflux transporter is involved in reduced sensitivity of *L. salmonis* to EMB. This observation may further be confirmed using gene knock-out strategies such as RNA interference (RNAi) technique. Changes in the expression of resistance-associated genes such as P-gp can be monitored and used in the diagnosis of resistance development to parasiticides. Although definitive diagnosis for clinical resistance can only be derived by determining changes in EC_{50}, it is important to predict when resistance is likely to occur and plan towards forestalling it. This could be achieved by monitoring markers or genes involved in reduced efficacy to the drug using such molecular tools as RT-qPCR (Williamson and Wolstenholme, 2011) and our studies have demonstrated that this molecular technique can be employed in monitoring resistance development to drugs used in aquaculture. Knowledge of the timing for resistance development will inform necessary changes to treatment options to prevent severe treatment failure. One of the greatest challenges to chemotherapy is that resistance to currently available classes of paraciticides already exists and there is an urgent need for the discovery of new classes of drugs for the control of parasites including *L. salmonis*. Although some studies have shown that MLs interact with other ABC transporters, P-gp is believed to be the major resistance mechanism for this family of parasiticides (Kerboeuf and Guégnard, 2011; Prichard and Roulet, 2007). Comparison between EMB sensitive and resistant strains of *L. salmonis* should be carried out to verify whether P-gp is involved in the loss of parasite sensitivity to the drug.

Using the P-gp competitive inhibitor, verapamil, we have demonstrated that the efflux transporter could be playing a major role in EMB resistance in *L. salmonis*. Also, results presented showed that EMB will induce overexpression of the transporter in *L. salmonis*. Further investigation is required to confirm the extent of P-gp involvement in reduced EMB efficacy in *L. salmonis*, and whether this can be targeted itself for therapy. The use of RT-qPCR as a drug resistance monitoring tool in aquaculture should also be explored further.

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