AN INVESTIGATION INTO POTENTIAL EMAMECTIN BENZOATE RESISTANCE MECHANISMS IN SEA LICE *LEPEOPHTHEIRUS SALMONIS*

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

in the Department of Biomedical Sciences

Atlantic Veterinary College

University of Prince Edward Island

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Charlottetown, P. E. I.

January, 2007

Your file Votre référence
ISBN: 978-0-494-32113-3
Our file Notre référence
ISBN: 978-0-494-32113-3

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ABSTRACT

Due to the limited chemotherapeutic options available, there is a continued reliance on emamectin benzoate (EMB) for the control of sea lice (*Lepeophtheirus salmonis*) infestation on farmed Atlantic salmon (*Salmo salar*), making resistance development a major concern. Previous studies indicate target site mutations and/or over-expression of P-glycoprotein (P-gp) to be the predominant mechanisms involved in the development of an avermectin resistance phenotype. This study primarily focused on the expression of P-gp in sea lice and its potential role in EMB resistance development. Two candidate genes, SL0525 and SL-Pgp1, were identified in sea lice from a sea lice expressed sequence tag (EST) database and RT-PCR on total RNA, respectively. Western blot analysis using mammalian P-gp-specific monoclonal antibodies, C219 and JSB-1, and the polyclonal antibody against SL0525 generated a signal at 160kDa which resembled P-gp with respect to the expected molecular mass. Immunohistochemistry using these antibodies localized P-gp-like immunoreactivity primarily in sea lice intestinal epithelial layer. The optimized Western blot analysis and immunohistochemistry methods offer novel tools for detection of P-gp in sea lice in future studies. Real time RT-PCR was used to examine both SL0525 and SL-Pgp1 mRNA levels in response to EMB exposure. The results of this study demonstrated significantly increased levels of both SL0525 and SL-Pgp1 in sea lice exposed to 10ppb EMB for 24 hours. These results represent the first evidence of increased levels of putative P-gps in *L. salmonis* in response to a xenobiotic, in this case EMB. This study is also the first to examine expression levels of P-gp in a parasitic organism in response to drug exposure using Q-RT-PCR. The information gained from this project

is the first evidence of P-gp expression in sea lice and the methods developed in this study allow for the detection of its over-expression, should resistance to EMB develop. In this study we also identified the genes for avermectin target sites, GluCl and GABA-gated chloride channel alpha subunits. These genes showed a high level of homology to their respective counterparts in other organisms and phylogenetic analysis also grouped these receptors within their respective channels. These receptor subunits are the primary binding regions for avermectins. The isolation of a GluCl-gated chloride channel gene in sea lice is the first to be identified in a crustacean species. Further identification of receptor polymorphisms in sea lice populations will assist in detecting resistance to avermectin resistance due to altered target sites. Additionally, receptor characterization will facilitate improved therapeutic efficacy. The knowledge gained from this study can be transferred to aquaculture to offer both surveillance methods for the early detection of resistance, and possibly advice for alternative treatments. Decreasing the selection pressure for such resistance, thus maintaining EMB use in an already limited available drug pool, is therefore important for the Atlantic salmon industry.

ACKNOWLEDGEMENTS

Firstly, a large amount of gratitude goes to the following sources of financial support during this study: AquaNet (Canadian Centre of Excellence in Aquaculture), Canadian Commonwealth International Scholarship, Atlantic Veterinary College Scholarship, Department of Biomedical Sciences, and NSERC.

Thank you to my supervisors, Drs. John Burka and Fred Kibenge, for your invaluable support, guidance and friendship. You both showed great faith in this project and in me for which I am forever grateful. Thanks to my supervisory committee for their insight and suggestions during this study. I would also like to express my thanks to all my colleagues who have given me a wide array of technical support, especially to Jillian Westcott and Nicole MacDonald.

I feel blessed to have met so many great individuals who truly enriched my life on Prince Edward Island. Thanks to Fortune, Dante, Charles, Javier and all the other members of the soccer team for all the years of success we had.

To my dearest friends Carolyn and Matt, thank you so much for giving me support (and food) when I was tired and lonely. A big kiss goes to my god child Jillian who will continue to prefer soccer over football even though I am no longer around.

Finally to Vicky for her endless love and support that continues to keep me strong.

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List of Abbreviations

ABC	ATP-binding cassette
AchE	Acetylcholinesterase
ALD	Adrenoleukodystrophy
ANOVA	Analysis of variance
AP-1	Activator protein 1
ATP	Adenosine triphosphate
AVC	Atlantic Veterinary College
BCIP	5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt
BLAST	Basic Logic Alignment Search Tool
bp	base pair
BSA	Bovine serum albumin
CFTR	Cystic fibrosis transmembrane conductance regulator
CNS	Central nervous system
Ct	Threshold cycle
CYP450	Cytochrome-P450
DAB	3,3`-diaminobenzidine tetrahydrochloride
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
eEF1α	Eukaryotic elongation factor 1 alpha
EMB	Emamectin benzoate
EST	Expressed Sequence Tag
GABA	γ-aminobutyric acid
GluCl	Glutamate gated chloride channel
GST	Glutathione-S-transferase
IgG	Immunoglobulin G
IPTG	Isopropyl-D-thiogalactopyranoside
IL	Interleukin

JNK	c-Jun-NH_2-terminal protein kinase
Kb	Kilobase
kDa	Kilodalton
LB	Luria Bertani
LNA	Locked nucleic acid
mAb	Monoclonal antibody
MDR	Multi-drug resistance
MEF1	Multi-drug resistance promoter enhancing factor 1
mRNA	messenger RNA
MRP1	Multi-drug resistance associated protein 1
NBD	Nucleotide binding domain
NBT	Nitro-blue-tetrazolium-chloride
NCBI	National Centre for Biotechnology Information
NF-Y	Nuclear factor Y
NHS	Normal horse serum
NZY	NZ amine (casein hydrolysate)
pAb	Polyclonal antibody
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween 20
PCAF	p300/coactivator associated arginine methyltransferase associated factor
PCR	Polymerase chain reaction
PEI	Prince Edward Island
P-gp	P-glycoprotein
PIPES	1,4-piperazinediethanesulfonic acid
PKC	Protein kinase C
Q-RT-PCR	Quantitative reverse transcription polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RT	Reverse transcription
SDS	Sodium dodecyl sulfate

Sp	Specific protein
SSC	Sodium trisodium citrate
ssDNA	Single stranded DNA
STE	Sodium, Tris, EDTA
TAMRA	6-carboxytetramethylrhodamine
TBS	Tris buffered saline
TBST	Tris buffered saline Tween 20
TMD	Transmembrane domain
TMS	Tricaine methanesulfonate
TNFα	Tumor necrosis factor α
tRNA	transfer RNA
UV	Ultraviolet

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CHAPTER 1. GENERAL INTRODUCTION

1.1. Salmon Aquaculture Industry

The rearing of salmon under controlled and supervised conditions from the egg to the market is called salmon aquaculture or salmon farming. Salmon aquaculture was developed in Norway, and has exploded in growth world wide since the 1980's (Monahan, 1993). An increase in the world population size, the increased recognition of the nutritional advantage of fish as a source of protein and omega-3 fatty acids in the diet, and the increased demands of food establishments for high quality fresh fish have strongly influenced the rapid expansion of salmon aquaculture throughout the world. United Nations studies indicate that aquaculture is the world's fastest growing food production industry (http://www.un.org/apps/news/story.asp?NewsID=6226&Cr=aquaculture&Cr1, accessed: 20 November 2006). As wild fish resources decline, aquaculture will continue to develop as a sustainable cost-effective solution to world fish supply. The culture of salmon is performed in an intensive system with a reliance on the use of commercial feeds. Densities of fish kept within such holding areas are limited by the species ability to grow at raised stocking densities and maintenance of environmental parameters (Fitzgerald *et al.,* 2002). The farming of salmon has become an increasingly competitive industry with narrowing profit margins putting further pressure on farmers to employ efficient production techniques (Stewart, 2001).

The Atlantic salmon farms of Eastern North America are located off the State of Maine in the US and the Provinces of New Brunswick and Nova Scotia in Canada. Until 1978, attempts at Atlantic salmon aquaculture along the Eastern North American coast had been unsuccessful because of poorly chosen sites which had episodes of lethally cold waters of -0.7°C (Heen *et al.,* 1993). Location of farm sites within the Passamaquoddy

Bay and Grand Manan regions of the Bay of Fundy demonstrated a greater protection from the extreme cold temperatures allowing salmon to thrive during the winter season (Heen *et al.,* 1993). The New Brunswick salmon farms have developed into the major producer of Atlantic salmon in North America, with output six times that of Nova Scotia and Maine combined. In 2000 the New Brunswick industry produced 29,100 tonnes of product with an estimated wholesale value of 223 million Canadian dollars (Stewart, 2001). The farming industry has offered a welcome supply of employment in regions of Canada (and the US) with above average unemployment rates and below average income levels. The New Brunswick salmon industry employment totaled 1,849 jobs in 2000, with full time positions comprising 80% of the jobs, which has had a significant impact on areas of New Brunswick with the highest non-seasonal unemployment rates within the province (Stewart, 2001).

1.2. Salmon Production

The production cycle of Atlantic salmon consists of a minimum of 18 months in fresh water and another 15 months or more in seawater before the fish are at a market size of 3.5-4.5kg. There are four main steps of Atlantic salmon production; (i) Collection and fertilization of eggs, (ii) Incubation and hatching of eggs, (iii) Grow out to produce 75g – 150g smolts which are transferred to sea cages, and (iv) Grow out to harvest. The broodstock used by the New Brunswick salmon farmers are developed by the Atlantic salmon broodstock development program which aims to provide high performance salmon using modern breeding techniques to select for favorable traits such as enhanced

growth, delayed sexual maturation and disease resistance (The Huntsman Marine Science Centre, 2006).

The salmon production cycle tries to mimic the natural salmon life cycle, with the main difference being the egg and fry stages where the water is maintained at a higher temperature than it would in nature (10-14°C) (Kindness, 2002). The higher temperature increases the rate of development and therefore compresses the time required between egg and smolt. Fish transferred from the hatchery at 6, 12, 18, 24, and 30 months post egg fertilization are known as S0 or S½, S1, S1½, S2 smolts respectively. In New Brunswick S1 and S2 smolts are transferred to sea cages from mid April to June; ideally the transfer is done when the temperature of fresh water is close to seawater (Fitzgerald *et al.,* 2002).

The most commonly used cage or pen in New Brunswick are floating circular cages 22 meters in diameter and 12-15 meters deep. These cages can have up to 40,000 – 50,000 smolt at transfer and 15,000 – 18,000 salmon at market size. It is important for farmers not to overstock the cage (maximum 18kg/m^3) due to increased stress levels and thus reduced feed conversion ratios, and also because of the higher risk of disease outbreaks in these cages (The Huntsman Marine Science Centre, 2006).

1.3. Sea Lice

Salmonids of the genera *Salmo*, *Salvelinus* and *Oncorhynchus* are known hosts to seven genera of crustacean parasites, six copepods and one branchiuran (Pike and Wadsworth, 1999). Sea lice are marine ectoparasites belonging to the phylum Arthropoda, class Crustacea and order Copepoda. Two genera of sea lice, *Caligus* Müller 1785 and *Lepeophtheirus* Nordmann 1832, and in particular two species *C. elongatus*

Nordmann 1832 and *L. salmonis* Krøyer 1837 are ectoparasites of wild salmonids and currently represent the greatest on-going health concern to salmonid culture in the marine environment (Pike and Wadsworth, 1999; Johnson *et al.*, 2004). Indirect and direct losses due to sea lice infestation in salmonid aquaculture globally are estimated at being greater than $123 million Canadian annually (Johnson and Fast, 2004).

L. salmonis is fundamentally a parasite of salmonids and has been recorded on *Salmo salar* (Atlantic salmon), *Salmo trutta* (sea trout), *Oncorhynchus mykiss* (rainbow or steelhead trout), *O. gorbuscha* (pink salmon), *O. keta* (chum salmon) and several other salmonids. On the other hand, *C. elongatus* has a wide host range and has been documented on over 80 species of elasmobranch and teleost fish belonging to 43 families (Pike and Wadsworth, 1999; Johnson *et al.*, 2004). *L. salmonis* appears to be restricted to the Northern Hemisphere where it is said to be circumpolar in distribution, whereas *C. elongatus* is widely distributed in both the Northern and Southern hemispheres (Pike and Wadsworth, 1999; Johnson *et al.*, 2004).

Although *C. elongatus* is quite prevalent, it is *L. salmonis* that has had, and continues to have, considerable effects on salmon farms in many areas of the world due to their larger size and aggressive feeding habits. Sea lice infestation of *L. salmonis* became a problem on farmed salmon in Norway in the 1960's, followed by Scotland and Ireland in the late 1970's, and the west and east coasts of Canada in the mid to late 1980's (Pike, 1989). In the fall of 1994, *L. salmonis* occurred on vast numbers of farms in the Bay of Fundy region, resulting in approximately $20 million of losses to the aquaculture industry in 1995, as a result of decreased fish quality, mortalities, and treatment costs (Johnson and Albright, 1991; MacKinnon, 1997). The development of

integrated pest management strategies over the years has reduced the impact of sea lice considerably. However, limited therapeutic options and concerns regarding farmed-wild salmon interactions still make sea lice a major concern in aquaculture.

1.4. Sea Lice Life Cycle

The life cycles of *L. salmonis* and *C. elongatus* are very similar; the focus of this thesis will be on *L. salmonis* as this is the primary parasite causing the most significant impact on Atlantic salmon production.

The life cycle of *L. salmonis* consists of 10 different stages (plus the egg stage), with a molt between each one (Fig. 1.1) (Schram, 1994). Molting is controlled by the endocrine and neurosecretory systems (Pike and Wadsworth, 1999). The rate of development between each stage is highly influenced by environmental temperature, such that a higher temperature will decrease the time between each stage. The number of larval stages is the same for both *L. salmonis* and *C. elongatus* and consists of two nauplius, one copepodid and four chalimus stages.

The first stages of the *L. salmonis* life cycle, after hatching from the egg, are the Nauplius I & II stages. During this period, they are non-feeding and are therefore dependent on internal reserves for nutrients. They are free swimming and have very noticeable appendages (Johannessen, 1978). Growth through nauplius I of *L. salmonis* lasts ½ day – 2 days, and naupluis II lasts 1½ days – 7 days depending on water temperature (Pike and Wadsworth, 1999). The late nauplius II becomes much less active

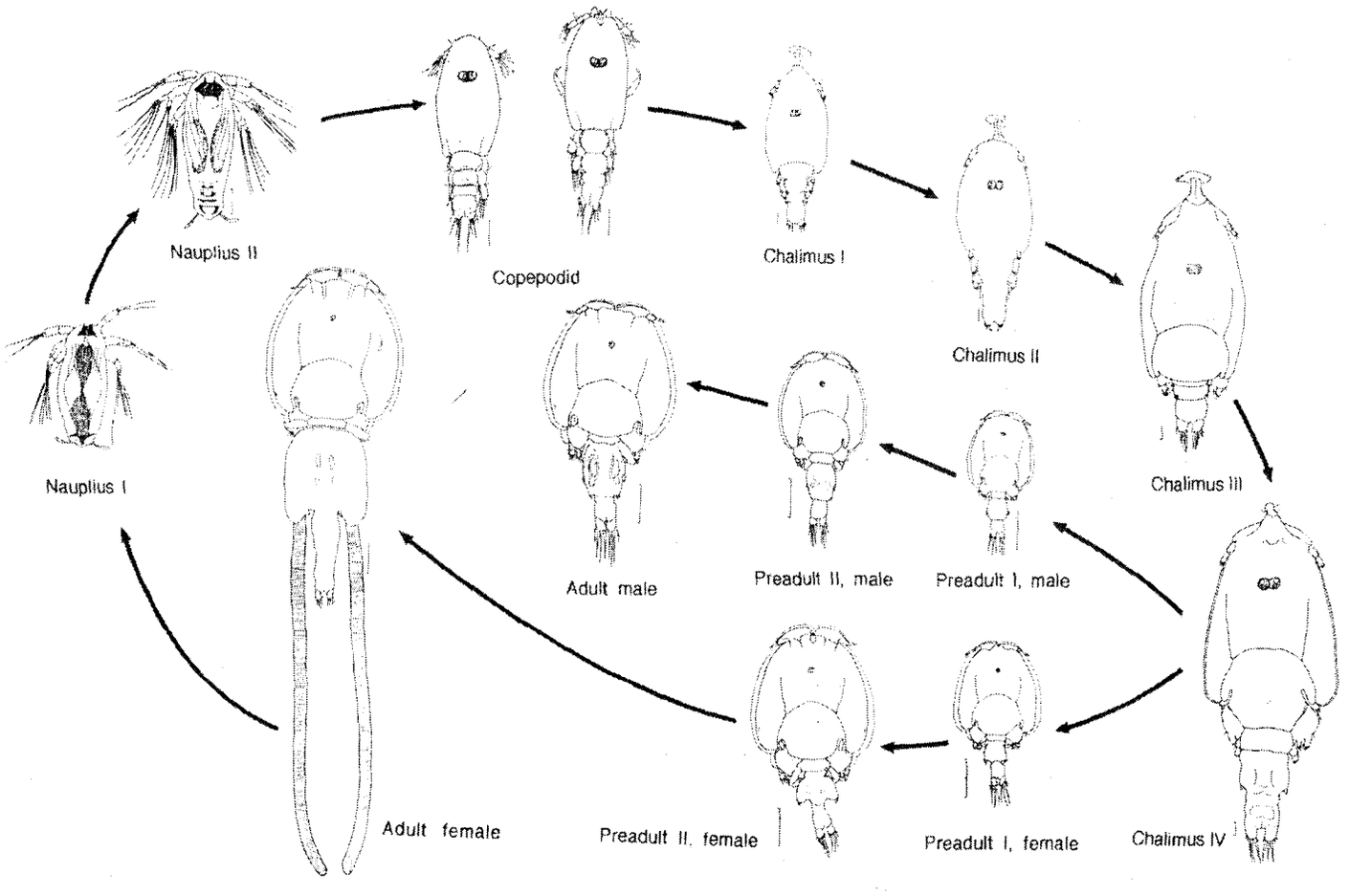


Figure 1.1. The life cycle of Lepeophtheirus salmonis consisting of 10 different stages (plus the egg stage) with arrows indicating a molt between each stage. (Schram, 1993)

and contains the clear outline of the copepodid within the body. This is the first of two major metamorphoses in the sea lice life cycle.

The molt to copepodid marks the end of the planktonic form to one that, although still planktonic, now possesses the capacity of infecting the fish host (Pike and Wadsworth, 1999). This is another free swimming stage, but the organism takes on a more rice-like shape, with less noticeable appendages. Survival of copepodid is dependent upon the energy reserves left within the yolk sac (Johannessen, 1978). No further development takes place until it settles on the host's skin; it is speculated that the stimulus to proceed with the on-host phase of the life cycle is provided by the host (Pike and Wadsworth, 1999). The copepodid initial attachment to the host is achieved with the prehensile antennae and the maxillipeds. After the copepodid has attached itself to the host's skin, it is able to feed due to the presence of an already functional mouth (Pike and Wadsworth, 1999). Without the ability of the copepodid to locate and attach to its host, the parasite would not be successful. The response of the copepodid to both light and pressure ensure that it is situated in the appropriate water column where host populations occur (Tully and Nolan, 2002).

After the copepodid stage come the first parasitic stages, called the chalimus I through to chalimus IV stages. This represents the second major metamorphosis in the life cycle of *L. salmonis* in which the planktonic copepodid transforms into a parasitic organism (Johnson and Albright, 1991). At this stage, the louse develops a structure called a frontal filament which allows the louse to attach to the fish. This frontal filament will anchor the larval stages to the host until the first pre-adult stage (Gonzalez-Alanis *et al.,* 2001). It is possible for larvae to be sexed at the chalimus IV stage based on

differences in shape and size (Schram, 1994). This stage is also the earliest at which *Lepeophtheirus* and *Caligus* species can be grossly distinguished (Johnson and Albright, 1991).

Adults of *C. elongatus* develop directly from chalimus IV, whereas those of *L. salmonis* develop through two preadult stages (Pike and Wadsworth, 1999). *L. salmonis* preadult and adult stages are not attached to the fish by a frontal filament, but are mobile and move freely over the surface of the host, and thus are often referred to as the "mobile" stages. Prior to molting, the pre-adults I and II form a temporary frontal filament that enable them to attach securely to the host (Schram, 1994). Pre-adult females are bigger than males in both the pre-adult I, (3.6mm x 1.9mm compared to 3.4mm x 1.6mm) and pre-adult II stages, (5.2mm x 3mm compared to 4.3mm x 2.2mm) (Schram, 1994).

The final molt for *L. salmonis* is that from pre-adult II to adult. Adult females are larger than their male counterparts (~11mm x 4mm compared to ~5mm x 3mm) (Schram, 1994). Both the pre-adults and adults can live off the host for a number of days and can successfully transfer between hosts (Tully and Nolan, 2002).

The rate of development, generation time, and reproductive output are all temperature dependent. At temperatures below 3°C the successful development of free-living stages is extremely low, and increasing temperatures lead to a greater number of lice per fish due to greater reproductive and development rates. The entire life cycle for *L. salmonis* is estimated at about 7-8 weeks at 10°C, and for *C. elongatus* it is about 6 weeks (Pike and Wadsworth, 1999).

1.5. Sea Lice Reproduction

The reproductive organs of both sexes are paired with the ovaries or testis situated in the cephalothorax (front body section) behind the level of the eyes. The development of adult male *L. salmonis* occurs earlier than adult females. The first adult males appear approximately one day before the adult females. Adult males generally mate with newly molted, virgin, adult females and will pair with these in preference to the more numerous preadult II females (Pike and Wadsworth, 1999). Once the male finds a suitable female, the male attaches to the pre-adult II female's genital segment where it remains until the final molt is completed (Ritchie, 2006). This behavior explains why the adult males develop ahead of the females.

Production of eggs occurs approximately nine days after copulation. Adult females that have egg strings are called gravid females (or just gravids). These paired egg strings can be more than twice the length of the female abdomen. The number of eggs contained in the egg strings varies greatly between individuals. Johannessen (1978) estimated a range of 100 – 500 eggs per egg string, and Wooten *et al.* (1982) estimated a figure of up to 350 eggs per egg string.

1.6. Sea Lice Feeding

Both *L. salmonis* and *C. elongatus* cause injury to the salmonid host, but it is *L. salmonis* that has a more severe impact. The degree of damage caused by *L. salmonis* differs depending on the parasitic stage. The newly attached copepodids will cause a local cellular response that can be seen with the naked eye as a small black spot caused by an accumulation of melanocytes (Pike and Wadsworth, 1999). Once the sea lice are attached

by means of the frontal filament the chalimus stages will cause further damage to the epidermis (Wooten *et al.,* 1982). The extent of damage caused by these two stages feeding on the mucus and epithelium is rather limited (Wooten *et al.,* 1982; Pike and Wadsworth, 1999). The mobile stages feed far more aggressively and are unrestricted in where they feed on the host. They will consume mucus, epithelium, subcutaneous tissue, and blood and thus are capable of causing significantly greater damage than the attached stages (Wooten *et al.,* 1982). It is not uncommon to see blood inside the gastrointestinal tract when collecting sea lice. The adult stages achieve close contact with the host in order to feed by the means of the paired thoracic appendages (Pike and Wadsworth, 1999). The body of the sea louse essentially becomes a kind of suction pad and the edge of the cephalothorax provides a good seal.

The feeding apparatus that is responsible for damaging the host skin is called the oral cone and first appears in the free-swimming copepodid stage. It is a muscular tube surrounding the foregut. The presence of a toothed ridge, called the strigil, which lies inside the mouth and across the entrance to the foregut give it the ability to disrupt the host's skin (Pike and Wadsworth, 1999). This organ is used to abrade the fish epidermis; the tissue so removed is conveyed to the foregut by another ridged plate, the mandible, lying further inside the mouth tube (Kabata, 1974).

1.7. Host Defensive Mechanisms

Infestation with either *L. salmonis* or *C. elongatus* only becomes life threatening when infestation levels are above the host's ability to compensate. The host-parasite interaction plays a vital role in the sea lice population. A successful host-parasite relationship is a balance between limiting the parasite through host defences and the

ability of the parasite to modulate, evade or resist the host's responses (Tully and Nolan, 2002).

Atlantic salmon have very limited cellular and humoral responses to *L. salmonis* infection (Johnson and Albright, 1991). There is variability by naïve Atlantic, Chinook, and Coho salmon in their cellular response to infection with *L. salmonis* (Johnson and Albright, 1992a, Fast *et al.,* 2002, 2003). Coho have been shown to be the most resistant to infection characterized by extensive epidermal hyperplasia and a well developed inflammatory response (Johnson and Albright, 1992a). As a consequence, chalimus stages are eliminated from the gills and markedly reduced in numbers on the fins within 10 days of infection. Atlantic salmon show a limited cellular response in which no reduction in chalimus numbers is seen. Chinook showed an intermediate response (Johnson and Albright, 1992a). In all species the main cell types involved in the cellular response to *L. salmonis* infestation were the neutrophil and macrophages, and lymphocyte presence was low (Johnson and Albright, 1992a). The difference in the rate of *L. salmonis* development on Atlantic and Chinook salmon could be due to a non-specific host response. Studies conducted using cortisol implants in Coho salmon resulted in the fish being unable to shed *L. salmonis* (Johnson and Albright, 1992b).

1.8. Physiological Consequences of Sea Lice Infestation

Osmoregulatory homeostasis may be greatly affected by the wearing away of the mucous and epidermal layers. This imbalance in plasma osmolarity and electrolyte concentrations can be regulated, short term, by physiological adaptation to counteract the

changes. This process is energetically demanding and stressful to the fish (Tully and Nolan, 2002). Osmoregulatory stress alone is not the sole cause of mortalities because the skin represents only 20% of the total surface area over which osmoregulation takes place, the other 80% being the gills. It is the loss of body fluids including blood, lymph, proteins and electrolytes as well as the osmoregulatory stress that all contribute to fish mortalities (Pike and Wadsworth, 1999). Fish compensate for this osmoregulatory imbalance with an increase in Na^+/K^+ ATPase activity that eliminates the excess sodium ions entering the fish through the disrupted epithelium (Wendelaar Bonga, 1997).

A heavy sea lice burden with the associated osmoregulatory issues all contribute to a number of stress responses in fish. The production of both catecholamines and cortisol in response to such stress has numerous effects on the fish. Increases in plasma glucose levels are the results of catecholamines and glucocorticoids on glycogenolysis and glucose release from the liver; this mobilizes energy resources to fuel the stress response (Wendelaar Bonga, 1997; Tully and Nolan, 2002). Catecholamines also have a negative impact on the hydromineral balance, causing the tight junctions of the gills to become more permeable, causing an increase in ion diffusion rate through the branchial epithelium (Karnaky, 1997; Tully and Nolan, 2002). The presence of cortisol has been implicated in mediating inhibitory effects on the immune system, thereby decreasing the ability to respond to other infectious diseases (Wendelaar Bonga, 1997). The level of cortisol present in the blood has been used as a primary stress parameter to evaluate stress in fish. Its presence in the blood is associated with reduced circulating lymphocyte levels and antibody production, lower mitogen induced proliferation of these cells and inhibition of phagocytotic activity (Wendelaar Bonga, 1997; Tully and Nolan, 2002).

The tissue damage associated with *L. salmonis* feeding on the epithelium, along with the associated physiological effects (i.e. immunosuppression) allow for opportunistic secondary infections with *Vibrio salmonis* and *Aeromonas salmonicida* (Pike and Wadsworth, 1999). It has been suggested that sea lice also have the potential to act as vectors for different diseases, including infectious salmon anemia virus (Nylund *et al.,* 1994).

1.9. Sea Lice Control

There are a variety of methods to prevent and treat sea lice outbreaks. These methods fall into three major categories; chemical, biological, and physical, but no single method can be used to permanently prevent sea lice. All fish farms reside in the marine environment, and thus sea lice are able to be reintroduced from wild hosts. Presently the use of good farm maintenance and husbandry help prevent sea lice outbreaks, assisted by the use of chemicals and drugs to treat salmon to control sea lice numbers on any given farm after an outbreak has occurred.

1.9.1 Physical Control

It is important that management plans be followed by all companies operating in the Bay for it to be successful. Effective prevention of sea lice infestation involves a coordinated Bay management that includes many of the following techniques: Single year classes, fallowing, site location, clean nets, lice monitoring, grilse removal, mortality removal, lower fish densities and introduction of healthy smolts. These techniques will now be discussed in greater detail.

A fallow period is a time when no fish are stocked on the farm. Copepods have up to eight days to find a host; thus leaving a site fallow will effectively break the life cycle of sea lice originating from the farm, allowing only for re-infestation from wild salmon populations. The fallow period should cover the life cycle of the parasite from the egg stage to the maximum time of survival of *copepodids*, as well as the maximum period that mobile stages can survive off the host (at least 30 days during winter) (Hogans, 1995). The refractory period before the lice numbers rise to a level requiring chemical intervention increases with longer fallow periods. The effectiveness of this method is dependent on the density of larvae dispersal in the area; it is assumed to be low if all sites in the bay fallow concurrently (Bron *et al.,* 1993).

The use of a single year class and an all-in all-out policy has also contributed to a significant reduction in sea lice infestation. Smolts that are introduced into a multiyear class site in April are quickly infested with lice and chemical intervention is required around June. If, however, fish are introduced into a single class site after the site has been fallowed there is a lower level of infestation with treatment not being required until the following winter period (Pike and Wadsworth, 1999).

In terms of sources of infection and water quality, location of the site is an important factor. Adequate water flow can help prevent build-up of sea lice larvae in a site and allow for the dispersal of chemotherapeutants after treatment. Ideally farms should be sited away from potential sources of infection, including locations where wild salmon are known to concentrate (e.g. salmon-spawning routes and adjacent farms) which may be infested with the parasite (Pike and Wadsworth, 1999).

Other routine practices are also important in the prevention of sea lice. The

monitoring of sea lice numbers should be performed regularly so that treatment decisions can be made based on species, stages, and numbers present. The removal of moribund fish should be conducted frequently because these fish will be more susceptible to sea lice infestation, and mortalities should be collected to check if sea lice were implicated in the cause of death. Grilse will no longer feed as they mature and should be removed as they can continue to harbour sea lice during in-feed treatment. Lastly it is good practice to keep the nets in good condition, cleaning and changing them regularly, allowing for a good flow of water. Sea lice infestation has been indicated to be lower where nets are kept clean (Health Canada, 2003).

Fish maintained at a depth of 0-4 meters have a far greater chance of being infected by sea lice (Hevrøy *et al.,* 2003). As feeding technologies continue to develop, it may be possible to maintain and feed the fish at greatest depths, subsequently reducing the potential lice burden.

1.9.2. Biological Control – Cleaner fish (Wrasse)

A means of controlling sea lice infestation that doesn't involve the use of chemotherapeutic agents is highly desirable. The use of wrasse, a cleaner fish, has been adopted in some fish farms in Scotland, Norway, and Ireland. There are four species of wrasse that are effective in clearing sea lice off salmon, these are; goldsinny (*Ctenolabrus rupestris*), rock cook (*Centrolbrus exoletus*), corkwing (*Crenilabrus melops*), and cuckoo (*Labrus mixtus*). It has been suggested that the rock cook wrasse are the most active cleaners of these fish (Borjordal, 1991).

Wrasse will generally only feed on the large adults and pre-adult II females and ignore the other stages. Various studies have indicated that stocking 1 wrasse to 25 – 150 salmon, can reduce the burden to one to eight lice through the first year compared with up to 40 lice per fish on unprotected and untreated fish (Treasurer, 1994, 2002)

There are unfortunately many problems associated with the use of wrasse. Due to the small size of these fish, many are able to escape from the net pens and, unless restocking occurs, most will escape within the first year the salmon are at sea. Observations also indicate that only a few wrasse conduct the majority of the feeding (1 in 30), while most of the population gain their nutritional requirements from the fouling on the cage nets (Pike and Wadsworth, 1999).

1.9.3. Biological Control – Vaccination

The immune system of fish has the capacity to respond to parasitic infections. Coho salmon demonstrate a strong non-specific response against *L. salmonis* infection, which is characterized by an acute inflammatory response and hyperplasia (Woo, 1992; Reilly and Mulcahy, 1993). The ability to vaccinate Atlantic salmon against sea lice would be strongly desired by fish farmers. Vaccination offers significant advantages over a chemotherapeutic approach, giving sustained action and having no residual drugs within the fish.

The development of vaccines against ectoparasites is at a very early stage. The only commercially available vaccine against any ectoparasite is against the tropical cattle tick (*Boophilus microplus*) (Opdebeeck *et al.,* 1988a). The principle of this strategy is to

immunize the host with essential internal parasite antigens (concealed antigens) not normally exposed during typical host-parasite interactions so as to induce a potent immune response to the hidden antigens (Opdebeeck *et al.,* 1988b, 1988c, 1994; Fast *et al.,* 2003; Kvamme *et al.,* 2004). Ingestion of host immunological components, such as antibody and complement, during a blood meal would result in immune-mediated damage to the parasite such that viability and fecundity are compromised. This approach has been investigated in the development of a vaccine against *L. salmonis* (Grayson *et al.,* 1995, Raynard *et al.,* 2002). These vaccines would be based on sea lice ingesting salmon antibodies that bind antigens in the intestinal tract, and thereby impair gut function to the point where louse nutrition, physiology, and survival are reduced.

However, there are problems inherent to vaccine development against sea lice. Sea lice are not known to require blood in their diet, and only a small proportion of a given population will have ingested blood. In comparison to the cattle tick, the amount of blood ingested relative to body size is rather low. These factors are not useful if you require sea lice to ingest significant amounts of antibody in the blood.

The use of vaccines can be complicated further due to the biology of sea lice. It is known that sea lice are able to produce various proteolytic enzymes to aid in digestion of proteins for nutrition (Fast *et al.,* 2003). The presence of proteases in the lumen could decrease the half-life of salmon immunoglobulins, meaning that sea lice will potentially need to ingest large volumes of blood. The pH and osmolarity of the sea louse intestine will also have a significant effect on antibody function, as it has been found that salmon immunoglobulin has a greatly reduced antigen binding at osmolarities above 550 mOsmol. It is predicted that the osmolarity of the sea louse intestine is very close to that

of seawater; if this were the case then binding of salmon antibodies would be greatly reduced (Grayson *et al.,* 1995; Raynard *et al.,* 2002).

There have been several studies using various antigens to immunize Atlantic salmon against sea lice infection. Immunization using purified gut antigens of *L. salmonis* was shown to reduce the number of ovigerous females significantly; there was however, no reduction in number of other mobile stages observed (Grayson *et al.,* 1995). Studies using other antigens such as trypsins may prove more effective in the future, and it is hoped that the successful development of a sea lice specific vaccine will eliminate the need to use chemotherapeutants to treat sea lice infestation (Fast *et al.,* 200; Kvamme *et al.,* 2004).

1.9.4. Chemical Treatment

Organophosphates, pyrethroids, H_2O_2, avermectins, and insect growth regulators (IGR's) represent the five major classes of antiparasitic agents used against sea lice infestation on Atlantic salmon farms. These groups can be further divided into topical (bath) and oral (in-feed) chemotherapeutants.

If chemotherapeutants are used to control sea lice it is important that there are no residues detectable in fish tissues when the fish goes to market. It is for this reason many compounds will have a set withdrawal period, theoretically the time that passes between the last dose given to the fish and the time when the level of residues in the tissues fall below the Maximum Residue Limit. However, in reality, political decisions dictate the withdrawal period and, subsequently, withdrawal times vary between compounds and

countries. Until the withdrawal period has elapsed, the fish cannot be harvested for human consumption (Grant, 2002; Grave *et al.,* 2004).

1.9.5. Topical Administration

All topical treatments involve the enclosure of large volumes of water prior to administration of the compound. Topical administration is advantageous in that all the sea lice in the net pen are, in principal, exposed to the same concentration of chemotherapeutant. The large variability in the enclosed volume of water can significantly affect the final concentration, which may result in reduced efficacy (Roth *et al.,* 1993). The actual volumes of water contained in the tarpaulins vary significantly and this exacerbates the small therapeutic margin of some drugs; for example, observed dichlorvos concentrations within treatment pens vary from 0.55mg/L to 3.5mg/L (Pike and Wadsworth, 1999). Some operators have resorted to the use of skirts rather than fully enclosed tarpaulins due to the large size of the fish pens. This may expose the sea lice to sublethal concentrations of the compounds and increase the risk of resistance. Topical administration is associated with high labor costs, vulnerability to environmental conditions, and stress to the fish.

1.9.5.1. Organophosphates

Organophosphate compounds act by inhibiting cholinesterases and subsequently interfere with neuromuscular transmission in the parasite and possibly the host (Burka *et al.,* 1997). They are effective against all mobile stages of sea lice, but are ineffective

against the chalimus stages. All stages appear to have acetylcholinesterase (AchE) and it is unclear why chalimus are unaffected. It is possible the ineffectiveness could be due to differing pharmacokinetics of organophosphates in the larval stage. Treatment with organophosphates should not begin until chalimus IV have molted through to pre-adult. Frequently repeated treatments are required to effectively clear the sea lice as they reach the susceptible stages. The organophosphates used to control sea lice infestation can be absorbed by the fish, as well as the sea lice, but are rapidly eliminated from the fish tissues 24 h post treatment, subsequently requiring relatively short withdrawal times. The withdrawal period for dichlorvos is 14 days in Norway but only 4 days in Scotland, and for azemethiphos is 7 days in Norway and only 2 days in Canada (Pike and Wadsworth, 1999; Grant, 2002). Both compounds have been reported to have little, if any, effect on non-target organisms and, thus, WHO determined that their proper use constituted neither a human health nor environmental hazard (WHO, 1989; Burridge, 2003).

The organophosphate, dichlorvos, was the first compound ever used against sea lice infestation. Dichlorvos is registered as Aquagard SLT® (Novartis) and is currently available for use in Norway and Scotland. It is effective against all mobile stages at a concentration of 1 mg L^{-1} for 1 hour between 5°C and 16°C. In healthy salmon never previously exposed to dichlorvos the therapeutic index is four times the recommended dose (4mg L^{-1}); this could decrease in fish having been exposed to repeated treatments (Wooten *et al.*, 1982; Rae, 1979).

Azamethiphos (Salmosan®) is a newer organophophate that is highly efficacious against all mobile stages at concentrations of 0.01-0.05mg L^{-1} (100 times less than dichlorvos) (Roth *et al.,* 1996; Grant, 2002). The therapeutic margin is ten times the

recommended dose, and this margin does not alter significantly in fish exposed to repeated treatments (Burka *et al.,* 1997). As well as a low toxicity to fish, the use of azamethiphos has a low environmental impact on other marine organisms including mussels, lobsters (and larvae), and crabs. Sea lice resistance development to azamethiphos has been reported in Norway and Scotland. There are numerous mechanisms that could be involved in organophosphate resistance, including decreased penetration, enzymatic detoxification and alteration of the target enzyme acetylcholinesterase. A study by Fallang *et al.* (2004) demonstrated that modified acetylcholinesterase was a mechanism involved in organophosphate resistance. The occurrence of a modified form of the acetylcholinesterase can be explained by either novel point mutations or post-transcriptional modifications which alters the kinetic parameters of acetylcholinesterase hydrolysis.

1.9.5.2. Pyrethrins and Pyrethroids

Pyrethrum is a petroleum extract of naturally occurring pyrethrins obtained from the flower heads of chrysanthemum and African daisies. The drug acts by binding to sodium channels and maintaining them in an open state (Narahashi, 1971). This causes neuronal depolarization and subsequent paralysis due to the inability to repolarize. Pyrethroids are synthetic analogues of pyrethrins and have similar pharmacological properties. Pyrethrins and pyrethroids are very safe for mammals due to the ~1000 times greater affinity for insect sodium channels. However, this safety margin is considerably smaller in fish and toxicity has been documented (Burka *et al.,* 1997; Grant, 2002). Only

cypermethrin is capable of killing both the mobile and all chalimus stages of sea lice; other pyrethroids do not significantly reduce the chalimus I and II stages.

These compounds are photosensitive and are hydrolysed in water giving them a short residency in the water and therefore reduced potential toxicity to other aquatic organisms. They do not accumulate in fish as they are readily metabolised when absorbed. Bioaccumulation will be greater in shellfish, which can be exposed through feeding on particulates or though direct uptake, due to slower rates of metabolism (Pike and Wadsworth, 1999).

Pyrethrum is not water soluble, yet has been used to control lice infestation in several countries including Canada, Norway, and Ireland. It was administered as an oil layer on the water surface of the enclosed pen and relied on the fish jumping through this layer for the lice to be exposed. Due to its highly variable efficacy this compound is now only in clinical use in Norway, where the withdrawal period is 7 days (Pike and Wadsworth, 1999), although the use of this compound is now outdated by the use of new synthetic pyrethroids.

Deltamethrin (Alphamax®) is a synthetic type II pyrethroid that can be used to control sea lice at a dose of 0.003mg L^{-1} for 40 min. The therapeutic margin is small, reported to be approximately 3.5 times the recommended dose, and varies with temperature (Burka *et al.,* 1997; Grant, 2002).

Cypermethrin (Exis®) is administered at a concentration of 0.005mg L^{-1} for 1 h. Toxic effects of cypermethrin in Atlantic salmon start to be seen at concentrations of 0.5mg L^{-1}, giving this drug a large therapeutic index (Burka *et al.,* 1997; Pike and Wadsworth, 1999; Grant, 2002). Many countries are currently using cypermethrin,

including Norway, UK, and Ireland. This compound is used in a rotation system with emamectin benzoate in Norway, where the first year fish receive emamectin benzoate and the second year fish receive cypermethrin (Roth, 2000; Horsberg, 2003; Grave *et al.,* 2004). Resistance to pyrethroid insecticides is caused by point mutations causing a conformational alteration in the pyrethroid target site (Fallang *et al.,* 2005). The rotation of two different compounds in Norway limits the potential for drug resistance.

1.9.5.3. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a strong oxidizing agent that has been used in aquaculture as a bacteriostat, bactericide, and fungicide, but can also be used to control chalimus and mobile stages of sea lice. The impact of hydrogen peroxide on sea lice is thought to be due to large amounts of oxygen accumulating in the haemolymph and intestine, causing mobile stages of sea lice to float to the surface (Grant, 2002). It is administered at a concentration of 1500mg L^{-1} for 20 min at 8 – 12°C (Thomassen, 1994; Burka *et al.,* 1997). Treatment results vary considerably, and there have been numerous cases of sea lice recovering post treatment, even at concentrations high enough to cause mortalities to Atlantic salmon.

The therapeutic margin of hydrogen peroxide is very narrow and temperature dependent. The therapeutic margin is five times at 6°C and close to zero at 14°C. Thus H_2O_2 is not recommended for use in seawater above 14°C. The environmental effects of hydrogen peroxide are extremely low because this agent rapidly breaks down into water and oxygen (Pike and Wadsworth, 1999).

1.9.6. Oral Administration

Compounds that are administered orally overcome many of the difficulties of topical treatments. These treatments offer longer lasting protection against sea lice infestation, are less labour intensive to administer, are easier to treat numerous pens at once, and treatment can be coordinated easily between adjacent sites. These compounds are classified as drugs by Health Canada and not pesticides, because they are fed to the fish rather than applied externally.

1.9.6.1. Benzoylureas

Benzoylureas are commonly referred to as insect growth regulators (IGR's) and act by inhibiting the incorporation of chitin into the exoskeleton, thereby preventing molting (Burka *et al.,* 1997; Pike and Wadsworth, 1999). These drugs have no toxic effect on vertebrates due to the absence of chitin, and so are safe for fish and fish farm personnel. These drugs are not useful against adult sea lice due to these lice having completed the molting stages of their life cycle and subsequently will not be killed by such treatment. These compounds will bind to marine sediments and remain in the environment for lengthy periods, which raises the concern of harming other marine crustaceans (Burridge, 2003).

Diflubenzuron (Dimilin® in the US and Lepsidon® in Norway), given at a dose of 75mg kg^{-1} body weight for a period of 14 days will produce a significant reduction in mobile and larval stages of sea lice (Burka *et al.,* 1997). This dose is high when compared to the avermectins. This compound is poorly absorbed in the salmon intestine, but despite this, concentrations of 4μg g^{-1} can be detected in the epidermal mucus layer

48 h post treatment. This compound is metabolized quickly by salmon with up to 37% being metabolized and excreted within 6 h of administration (Pike and Wadsworth, 1999). The withdrawal period for this drug is 60 days in Norway (Roth, 2000; Grave *et al.,* 2004).

Teflubenzuron (Ektobann® in Norway and Calicide® in the UK and Canada) is administered at a dose of 10mg kg^{-1} body weight for a period of 7 days, a dose lower than diflubenzuron but still significantly higher than the avermectins (Burka *et al.,* 1997; Grant, 2002). The metabolism of teflubenzuron is similar to deflubenzuron and has been given a withdrawal period of 60 days in Norway (Roth, 2000). This treatment has been very successful and shows a high level of efficacy against all molting stages of sea lice. Sea lice around the gills of salmonids appear to be reduced more significantly than those in other areas possibly due to greater exposure to the compound from the richer blood supply (Pike and Wadsworth, 1999). The efficacy of teflubenzuron is however limited to 3 wk post treatment (Campbell *et al.,* 2006a, 2006b). Exposure to teflubenzuron also has significant negative impacts on eggs string development causing either little to no development or large deformities and defects (Grontvedt, 1997).

Very little is known concerning the environmental fate of teflubenzuron. Due to its mode of action, this compound is a risk to sediment dwelling crustaceans. It is predicted that the marine sediment will act as a sink for this compound and an estimated half-life varies from 35 days to 6 months (Scottish Environmental Agency Fish Farm Advisory Group, 1999b; Burridge, 2003).

1.9.6.2. Avermectins

Avermectins are structurally similar compounds that belong to a family of macrocyclic lactones isolated as natural fermentation products from the gram positive bacteria *Streptomyces avermitilis* and represent a group of potent anthelmintic and insecticidal natural compounds with low toxicity in mammals. Avermectins exert their anthelminthic and insecticidal activities by irreversibly binding to, and possibly activating, both the gamma-aminobutyric acid (GABA) and glutamate gated chloride channels (GluCl's) (Schaeffer and Haines, 1989; Borgsteede *et al.,* 1996). GABA and GluCl channels belong to a gene superfamily of ligand-gated ion channels which share certain structural and functional characteristics. The genes encoding for all the subunits for the various members of the superfamily share common sequence homology. Structurally all subunits are composed of a large N-terminal extracellular domain and four membrane spanning domains (M1-M4) with a large intracellular loop containing sites for regulation (Smith and Olsen, 1995; Etter *et al.,* 1996). GABA receptors can be found in both vertebrate and invertebrates whereas GluCl channels are uniquely invertebrate and mediate fast inhibitory (hyperpolarizing) responses to glutamate (Bloomquist, 2003). Avermectins, therefore, result in an increased flow of chloride ions into nerve cells, with a consequent hyperpolarization and elimination of signal transduction, resulting in an inhibition of neurotransmission (Grant, 2000, 2002). The exposure to avermectins can cause paralysis and subsequent death to numerous nematode species (McKellar and Benchaoui, 1996). It is due to the novel mode of action that avermectins represent an important resource for sea lice control. GluClα and GluClβ are subunits of GluCl found in numerous invertebrates. *Caenorhabditis elegans* GluClα have

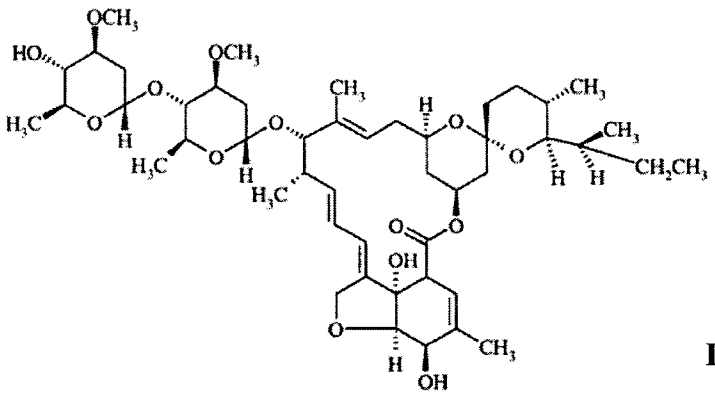
been expressed as homomeric channels in *Xenopus* oocytes, and were demonstrated to be highly sensitive to ivermectin. The GluClβ subunits contain a binding site for glutamate (Cully *et al.,* 1994; Laughton, 1995). The binding of glutamate to this β-subunit is reversible and thus allows for reversible chloride currents through the channel. Avermectin binding to the α-subunit is irreversible and thus chloride will continue to enter though this channel causing permanent hyperpolarization of nerve cells. Studies have also identified avermectin binding sites to be located on the α-subunits of GABA receptors (Stephenson, 1995; Laughton *et al.,* 1995, 1997; Boileau *et al.,* 1999; Dent *et al.,* 2000).

Avermectins pose a potential environmental risk, as their breakdown is dependent upon light and temperature, which can cause long residence time in sediments. Studies have shown that the half life of Emamectin benzoate under autumn conditions (10 h light/ 14 h dark) is 1.4 – 22.4 days (Scottish Environmental Protection Agency Fish Farm Advisory Group, 1999a). Using radiolabelled emamectin in marine sediment under either aerobic or anaerobic conditions show the relative estimated half life to be 193 and 427 days, respectively. Avermectins do not possess any anti-microbial or anti-fungal activity, and also have not been shown to have any adverse effect on marine macro-algae (Willis *et al.,* 2005). When looking at both emamectin benzoate and ivermectin it is clear that emamectin benzoate is less toxic to marine invertebrates by an order of magnitude than ivermectin (Scottish Environmental Protection Agency Fish Farm Advisory Group, 1999a). Although the environmental risk to the marine environment is considered to be low, emamectin benzoate does have the potential to be toxic to a number of marine invertebrates. It has been suggested that the sediment will act as the environmental sink

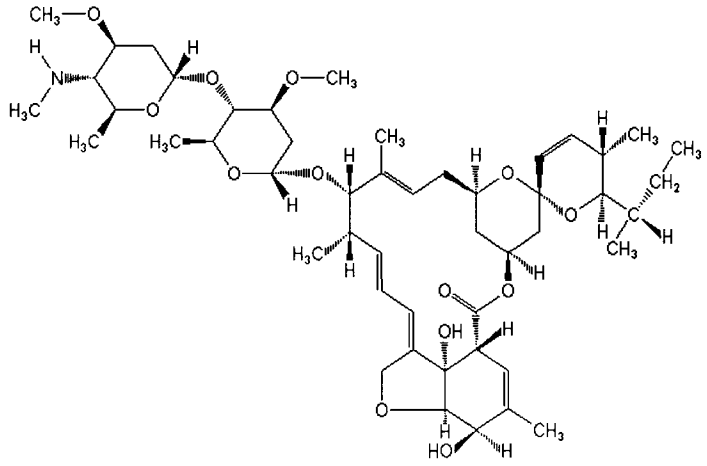
for avermectin compounds. There is no information available on the long-term impact emamectin benzoate use on fish farms will have on the surrounding benthic communities.

Ivermectin (structure shown in figure 1.2) has been widely used in the treatment of ectoparasites in companion animals, livestock and horses, and has also been used to control sea lice infestation on salmon farms prior to the introduction of emamectin benzoate. Ivermectin administered orally at a dose of 0.2mg kg^{-1} body weight four times a week for 2 weeks will cause significant reductions in all stages of sea lice (Roth *et al.,* 1993; Burka *et al.,* 1997). CNS depression and subsequent mortalities have been documented in salmon at only 2 times the therapeutic dose (Burka *et al.,* 1997; Pike and Wadsworth, 1999). Ivermectin is not easily excreted and is retained for lengthy periods in the liver, kidneys, muscle and skin. Following an oral treatment of 0.05mg kg^{-1} once a week for 9 weeks, it will take approximately 1000-degree days to be completely eliminated. This compound is no longer used for the control of sea lice due to the introduction of emamectin benzoate (SLICE®) (Westcott *et al.,* 2004).

SLICE® (containing the active ingredient emamectin, structure shown in figure 1.2) was developed by Schering Plough Animal Health and is currently the most effective chemotherapeutant administered against sea lice infestation and has replaced the use of ivermectin. SLICE® is composed of 0.2% emamectin benzoate, 0.01% butylated hydroxyanisole, 2.5% propylene glycol, 47.4% maltodextrin, and cornstarch (Bright *et al.,* 2005). SLICE® was originally developed for use against *L. salmonis* and *C. elongatus*, but has also been used very successfully against *C. teres* and *C. rogercressyi* in Chile (Schering Plough Animal Health, 2001). The therapeutic index is in excess of 3.5 times the therapeutic dose, larger than ivermectin.



Ivermectin



Emamectin

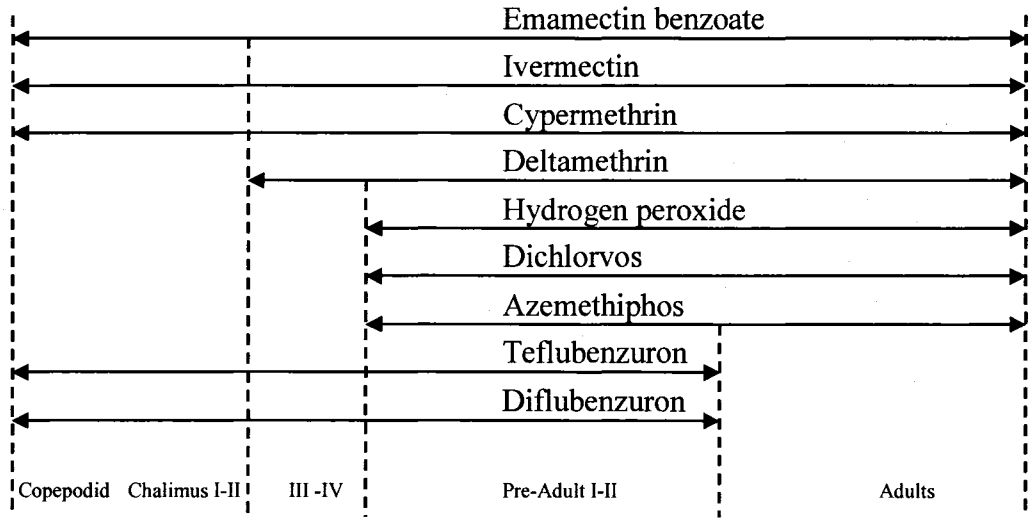
Figure 1.2. Chemical structures of ivermectin and emamectin.

Emamectin benzoate is safe for salmon when administered at a dose of 0.05mg kg^{-1} for a seven-day period, which is considerably lower than ivermectin (Ramstad *et al.,* 2002). At this dose level, emamectin benzoate is highly efficacious (99%) and will kill all parasitic stages of sea lice, i.e. chalimus, pre-adults and adults including gravid females (Stone *et al.,* 2000). Unfortunately there appears to be a continued reliance on emamectin benzoate for the control of sea lice infestation in Atlantic Canada, which raises concerns of potential resistance development (Westcott *et al.,* 2004).

When emamectin benzoate is administered to fish, it is readily absorbed in the gut due to its highly lipophilic nature, and distributed into the tissues of the fish, including the skin and mucus (Kim-Kang *et al.,* 2004; Sevatdal *et al.,* 2005). Elimination studies using Atlantic salmon estimate the half life of emamectin benzoate to be 10 days in plasma, 11.3 days in mucus, and 9.2 days in muscle (Sevatdal *et al.,* 2005). Administered at the recommended dose (50μg/kg/day for seven days) the maximum concentration of emamectin benzoate in the mucus, muscle and blood is 105, 68 and 128 ng/g (ppb) respectively. These levels are reached at day 7, the last day of treatment (Sevatdal *et al.,* 2005). Sea lice are exposed to emamectin benzoate when they feed upon the skin, mucus, blood, and muscle of the fish. The slow depletion of emamectin from the skin provides fish protection from sea lice for an extended time after the medication period (up to 9 weeks from the start of treatment). The withdrawal period for treated salmon in the European Union and Chile is zero, regardless of temperature. The withdrawal period in Norway is 175 degree-days, and 68 degree-days in Canada, with the maximum residue limit of 100ppb or 42ppb, respectively (Health Canada online: www.hc-sc.gc.ca/dhp-mps/vet/faq/faq_slice_e.html; accessed 21 Nov 2006).

1.9.7. Sea Lice Control – Summary

Each method mentioned has various advantages and disadvantages associated with its use. Ideally the control of sea lice infestation should be as much a preventative approach as it is a treatment measure, but even the best management practices will require treatment from time to time. A summary of the spectrum of action against the different stages of sea lice for each chemical treatment is shown below:



Both EMB and cypermethrin are the only compounds available to date that have low toxicity for salmonids and are also effective in the removal of all parasitic stages of sea lice. Emamectin benzoate has the advantage of giving the fish a longer lasting protection. Orally administered compounds are only effective against sea lice if the fish are feeding. Therefore the level of feeding may be significantly reduced in situations where the fish may be stressed (e.g. transfer to salt water). The use of topically administered compounds eliminates this problem but, again, have their own

disadvantages. Topical treatments are highly labor intensive and the final water volume in the tarpaulin is extremely difficult to estimate; this can causes either over or under dosing.

When treating fish, the use of only a single compound is extremely unfavorable. The ideal is to rotate treatment between different drug groups to reduce the risk of resistance development. Norway appears to be the pioneers of this approach, adopting a good drug rotation system between first and second year fish. Unfortunately, many other countries (including Canada) rely on emamectin benzoate as the sole method of sea lice control, which consequently increases the potential of resistance development to what is one of the most effective compound currently available.

1.10. Resistance to Chemotherapeutants

Control of sea lice infections has been largely through the use of chemotherapeutic drugs that are toxic to the parasite (Pike and Wadsworth, 1999; Raynard *et al.,* 2002). Prevention of infection using vaccinations would reduce the use of pesticides and drugs; however vaccines to date have been largely unsuccessful (Raynard *et al.,* 2002).

With the use of chemotherapeutants as the method for parasite control, resistance is a major concern. However, drug resistance is not necessarily the result of chemotherapeutic exposure. Cells and organisms can be intrinsically resistant (i.e. resistant before drug treatment) because of the lack of target receptors. For example flatworms lack GABAergic innervation and are therefore intrinsically resistant to

ivermectin (Eriksson *et al.,* 1995). Intrinsic resistance can also be due to the localization of tissue cells in an organism. For example, brain tumors can be resistant to chemotherapy due to the blood-brain barrier preventing the drug reaching the target cells (Stewart, 1994; Kantharidis *et al.,* 2000). Intrinsic drug resistance may also be connected with a genetic change that initiated the tumor. For example, chromosome translocation from chromosome 9 to 22 results in the occurrence of fused breakpoint cluster region/abelson (BCR/ABL) protein and causes chronic myeloid leukemia; this translocation can also abolish apoptosis and so cause multidrug resistance (MDR) (Brunel *et al.,* 1995).

Organisms and cells, when exposed to potentially harmful conditions have the ability to temporarily upregulate various cellular defense mechanisms (i.e. increase in either transport proteins or metabolic enzymes such as cytochrome P450 (CYP450)). These changes can lead to adaptive drug resistance (resistance in response to exposure to drug) (Devine *et al.,* 1999).

Acquired resistance to compounds can be defined as an increase in the quantity or dose rate of a chemotherapeutant required to elicit a given response due to a change in gene frequency in a population of the gene(s) that control susceptibility (Kantharidis *et al.,* 2000). Like all adaptive traits, resistance evolves through a process of natural selection. Genes conferring resistance probably arise repeatedly by mutation, but in the absence of selection will remain at low numbers (Prichard and Tait, 2001). With the onset of exposure to chemotherapeutants, individuals possessing such genes are favored and increase in frequency (Devonshire and Field, 1991; Devine *et al.,* 1999).

Over time organisms have developed various biological mechanisms giving them the ability to defend themselves against a variety of chemotherapeutants (Borgsteede *et al.,* 1996; Köhler, 2001). There is much scientific effort spent on elucidating the specific mechanism(s) underlying this resistance with the intention of improving the efficacy of existing drugs and of developing new drugs that can bypass the resistance mechanisms. The major biological mechanisms that provide a defense against chemotherapeutants include (i) changes in the binding site of a drug, (ii) detoxifying processes involving increased activity of specific enzymes and, (iii) increased drug efflux by membrane transporters. There are many examples where a mutation in a gene encoding a protein target for a chemotherapeutant reduces the subsequent affinity and thus reduces the toxicity to the target organism, making the organism resistant. Pyrethroid resistance can develop due to a point mutation in the gene encoding sodium channels. This point mutation reduces the affinity for pyrethroid binding and results in a resistant individual. Pyrethroid resistance due to target receptor mutations has been documented in numerous invertebrates including *Drosophila melanogaster*, *Cydria pomonella*, *Heliothis virescens*, and *L. salmonis* (Vassilev *et al.,* 1988; Taylor *et al.,* 1994; Martin *et al.,* 2000; Fallang *et al.,* 2005; Brun-Barale *et al.,* 2005). Another mechanism of resistance is the rapid metabolism and elimination of drugs before the target site is reached and any pharmacological effect is achieved. This can be achieved by the increased expression of certain CYP450 enzymes. These enzymes belong to a large family of proteins responsible for the metabolism of many compounds including various drugs. Resistance to pyrethroids has been documented in certain species due to an increase in the expression of CYP450 enzymes (e.g. *D. melanogaster*, *Anopheles gambiae*, and *Triatoma dimidiate*)

(Amenya *et al.,* 2005; Picollo *et al.,* 2005; Vontas *et al.,* 2005). Resistance can also arise due to the over-expression of transporter proteins that act to pump drugs out of cells thus limiting the amount of drug reaching its target site. The over-expression of these proteins has conferred drug resistant phenotypes in numerous cases from human cancer cells to insects and bacteria (Lage, 2003). Of the three major potential mechanisms involved in the development of drug resistance, it is possible that more than one could play a role in resistance development. It has, however, been suggested that among these various mechanisms an over-expression of drug transporters plays a key role in drug resistance development in numerous parasites (Sangster, 1994; Ambudkar *et al.,* 1999; Lage, 2003).

The spectrum of resistance that an organism possesses may be limited to a single drug class or can be extremely broad and may include cross-resistance to a variety of unrelated drugs that differ in their molecular structure as well as their mechanism of action (Köhler, 2001). This phenomenon, termed multiple drug resistance (MDR), has been described in many organisms from invertebrates to humans (Conder *et al.,* 1993; Bard, 2000; Lage, 2003). It is understood that the presence of plasma membrane-spanning multidrug transport proteins, specifically P-glycoproteins (P-gps), plays a significant role in resistance to a broad spectrum of drugs (Lage, 2003). Although some drug transporters are more or less specific to a certain class of drugs, the multidrug transporters mediate a unidirectional transport of a broad range of compounds across biological membranes. This association of drug resistance with P-gp has been described in vertebrates, helminths, protozoa, and bacteria. Various genetic studies indicate that the helminth *C. elegans* has at least fourteen P-gp homologs, with humans possessing two P-gp genes and rodents three genes (Bard, 2000). The presence of many P-gps in an

organism with a comparatively small genome may be a reflection of the needs of such organisms to protect themselves against the multitude of environmental toxins that it constantly encounters, without the benefit of the more sophisticated protective measures a mammal possesses (Bard, 2000).

1.11. Avermectin Resistance

Macrocyclic lactones have been used to treat various parasites of humans, pets, and livestock. Resistance to these compounds has become increasingly documented in numerous organisms, most commonly in nematodes (Echevarria *et al.,* 1992; Watson *et al.,* 1993; Vermunt *et al.,* 1995; Barnes and Hekimi, 1997; Gopal *et al.,* 1999; Anziani *et al.,* 2001; Kaplan, 2002). The resistance mechanisms involved for different species, particularly gastrointestinal nematodes of ruminants and horses, has been extensively investigated. Understanding these resistance mechanisms as well as the factors influencing the spread of resistance development, is desirable when implementing management programs.

As the various avermectins and milbemycins share the same mechanism of action, an organism resistant to one macrocyclic lactone compound is likely to be resistant to another. This co-resistance has been demonstrated in *H. contortus* using both ivermectin and moxidectin (Conder *et al.,* 1993; Shoop *et al.,* 1993). Other studies demonstrate cross-resistance to abamectin in pyrethroid resistant houseflies and diamondback moths. This cross-resistance is due to an increase in drug metabolism by elevated levels of cytochrome P450 monooxygenase as well as an increase in excretion (Clark *et al.,* 1995).

Genetic studies all indicate that avermectin resistance is polygenic, determined by autosomal factors but not sex-linked or caused by cytoplasmic factors. Studies involving the Colorado potato beetle suggest that resistance is incompletely recessive, whereas other studies investigating *H. contortus* suggest that resistance is a completely dominant trait which would therefore increase the risk of rapid resistance development in this species (Le Jambre *et al.,* 1999, 2000).

Potential resistance mechanisms involved against the toxicity of avermectins and milbemycins in parasitic organisms include, decreased penetration, increased excretion, increased oxidative metabolism, altered target site, and GST (glutathione S-transferase)-dependent conjugation (Clark *et al.,* 1995; Grant, 2000).

Studies using *C. elegans* to which 3 GluCl genes were knocked-out demonstrated a lack of sensitivity to ivermectin in these strains compared to wild type strains (Dent, 2000). Population studies have also found evidence for selection at GluCl and GABA receptor genes, with significant differences in allele frequencies detected between ivermectin selected and unselected genes in *H. contortus* and *C. oncophora* (Blackhall *et al.,* 1998, 2003; Njue and Prichard, 2004). Other studies, however, suggest that avermectin resistance does not involve target site mutation due to the lack of any consistent changes in ivermectin binding between resistant and susceptible strains of both *H. contortus* and *T. circumcincta* (Roher, 1990, Hejmadi *et al.,* 2000). This does not, however, hold true for *D. melanogaster* ivermectin resistant strains studied by Kane (2000). These *Drosophila* strains showed a much lower ivermectin affinity for the GluClα subunits from ivermectin resistant strains.

Metabolizing enzymes, specifically cytochrome P450's, have been identified in

invertebrate species. If the correct metabolizing system exists in the parasite for macrocyclic lactone breakdown, then alterations leading to enhanced metabolism could cause drug resistance.

Of all the potential resistance mechanisms, several studies indicate the most prevalent in terms of macrocyclic lactone resistance, is the over-expression of P-gps (Xu *et al.,* 1998; Molento and Prichard, 2001). Avermectins are excellent substrates for P-gp transporters (Pouliot *et al.,* 1997), whereas milbemycins are poor substrates (Griffin *et al.,* 2005). Upregulation of these proteins would serve to eliminate avermectins from the parasite limiting the accumulation of a toxic concentration at the target receptors. Ivermectin selected *H. contortus* have been demonstrated to have higher mRNA P-gp levels, suggesting an over-expression of this protein confers drug resistance in these nematodes (Xu *et al.,* 1998). Other studies have demonstrated the selection for specific alleles of P-gp in ivermectin selected *H. contortus* and *Onchorcerca volvulus* (Blackhall *et al.,* 1998; Sangster *et al.,* 1999b; Ardelli *et al.,* 2005; Eng and Prichard, 2005; Ardelli *et al.,* 2006). A recent study has also demonstrated that an alternative energy driven transporter was correlated with ivermectin resistance in O. *volvulus (*Ardelli *et al., 2006).* This transporter is not expressed as one large fused peptide but rather two peptides expressed separately; these proteins are known as "half transporters".

1.12. The ABC Transporter P-glycoprotein

Many of the proteins involved in the transport of substances across biological membranes are members of the ATP-binding cassette (ABC) superfamily. ABC

transporters have been identified in every organism examined so far, from bacteria to man. Most ABC transporters possess a structure that is evolutionarily well conserved. A typical ABC transporter consists of four units: two nucleotide binding domains (NBD's) and two transmembrane domains (TMD's) (Klein *et al.,* 1999). These four units can be expressed as individual polypeptides ("half transporters") or can be fused together ("full transporters") (Holland and Blight, 1999; Klein *et al.,* 1999; Váradi *et al.,* 2003). The mobilization of compounds by the various ABC transporters involves the interaction between the two NBD's and a strong coupling of the TMD's to the NBD's which ensures the transmission of the conformational changes caused by substrate binding and hydrolysis of ATP (Sharom 1997, 1999; Borges-Walmsley 2003). The NBD of the all ABC transporters contain several conserved regions; Walker A (GXXGXGK[S/T]), Walker B (XXXXD[E/D]), Signature (LSGGQ), glutamine loop and histidine loop (Qian *et al.,* 2006).

ABC proteins have been categorized into seven major subfamilies (A-G). The following is a brief description of each of these subfamilies identifying some of the major proteins within each. ABC-A have two members, ABC1 and ABCR; these are both "full transporters" which are involved in engulfment of apoptotic cells by macrophages and photoreception in the eye, respectively (Albrecht and Viturro, 2006). The subfamily ABC-B contains both full and half transporters. Importantly this family contains *MDR1* (or P-glycoprotein) the multi drug transporter (Sarkadi *et al.,* 2006). The ABC-C family contains the full transporter multi drug resistance associated protein (MRP1). This MRP1 protein is important in adaptive resistance but, as yet not, in acquired resistance, meaning increased levels can be induced, but not over-expressed, as is seen in inherited resistance

(Teodori *et al.,* 2006). The ABC-D family consists of the adrenoleukodystrophy (ALD) subfamily, mutation to these proteins, as the name suggests, are involved in the X-linked neurogenerative disorder ALD (Kemp and Wanders, 2006). Subfamilies ABC-E and ABC-F are not transporters. These proteins consist of two fused NBD with the absence of TMDs. These families are involved in RNase L inhibition and aminoacyl-tRNA binding, respectfully (Sauvage *et al.,* 2006). The last subfamily, ABC-G contains the clinically important breast cancer resistance protein which is responsible for anthracycline resistance without the upregulation of *MDR1* (Sarkadi *et al.,* 2006). Table 1.1 summarizes the ABC protein groups, their functions, and their topologies.

ABC proteins share a remarkable degree of homology within and between species. These protein have been grouped into subfamilies primarily based on their NBD amino acid sequence homologies, protein topology, with function having a smaller influence in some cases (Dassa, 2003). Although characterization appears to be influenced heavily by sequence homology, the use of sequence analysis is a poor guide for the prediction of protein function and numerous misassignments have been made based on this approach. Therefore, sequence homology to a particular ABC protein does not always imply its functional counterpart. In humans the protein encoded by *MDR3* is the closest homologue to the human multi-drug transporter P-gp encoded by *MDR1*, with

Table 1.1. Summary of the major ABC protein subfamilies, their functions and topology.

Subfamily	Name	Function	Topology
ABC-A	ABC1	Engulfment of apoptotic cells by macrophages.	(TMD-NBD) x2
	ABCR	Retinal transporters found exclusively in the retinal rod photoreceptors	(TMD-NBD) x2
ABC-B	MDR1	Drug resistance to a wide range of structurally unrelated hydrophobic toxic compounds	(TMD-NBD) x2
	MDR2	Bile salt export pump	(TMD-NBD) x2
	MDR3	Phospholipid transporter	(TMB-NBD) x2
	TAP1/2	Antigen presentation to T-lymphocytes	TMD-NBD
ABC-C	MRP1	Glutathione and glycoronate conjugate pump and resistance factor	TMD-(TMD-NBD) x2
	MRP2	Canalicular efflux pump for amphipathic anions and resistance factor for anticancer agents	TMD-(TMD-NBD) x2
	MRP3	Resistance to etoposide (anticancer drug)	TMD-(TMD-NBD) x2
	SUR	Promote insulin secretion	TMD-(TMD-NBD) x2
	CFTR	cAMP activated chloride channel - mutations cause Cystic fibrosis	TMD-NBD-R-TMD-NBD
ABC-D	ALD	Import and anchor the required enzymes for very long chain fatty acid activation	TMD-NBD
ABC-E	OABP	RNase L inhibitor	NBD-NBD
ABC-F	GCN20	Protein kinase that phosphorylates the alpha-subunit of translation initiation factor 2	NBD-NBD
ABC-G	White family	Transport of eye pigment precursors (interestingly was the first gene ever mapped)	TMD-(TMD-NBD) x2
	ABC8	Possible role in tryptophan and guanine uptake	TMD-(TMD-NBD) x2
	BCRP	Responsible for anthracycline resistance	TMD-(TMD-NBD) x2

Transmembrane domain (TMD), Nucleotide binding domain (NBD), Regulator region (R)

approximately 80% identity. The protein encoded by *MDR3* is not, however, involved in multi-drug transport. Instead this protein functions as a phosphatidylcholine transporter (van Helvoort *et al.,* 1996). Making assumptions based on homology between species is is also prone to problems. The bacterial protein LmrA shows only 40% homology to human *MDR1*, but, after expression in human lung fibroblast cells, was shown to be targeted to the plasma membrane and conferred typical multidrug resistance in these human cells. The pharmacological characteristics of LmrA were very similar to human P-gp and the affinities of both proteins for vinblastine and magnesium-ATP were indistinguishable (van Veen *et al.,* 1998).

P-gps are 110 – 190 kDa in size belonging to the ABC-B subfamily, and have all four domains fused into one single polypeptide (Váradi *et al.,* 2003). Figure 1.2. shows a cartoon representation of the structure of a P-gp within a cell membrane. P-gps function as an ATP-dependent transporter for a wide number of structurally diverse compounds including anticancer drugs (anthracyclines, taxanes, etc), steroid hormones (cortisol and dexamethasone), cardiac drugs (digoxin and diltiazem), immunosuppressants (cyclosporin and tacrolimus), antimicrobial agents (tetracycline and erythromycin) and other miscellaneous agents including the macrocyclic lactone avermectins. Exactly how P-gps are able to transport such a diverse array of compounds is not understood, which makes it difficult predicting substrates for this transporter (Holland and Blight, 1999; Klein *et al.,* 1999; Lage, 2003; Ouellette and Léharé, 2003).

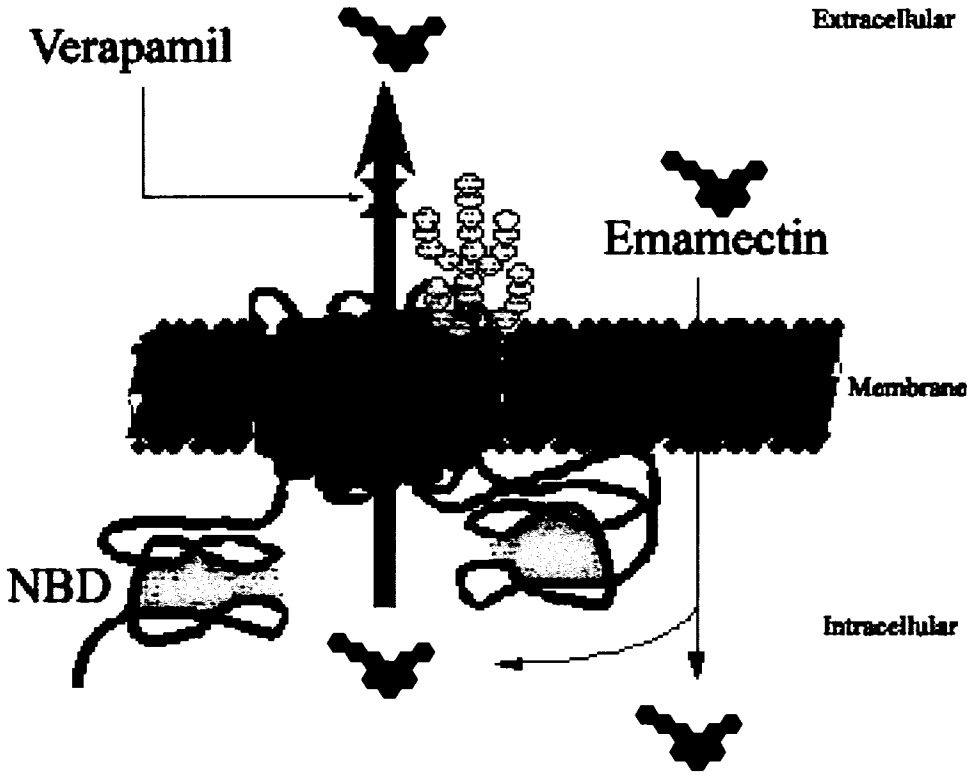


Figure 1.3. A cartoon representation of a single P-glycoprotein (P-gp). These proteins have two intracellular nucleotide binding domains (NBD) and two transmembrane domains (TMD) each containing 6 membrane spanning helicies. Avermectins are good substrates for P-pg and can be actively transported out of the cell. The action of P-gp can be competitively inhibited by verapamil.

It is clear, however, that many of the substrates transported by P-gps are either naturally occurring compounds or synthetic derivatives of natural compounds.

P-gps are expressed constitutively at high levels in many tissues including the apical border of the intestinal epithelial cells, blood brain barrier, bile canaliculus, and renal tubule. The use of *mdr1* knock-out CF-1 mice has demonstrated that P-gps are not essential for the basic physiological functioning of an organism, but their absence in regions such as the blood brain barrier and colon epithelium affects drug pharmacokinetics and biodistribution, resulting in increased toxicity to various chemotherapeutic agents (Schinkel, 1997, 1998).

1.13. Pharmacokinetics and P-glycoproteins

Due to the wide variety of compounds recognized by P-gps, this protein plays a major role in the absorption, distribution, metabolism, and excretion of many drugs.

P-gps are expressed on enterocytes and can therefore alter oral absorption of substrate drugs. Oral absorption can be increased by the addition of P-gp inhibitors (e.g. verapamil or vinblastine) which will result in increased drug plasma concentrations. Vinblastine acts as a non-competitive inhibitor binding to a specific modulating site on P-gps which inhibits ATPase activity. Verapamil is a competitive inhibitor and thus will compete with other compounds for transport by P-gp (Garrigos *et al.,* 1997). This classical P-gp modulator has been shown to reduce resistance to both benzimidazole or avermectin resistance in resistance nematode populations (Kerboeuf *et al.,* 2003). The use of P-gp blockers to counter resistance in parasites has to date been unsuccessful due to

the systemic side effects in the host (von Samson-Himmelstjerna and Blackhall, 2005).

Compounds that are absorbed from the intestinal tract are restricted in their distribution by physiological barriers. In mammals, P-gp expressed on brain capillary endothelial cells is an important component of the blood-brain barrier and is responsible for limiting the entry of many compounds, including avermectins. There is a subpopulation of collies and other herding breeds that are extremely sensitive to neurotoxicity induced by ivermectin. This increased sensitivity is due to a deletion mutation, which produces a frame shift that generates a premature stop codon in the *mdr1* gene resulting in non-functional proteins (Mealey *et al.,* 2001). Therefore P-gp will no longer be able to limit the concentrations of various compounds in undesired areas such, as the CNS, causing an increased sensitivity to various compounds previously exported by these proteins.

P-gps do not possess any intrinsic metabolic activity, but are important elements of intestinal drug metabolism. P-gps and major phase I drug metabolizing enzymes (such as CYP3A in mammals) are expressed at high levels in the villi tips of the enterocytes in the gastrointestinal tract, the primary site of absorption for orally administered drugs. Once inside the enterocyte there are three possible outcomes: (i) the drug may enter the portal circulation (ii) the drug may be metabolized or (iii) the drug may be extruded by P-gps back into the lumen. This means that drugs that are not substrates of P-gps will only enter the enterocytes once, while substrates for P-gps can be continually cycled between the enterocyte and the lumen.

Drugs are eliminated from an organism either unchanged or as metabolites. Renal and biliary excretion are the two most important pathways of drug elimination in

mammals and P-gp expression on the surface of both renal tubule eplithelium and hepatic canaliculi suggest that they may play a role in drug excretion. It is fair to assume that P-gps could also play a role in the excretion of compounds in invertebrates, but the extent of this role is largely unknown.

1.14. P-glycoprotein and avermectins in mammals

A number of macrocyclic lactones (avermectins and milbemycins) are used in human and veterinary medicine for the control of both endo- and ectoparasites. Ivermectin is the most commonly used of the avermectin compounds, used mainly in the treatment of onchocerciasis in humans, as well as for strongyloidiasis, ascariasis, trichuriasis and enterobiasis.

In mammals, avermetins are moderately well absorbed following oral administration with the highest tissue concentrations occurring in the liver and the fat. Very little ivermectin is metabolized by mammals (rats, cattle, and sheep); approximately 90% of an administered dose is excreted in the feces, with less than 2% appearing in the urine (Chiu *et al.,* 1990). This lack of metabolism demonstrates the importance of P-gp in limiting penetration of avermectin compounds.

With the exception of the studies on collies (Mealey, 2003), the majority of the mammalian work on avermectin toxicity has been conducted on CF-1 mice (Lankas *et al.,* 1997; Kwei *et al.,* 1999). These mice have a 100X higher sensitivity to abamectin and ivermectin due to deficient P-gp in the intestine and blood brain barrier. Avermectins irreversibly bind to and open invertebrate GluCl and GABA receptors suppressing both neural and neuromuscular transmission. In contrast mammals only have GABA receptors

located in the CNS that are protected by the blood-brain barrier and are thus unaffected by avermectins. Some penetration of the blood-brain barrier does occur at relatively high doses, but at recommended doses, the presence of P-gp will not allow for avermectins to enter the CNS of mammals.

1.15. Regulation of P-glycoproteins

Over the past two decades much work has been directed at understanding P-gp expression and regulation in vertebrate tissues. Avermectin induced P-gp expression and the specific mechanisms of induction have not been elucidated; however, studies with other xenobiotics can give an indication into how this induction might occur. This review is based on these works, with the understanding that the following mechanisms may or may not exist in lower eukaryotic organisms such as crustaceans.

P-glycoprotein expression is controlled by a highly complex interplay of a variety of factors that are involved in multiple regulatory pathways. The response to potentially toxic compound is often not a single response but more a coordinated expression of both drug transporter proteins (P-gp) and detoxification proteins (such as cytochrome P450's) (Schuetz and Strom, 2001). When an organism is exposed to increased levels of certain toxicants, the up-regulation of both transport proteins and metabolizing enzymes appears to strengthen the barrier function especially in tissues such as the liver and intestine.

P-gp conferring multi-drug resistance is encoded by one gene in humans (*MDR1*) and two genes in rodents (*mdr1* and *mdr3*). The rodent genes are expressed in a tissue specific manner with *mdr1* expression primarily in the adrenal cortex, kidney, liver, and uterus. *mdr3* expression is seen in the intestine, heart, liver, lung, and brain capillaries.

The human *MDR1* belongs to a group of protein-encoding genes that lack a consensus TATA box within the proximal promoter region. Instead, basal transcription is directed by an initiator sequence that encompasses the transcription start site (+1) and most likely acts as the position of nucleation of the RNA Pol II preinitiation complex. Like other "TATA-less" genes, the *MDR1* promoter includes both an inverted CCAAT element or Y-box (-82 to -73) and a GC-rich element (-56 to -43) that have been shown to interact with members of the Sp family of transcription factors (Sukhai and Piquette-Miller, 2000). Transfection analyses of promoter constructs mutated in one or both of these elements indicate the involvement of the two elements in the constitutive (i.e. operative under normal growth conditions) expression of *MDR1* in some cell lines. Moreover, these elements and their cognate binding factors are also involved in the activation of *MDR1* transcription by a variety of inducers (Sukhai and Piquette-Miller, 2000; Scotto and Johnson, 2001).

A 130-kDa *MDR1* promoter–enhancing factor 1 (MEF1) has been shown to interact with an *MDR1* promoter element to mediate upregulation. Interestingly it appears that the same promoter element binds an inhibitory complex for transcription factors NF-kB and c-Fos. The mechanism by which MEF1 regulates *MDR1* transcription, the interplay between MEF1 and NF-kB/c-Fos, and the frequency with which these complexes are involved in *MDR1* activation remain to be determined (Kuwano *et al.*, 2004).

In addition to cell- and tissue-specific expression, there are a number of factors that can influence the induction of P-gp expression. In mammals *MDR1* induction has been observed in response to xenobiotics (regardless if the drug is a P-gp substrate or

not), cellular damage, DNA damage, heat shock, pollutants (e.g. PAH's), protein kinase C agonists (PKC), and the inflammatory cytokine tumor necrosis factor α (TNF-α) (Sukhai and Piquette-Miller, 2000; Scotto and Johnson, 2001). There are numerous possible mechanisms that can serve to increase the expression of P-gps. Increase in expression of P-gp may occur via an increase in transcription of P-gp mRNA. Post-transcriptional control such as increased mRNA stability, phosphorylation state, and plasma membrane incorporation are also important in the expression of P-gp. Of all the mechanisms, alterations in P-gp expression that occur at the level of mRNA are the most frequently observed, although the transcriptional events that underlie these responses are not completely understood.

Heat shock proteins are expressed in response to stressful environmental stimuli such as heat. These proteins aid in the stabilization and repair of cellular damage. It is likely that transport proteins such as P-gp, which are involved in removal of toxic metabolites and by-products, play an active role in this protection mechanism. It has been shown that basal activity of the P-gp is controlled by heat shock elements which requires the binding of heat shock transcription factor (Vilaboa *et al.,* 2000; Scotto and Johnson, 2001).

Polycyclic aromatic hydrocarbon (PAH) compounds have been demonstrated to induce *mdr1* in mice liver cells (Mathieu *et al.,* 2001). Certain PAH's such as 3-methylcholanthrene (3-MC) have been shown to bind to aromatic hydrocarbon receptors (AhR) in the cytoplasm. This ligand bound receptor then translocates to the nucleus, where it forms a heterodimer complex with a AhR nuclear translocator (Arnt) which bind specific regulatory DNA sequences located in the promoter regions of target response

elements, including p53, which subsequently enhances the transcription of both *mdr1* and CYP3A (Mathieu *et al.,* 2001).

Other studies have demonstrated pregnane X receptors (PXR, also known as steroid xenobiotic receptors (SXR)) to be a dominant regulator of numerous genes, in particular those involved in drug transport and metabolism (Bertilsson *et al.,* 1998; Geick *et al.,* 2001). PXR binds as a heterodimer to retinoid X receptor to specific DNA sequences, PXR-response elements, including the upstream regions of CYP450 3A gene family and *MDR1* (Masuyama *et al.*, 2005). PXR regulates its target gene transcription in a ligand-and promoter-selective manner (Matheny *et al.*, 2004; Chen *et al.*, 2005; Masuyama *et al.*, 2005). Signals from any of the inducers of *MDR1* (i.e. xenobiotics, UV light, etc) converge on a region of the *MDR1* promoter known as the *MDR1* enhanceosome. This region includes binding sites for the trimeric transcription factor, Nuclear factor Y (NF-Y), and the specific protein (Sp) family of GC-binding transcription factors. Together, these DNA-binding proteins recruit the histone acetyltransferase p300/coactivator associated arginine methyltransferase associated factor (PCAF) to the *MDR1* promoter, resulting in the acetylation of promoter-proximal histones and subsequent transcriptional activation that is likely mediated by further chromatin remodeling (Scotto and Johnson, 2001). Although the mechanism by which each agent produces the signal that results in promoter activation has not been determined, the role of the *MDR1* enhanceosome in the regulation of transcription by a variety of stimuli makes it an attractive target for therapeutic intervention.

c-Jun NH_2-terminal protein kinase (JNK) is involved in the mitogen activating protein kinase cascade. JNK is activated in response to many stressful stimuli and its

expression has been correlated with an increase in *MDR1* expression. JNK is known to phosphorylate and activate c-jun, which is half of the heterodimeric activator protein 1 (AP-1) transcription factor. AP-1 binding sites have been reported on the promoter regions of *MDR1* genes, and a positive correlation between AP-1 activation and *MDR1* expression has been reported (Sukhai and Piquette-Miller, 2000).

It has also been observed that a region of the *MDR1* promoter in the multidrug-resistant KB/VJ300 (over-expresses P-gp at the level of transcription) and KB-C1 (over-expresses P-gp due to gene amplification) cell lines was shown to be hypomethylated relative to the promoter in parental KB3-1 cells (Kusaba *et al.,* 1999); the mechanism underlying this intriguing correlation between methylation status and *MDR1* transcript levels is not yet understood.

An acute inflammatory response is associated with the release of pro-inflammatory cytokines, such as interleukin (IL) 1β and IL-6, and to a lesser extent TNF-α. These factors have been shown to decrease or suppress P-gp expression and activity *in vitro*. Investigations have indicated that TNF-α and IL-6 influence P-gp expression by either reducing mRNA transcription or decreasing mRNA stability, whereas IL-1β mediates effects of P-glycoprotien via post-transcriptional mechanisms (Sukhai and Piquette-Miller, 2000; Scotto and Johnson, 2001; Sukhai *et al.,* 2001).

P-gp expression may also be regulated by PKC mediated phosphorylation (Castro *et al.,* 1999). An increased phosphylation state is associated with increased P-glycoprotein function. *In vitro*, P-gp is phosphorylated by PKC at serine residues, primarily in the linker region of the protein. The use of specific PKC inhibitors (e.g.

staurosporine) have been shown to inhibit P-gp activity in the marine mussel M*ytilus galloprovincialis* (Smital and Kurelec, 1998).

Studies have shown an interesting correlation between *MDR1* and the cystic fibrosis transmembrane conductance regulator (CFTR) expression. In rodents, *mdr1* and CFTR show complementary patterns of tissue expression *in vitro*. Studies of CFTR knockout transgenic mice show a fourfold decrease of intestinal CFTR is accompanied by a fourfold increase in *mdr1* expression compared to wild type mice (Trezise *et al.,* 1997). This may suggest that CFTR and P-gps may serve analogous roles in epithelial cells, with suggestions that P-gps are capable of transporting chloride.

There is a clear lack of knowledge concerning the mechanism(s) by which avermectins might induce *MDR1*. The available knowledge suggests the role of various nuclear factors, including PXR, although it still remains to be seen if invertebrate species share these induction mechanisms.

1.16. Over-expression of P-glycoprotein

It is evident that P-gps are over-expressed in resistant phenotypes, and transfection of susceptible cells with P-gp genes can confer resistance (Fromm, 2000). Over-expression is a term used when a protein has increased dramatically in the basal level of expression compared to that in an identical cell or organ. The potential mechanisms that have been proposed to lead to over-expression of the P-gp gene include changes at the level of the gene by amplification, duplication or mutation, or in the stability of mRNA (Stein, 1997).

Gene amplification is a result of a fault in DNA replication. Instead of making a single copy of a region of a chromosome, many copies are produced. This leads to the production of many copies of the genes that are located on that region of the chromosome (Devonshire and Field, 1991). Sometimes, so many copies of the amplified region are produced that they can actually form their own small pseudo-chromosomes called double-minute chromosomes. Gene amplification, with the consequence of multiple copies of a *MDR1* located in extrachromosomal elements or within expanded chromosomal regions, is seen in some mefloquine resistant strains of *Plasmodium falciparum* (Cowman *et al.,* 1994).

Gene duplication occurs via replication errors, whereby a gene or group of genes may be copied more than one time within a chromosome. This is different from gene amplification in that the genes are not replicated outside the chromosome and they are only copied one extra time, not hundreds or thousands of times. It has been speculated that the various isoforms of P-gp arose from gene duplication and it is possible that this mechanism could also play a role in the over-expression of P-gp (William *et al.,* 1989).

Allelic differences in individual *MDR1* gene sequences may be associated with or even causative for differential expression levels. There are a number of regions on the genomic DNA where sequence alterations could influence gene expression. These regions include the promoter and/or enhancer, sequences that influence the efficacy of processing the pre-mRNA. Changes that occur within the *MDR1* gene, such as single nucleotide polymorphisms that are either 'silent' (resulting in no change in amino acid sequence) or result in alterations of single amino acids, have been shown to alter the drug resistance phenotype in various organisms (Devine *et al.,* 1992). Many of the observed

changes are located in or just upstream of the transmembrane regions and possibly confer substrate specificity.

Changes in the levels of transcription due to the use of a different promoter can also be responsible for achieving an increased P-gp expression (Riordan *et al.,* 1985; Ekong *et al.,* 1993; Sangster *et al.,* 1999). The promoter regions responsible for over-expression have proved extremely difficult to identify unequivocally. There is an alternative region 70 to 85 nucleotide bases upstream from the start site that may be involved and xenobiotics may bind to promoters of the P-gp genes (Sukhai and Piquette-Miller, 2000; Scotto, 2003).

It is also possible that mutations in the repressor sequences of the *MDR1* gene could play a role in the over-expression of P-gp. Mutations in the NF-KB/c-Fos binding regions, or in the genes that encode for or regulate these suppressor proteins could be responsible for the observed over-expression in resistant phenotypes.

Although there are numerous mechanisms by which P-gp can be over-expressed the end result will result in the ability to transport more substrate out of the cell. For numerous parasites this increased expression can result in resistance development to various drugs including avermectins.

1.17. Research Goals

Finding solutions to the spread of resistance requires both knowledge of the drug's mode of action and the mechanisms of resistance. This information can then be applied to both detect and monitor the state of resistance. It is therefore important to gain a more comprehensive understanding of the mechanisms resulting in avermectin

resistance in sea lice. This would allow for the development of new (or use of different) therapeutic agents that will successfully by-pass such resistance mechanisms. The early detection of resistance would also be a useful tool so that alternative drug groups could be used, thereby decreasing the selection pressure for resistance to emamectin benzoate.

The objectives of this research were to examine the presence of both the multiple drug transporters (P-gp) and the avermectin target receptor GABAα and GluClα subunits in sea lice and determine if P-gp expression is related to avermectin tolerance.

The hypothesis of this study was that sea lice express multidrug transporter P-gps as well as avermectin target GABA and GluCl receptors. Further to this it is hypothesized that P-gps are localized to the gastrointestinal tract. It is also hypothesized that sea lice surviving an exposure to EMB will have higher mRNA levels than control lice (no EMB exposure).

To test the hypotheses this project had three specific main objectives:

(i) a. Isolation of the gene(s) encoding for P-glycoprotein in sea lice and detection of the putative protein using Western blot analysis

b. Identify the genes encoding GABA- and glutamate-gated chloride channel alpha receptor subunits

(ii) Localize P-gp in sea lice tissue sections

(iii) Determine if P-gp mRNA levels in sea lice are altered with exposure to emamectin benzoate

1.18. References

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CHAPTER 2. CLONING OF PUTATIVE P-GLYCOPROTEIN GENES IN SEA LICE (*LEPEOPHTHEIRUS SALMONIS*) AND DETECTION USING WESTERN BLOT ANALYSIS

2.1. Abstract

Sea lice (*Lepeophtheirus salmonis*) are ectoparasites of Atlantic salmon that if left uncontrolled can have a significant impact on the health of farmed Atlantic salmon. The continual reliance on emamectin benzoate (EMB) against sea lice makes resistance development a major concern. Avermectin resistance has been documented in many organisms including numerous nematode species, and of the mechanisms responsible for such resistance, over-expression of P-glycoprotein (P-gp) has been suggested to be the most influential. This study reports the identification of putative P-gp in sea lice *L. salmonis* using molecular and immunological methods. The use of degenerate primers in RT-PCR and the screening of a sea lice EST database identified two partial genes of interest. The SL-Pgp1 gene showed a higher level of similarity to other eukaryotic P-gp than the SL0525 gene. The SL0525 gene was expressed as a GST-fusion protein and used to inoculate a rabbit for the production of polyclonal antibodies against this sea lice transporter. Western blot analysis using this antibody and P-gp specific commercially available monoclonal antibodies C219 and JSB-1, detected a 160kDa protein in sea lice membrane protein fractions indicating that the SL0525 clone is part of a "full transporter" containing two transmembrane domains and two nucleotide binding domains found in sea lice. The identification of these two genes and the detection of P-gp using Western blot offer the first evidence for the presence of multi-drug transporters in sea lice. The gene sequences and production of the specific antibody provide useful tools for further research to investigate the biological function and distribution of P-gps in sea lice.

2.2. Introduction

Sea lice (*Lepeophtheirus salmonis*) are ectoparasitic crustaceans of Atlantic salmon that if left untreated will feed aggressively on the mucus, skin and muscle. They can cause osmoregulatory and secondary infection problems which can result in mortality (Johnson and Albright, 1991; Pike and Wadsworth, 1999). Physical methods alone have not been sufficient to control *L. salmonis* infestation and therefore chemotherapeutic approaches are heavily relied on to maintain low lice burdens on salmon farms (Burka *et al.,* 1997; Pike and Wadsworth, 1999). Due to stringent regulatory policies, and that few drugs available have the desirable characteristics, therapeutic options are limited (Storey, 2005). Emamectin benzoate (EMB), an avermectin, is an orally administered drug that when given at a dose of 50μg/kg/day for a seven day period will kill all parasitic stages of sea lice with no adverse toxic effects on the salmon (Stone *et al.,* 2000; Ramstad *et al.,* 2002) and limited negative impact on the environment (Burridge, 2003).

The limited chemotherapeutic options available have resulted in the continued reliance on EMB for the control of sea lice infestation on farmed Atlantic salmon which makes resistance development a major concern (Denholm *et al.,* 2002; Westcott *et al.,* 2004). Avermectin compounds have been used to control various parasites of humans, pets, and livestock (Vercruysse and Rew, 2002). Resistance to avermectin compounds has become increasingly documented, most commonly in various nematodes. Of the mechanisms identified, over-expression of the multiple drug transporters P-glycoproteins (P-gps) are suggested to be of critical importance in resistance development (Blackhall *et al.,* 1998; Xu *et al.,* 1998; Ardelli *et al.,* 2006).

P-gps are 110 – 190 kDa in size and belong to the ATP-binding cassette (ABC) superfamily. Most ABC transporters possess a structure that is evolutionarily well conserved. A typical ABC transporter consists of four segments: two nucleotide binding domains (NBD's) and two transmembrane domains (TMD's) (Klein *et al.,* 1999) fused into one single polypeptide (Váradi *et al.,* 2003). P-gps contain various conserved motifs within the NBD's. A potentially important motif is the "signature" sequence (LSGGQ). The function of this motif is unknown, but it appears to be present in all ABC transporters, and is absent in any other transporter (Loo *et al.,* 2002). Within the NBD are other conserved regions known as Walker A and B motifs; the function of these motifs is still unclear but it has been speculated that the "signature: motif may in some way interact with the Walker A motif in the opposing NBD to allow for ATPase activity (Loo *et al.,* 2003).

The aims of this study were to: (1) identify the putative gene(s) encoding for drug transporter P-gps in *L. salmonis*; and (2) detect the putative P-gp using Western blot analysis. This information will be used in further studies involving immunolocalization and quantitative RT-PCR. Detection of P-gp in sea lice may be useful in the future as a tool for early detection of resistance development to emamectin benzoate.

2.3. Materials and methods

2.3.1. Sea Lice

Adult *L. salmonis* were collected from the Atlantic salmon farms in the Bay of Fundy, New Brunswick, Canada. Lice were carefully removed using forceps and placed

in a glass container with the seawater from the location of the farm being sampled. Once in the laboratory, live lice were flash frozen and stored at -80°C.

2.3.2. Control Organisms/Tissues

Drosophila melanogaster were purchased from Wards Natural Science (Rochester, NY). *D. melanogaster* were cultured and flash frozen at -80°C. *Haemonchus contortus* were removed from the intestinal fluid of a goat and washed to remove any debris before flash freezing and storage at -80°C. Livers were removed from Sprague Dawley rats that had been euthanized in accordance to Canadian Council on Animal Care guidelines. The livers were flash frozen in liquid nitrogen and stored at -80°C until required.

2.3.3. RNA isolation

Total RNA was isolated using an RNeasy kit (Qiagen, Mississauga, ON). Approximately 100mg of tissue were placed in liquid nitrogen and ground thoroughly using a sterile mortar and pestle not allowing the liquid nitrogen to completely evaporate. The ground tissue in liquid nitrogen was poured into a sterile 50ml tube. Immediately following the evaporation of the liquid nitrogen, 3ml of RNeasy lysis thiocyanate (RLT) buffer was added and the tissue was further homogenized by passing the lysate 10 times through a sterile 22 gauge needle fitted with an RNase-free syringe. The tissue lysate was centrifuged for 10 min and the supernatant was carefully pipetted into a new sterile 15ml tube. After the addition of 70% ethanol the tissue lysate was applied to a Qiagen

Miniprep total RNA isolation column. After a series of washes, high quality sea lice total RNA was eluted.

2.3.4. The use of degenerate PCR primers to amplify the genes encoding P-glycoprotein in *L. salmonis*

Amino acid alignments using the following sequences were conducted using the ClustalW sequence alignment tool (Thompson *et al.,* 1994) and conserved homologous regions between species were identified (Fig. 2.1); *H. contortus* P-GP-A (accession number AF003908), *Onchocerca volvulus* P-GP-1 (accession number AF083642), and the four P-gp iosforms of *Caenorhabditis elegans* P-gp-1 – P-gp-4 (accession numbers NM_070012, NM_059306, NM_077500, NM_077501).

Degenerate primers designed previously by Xu *et al.* (1998) and Drogemuller *et al.* (2002) based on the conserved ATP-binding domains TVALVGS(S/H/Q/P)SGSG and DEATSALD were used (see Fig. 2.1). The primers and products based on Xu *et al.* (1998) or Drogemuller *et al.* (2002) will from herein be referred to as Xu/Drogemuller primers or products respectively. An additional primer based on the conserved region GGQKQRIAI was used in combination with both Xu and Drogemuller forward and reverse primers.

First-strand cDNA synthesis for all samples was primed using oligo-dT primers (Superscript III First Strand Synthesis Kit, Invitrogen, Burlington, ON). Both conventional and touchdown RT-PCR was performed in 50µl volumes and contained a

Figure 2.1. Amino acid alignment of nematode and arthropod P-glycoproteins. Sequences aligned are: *H.contortus* P-GP-A (accession number AF003908), *O.volvulus* P-GP-1 (accession number AF083642), and the four P-gp iosforms of *C.elegans* P-gp-1 – P-gp-4 (accession numbers NM_070012, NM_059306, NM_077500, NM_077501). Conserved regions are indicated by (*), conserved substitutions by (:), and semi conserved substitutions by (.). Conserved regions where degenerate primers were designed are identified in bold and underlined.

1X Buffer (50 mM KCl; 10 mM Tris-HCl; 1.5 mM $MgCl_2$), 250μM each deoxynucleoside triphosphate (dNTP), 12.5μM each primer, and 0.5μl (1 U) Taq Polymerase. The cycling conditions comprised of an initial 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 60 sec, annealing for the conventional was attempted at 45°C, 50°C, and 55°C for 90 sec in different reactions, whereas the touchdown started at 55°C and decreased by 1°C every second cycle to a "touchdown" annealing temperature of 40°C for 90 sec, extension at 72°C for 120 sec. A final extension step at 72°C for 10 min completed the cycle. From the resulting PCR product, 1μl was used as a template for re-amplification using the same primers and conditions as before. Subsequent PCR products were analyzed on a 1% agarose gel containing ethidium bromide extracted directly from the gel, purified and cloned into a TOPO2.1 TA vector (Invitrogen) in preparation for DNA sequencing (all sequencing was performed commercially by ACGT Corp., Toronto, ON).

2.3.4.1. Sea lice expressed sequence tag (EST) database search

Two *L. salmonis* EST databases were made available to screen for conserved motifs previously identified in the P-gp sequences of other invertebrate species. The EST libraries were located at the NRC Institute of Marine Biosciences, Halifax, NS, and at the Institute of Marine Research, Bergen, Norway. From these databases only a single clone, SL0525, was identified in the EST library in Halifax; the EST library in Bergen did not identify a clone with any homology. The SL0525 clone was sequenced commercially by ACGT Corp. (Toronto, Ontario, Canada).

2.3.4.2. 5`/3` Rapid amplification of cDNA ends (RACE)

The sequences of the Xu PCR product and the SL0525 clone were used to design gene–specific primers for use in 3` and 5` RACE reactions. These primers were initially used in combination in RT-PCR to confirm primer specificity. After ensuring primer specificity, the primers were applied to sea lice cDNA using the 5`/3` RACE kit (Roche, Laval, QC). All RACE products were re-amplified using either specific primers up or downstream of the last specific primer used in combination with an adaptor primer provided in the RACE kit. During each synthesis reaction (i.e. RNA-ssDNA-dsDNS-PCR) the template was checked using gene-specific primers. All RACE products were sequenced commercially by ACGT Corp. (Toronto, Ontario, Canada).

2.3.4.3. Blunt-end cloning

Specific primers based on SL0525 and Xu PCR product sequences were used to generate a single stranded cDNA strand. The 5` end was poly-A tailed and using oligo-dT primers, specific double stranded cDNA templates for cloning into a pEZseq cloning vector (Lucigen) were generated. To ensure a blunt ended template for cloning, a DNAterminator end repair kit was applied (Lucigen, Middleton, WI).

2.3.4.4. Splice-leader primer RT-PCR

To extend the SL0525 and Xu genes, the use of gene-specific primers combined with primers based on the conserved splice leader sequences SL1: 5`GGT TTA ATT ACC CAA GTT TGA G3` and SL2: 5`GGT TTT AAC CCA GTT ACT CAA G 3` located on the 5` ends of some or all mRNAs in numerous invertebrate species in a RT-

PCR was performed (Ross *et al.,* 1995). RT-PCR was performed in 50µl-volumes with cycling conditions of an initial 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 50°C for 90 sec, extension at 72°C for 120 sec. A final extension step at 72°C for 10 min completed the cycle.

2.3.4.5. Degenerate primer 5` extension

Degenerate primers were constructed based on conserved motifs of the P-gp nucleotide binding domains closest to the N terminal of the protein. These primers were used in combination with gene specific SL0525 and SL-Pgp1 reverse primers in an attempt to extend these sequences further. The gene specific reverse primers (designed using Primer Detective (Clonetech, Version 1.0)) for SL0525 and SL-Pgp1 gene extensions were: 5` AGA GCA ACG CGG GAC TTT 3` and 5` TCT CTC AAG GAG TGC TGC AA 3`, respectively. The degenerate primers used were: 5` GCN CAN WSN GCN CTN GA 3`, 5` CTN CTN CTN GAY GAR GCN AC 3`, and 5` CTN WSN GGN GGN CAR AAR CA 3` (based on the amino acid sequences ATSALD, LLLDEAT, and LSGGQKQ, respectively). PCR conditions were the same as those used previously for RT-PCR and "touchdown" RT-PCR.

2.3.4.6. Sequence analysis

A Basic Local Alignment Search Tool (BLAST) (Altschul *et al.,* 1990) was used to search for sequence similarity to the *L. salmonis* gene sequences in the Genbank database (National Centre for Biotechnology Information, NCBI). Amino acid sequences were alignment to corresponding proteins using ClustalW. The species used to align the

SL0525 and Xu product were *H.contortus* P-GP-A (accession number AF003908), *O.volvulus* P-pg-1 (accession number AF083642), and the four P-gp isoforms of *C.elegans* P-gp-1 – P-gp-4 (accession numbers NM_070012, NM_059306, NM_077500, NM_077501).

2.3.5. Sea Lice cDNA Library Construction

A sea lice cDNA library was constructed using a ZAP cDNA library construction kit (Qiagen) and sea lice mRNA. This library was used to screen for clones containing the full SL0525 gene sequence. The library construction consisted of cDNA synthesis and size fractionation, ligation and packaging of cDNA in phage heads, and library amplification. After construction, 10 randomly picked plaques were checked for the presence of DNA inserts. This was performed by PCR using vector specific T7 and T3 primers. The screening of this library applied either (1) a SL0525 specific antibody (production method described in section 2.3.6) or (2) an SL0525 digoxigenin (DIG) labeled probe.

2.3.5.1. Library screening using DIG labeled DNA probes

A DIG labeled SL0525 probe was generated using the DIG PCR labeling kit (Roche). XL1-Blue host cells were grown to OD_{600} 0.5 in LB broth at 37°C with shaking. 4µl of the amplified phage stock was added to 600µl of the XL1-Blue cells. After incubating for 15 min at 37°C, 8ml of NZ amine (NZY) top agar, melted and cooled to ~48°C was added to each tube and poured evenly onto a pre-warmed 150mm NZY agar

plate. Each plate was allowed to set for 10 min at room temperature before incubating at 37°C overnight.

After incubating, the plates were cooled at 4°C for at least 30 min. A nylon membrane was placed onto the surface of the pre-cooled plates avoiding air bubbles for 1 minute. The membrane was orientated by punching three holes into the membrane using a small needle and Indian ink. The membrane was then carefully removed and briefly air dried on filter paper. Using tweezers the membrane was then placed in a 0.5M NaOH, 1.5M NaCl denaturation solution for 2 min. The membrane was then removed, briefly air dried on filter paper, and placed in a 1.5M NaCl, 0.5M Tris-HCl (pH 8) neutralizing solution for 5 min. After the incubation period was complete the drying was repeated and the membrane placed in a 0.2M Tris-HCl (pH 7.5), 2X sodium trisodium citrate (SSC) washing solution for 30 sec. After briefly drying the membrane was placed in an ultraviolet (UV) Stratalinker (Stratagene, La Jolla, CA) (at 120mJ) to immobilize the DNA.

The membrane was placed in a clean glass hybridization tube and kept moist with hybridization buffer (2X PIPES, 50% Formamide, 0.5% SDS) pre-warmed to ~50°C. The appropriate amount of salmon sperm DNA (100µg/ml: 77µl was used for a 150mm membrane) was boiled for 10 min and added to 8ml hybridization buffer. This pre-hybridization buffer was added to the membrane and incubated for 1h at 42°C in a hybridization oven. The appropriate amount of salmon sperm DNA and DIG DNA probe (77µl salmon DNA and 32µl DIG probe) was boiled for 10 min and added to 8ml of hybridization buffer. The pre-hybridization buffer was poured off and the probe solution

added to the membrane. The membrane was allowed to incubate with the probe for 2h at 42°C.

The membrane was then washed twice with 30ml 2X SSC, 0.1% SDS for 5 min at 42°C followed by two high stringency washes with 30ml 0.5X SSC, 0.1% SDS for 15 min at 68°C. Colorimetric detection was performed using an anti-DIG antibody conjugated to alkaline phosphatase followed by incubation with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (NBT/BCIP) color substrates using a DIG-nucleic acid detection kit (Roche).

2.3.5.2. Library screening using antibodies

Polyclonal antibody SL0525 (method of production described in section 2.3.6.) was used to screen this library. Before screening the library this antibody was absorbed with XL-Blue bacterial protein to limit cross reactivity to any bacterial proteins that may be present on the membrane.

NZY phage plates were generated using the method described above. A nitrocellulose membrane was soaked in 0.01M isopropyl-D-thiogalactopyranoside (IPTG) for 30 min and allowed to air dried on filter paper. The membrane was placed on the phage plate avoiding any potential bubbles, and the plates were placed back at 37°C for 3½ h. Plates were removed from incubator and orientated to the plate using a small needle and Indian ink. The membrane was then carefully removed and placed in a 1% BSA in Tris-buffered saline (TBS) blocking solution for one hour. The membrane was then washed for 5 min in Tris buffered saline Tween 20 (TBST) three times and then incubated for 1h in a 1:10,000 dilution of SL0525 primary antibody. The membrane was

then washed with TPBS for 5 min four times and then incubated in the appropriate 1:16,000 concentration of secondary antibody for 1h. The membrane was then washed in TBST three time for 5 min each followed by two 5 min washes in TBS. The membrane was finally exposed to GE Healthcare Life Sciences (Piscataway, NJ) enzymatic chemiluminescence (ECL) detection agent according to the manufacturer's instructions

2.3.6. Expression of SL0525 protein

2.3.6.1. Subcloning of the SL0525 DNA insert in pGEX plasmid

The SL0525 nucleotide sequence was analyzed for restriction enzyme sites using CLC free workbench (CLC bio, Massachusetts). There were no restriction sites within the sequence that would be useful for insertion into a pGEX vector. BamHI and XhoI enzyme restriction sites were therefore incorporated into the SL0525 DNA by PCR, so that the gene would be in the correct reading frame for glutathione-S-transferase (GST)-fusion expression upon subcloning into the pGEX vector. The forward and reverse primers were 5`CGGATCCGAAACGTCGCATTTACAG (the BamHI site is underlined) and 5`GCTCGAGTTAAGATTGAACAACAGC (the XhoI site is underlined), respectively. The PCR product was ligated into a pCR®2.1 TOPO® vector (Invitrogen) and digested with BamHI and XhoI. The resulting digest was run on a 1% agarose gel and the insert was cut and purified using a High Pure PCR Product Purification Kit (Roche). The DNA digest was subcloned into the pGEX vector previously digested with BamHI and XhoI. This was confirmed by restriction enzyme digest and sequencing.

2.3.6.2. Expression of recombinant protein

The expression plasmid was transformed into the bacterial host *Escherichia coli* BL-21 strain following standard protocol. Clones harbouring the pGEX-4T-1/SL0525 plasmid were grown in 1ml Luria Bertani (LB) culture medium containing 100μg/ml ampicillin at 37°C with vigorous shaking overnight. Thirty microlitres of the overnight culture was added to 1.5ml LB broth containing 100μl/ml ampicillin and grown for 90 min. Expression of the fusion gene was induced with 0.1mM isopropyl-D-thiogalactopyranoside (IPTG) for 4 h at 37°C with vigorous shaking. The cells were harvested by centrifugation at 14,000 xg and washed once with 200μl of ice cold sodium, tris, EDTA (STE) buffer. The bacteria were resuspended in 135μl of STE and placed on ice for 15 min. The bacteria were lysed by the addition of N-laurylsarcosine (sarkosyl) to a 1.5% final concentration from a 10% stock solution in STE. The cells were sonicated on ice using short bursts of 10 sec allowing the protein/bacterial suspension to cool on ice between each burst. The lysate was centrifuged at 14,000 xg for 5 min and the supernatant was transferred into a new tube. Triton X-100 was added to a 4% final concentration from a 10% stock in STE. Protein expression was confirmed by running the sample in a 12% SDS gel and staining with Coomassie Blue dye.

Preparative scale preparation of GST fusion proteins were treated identically to analytical scale preparation except that 2ml of an overnight culture was used to inoculate 100ml LB broth. Bacteria were pelleted by centrifugation at 7000 xg for 10 min using a JA-14 rotor and washed with 6ml STE. The pellet was resuspended in 6ml STE and placed on ice for 15 min. Dithiothreitol (DTT) was added to a final concentration of 5mM from a 10mM stock solution. Lysis was achieved using an identical method as the

analytical preparation. The lysate was centrifuged for 5 min at 10,000xg using a JA-21 rotor. The supernatant was transferred into a clean 15ml tube and Triton X-100 was added to a 4% final concentration from a 10% stock in STE. Samples were analyzed on a 12% SDS gel stained with Coomassie Blue dye.

2.3.6.3. SL0525 protein purification

The SL0525 GST-fusion protein was purified from the bacterial sonicate by the use of a glutathione sepharose column. The sonicate was applied to the column and allowed to flow through. A sample of the elution was retained for analysis on an SDS gel to measure the efficacy of binding to the matrix. The matrix was washed three times by the addition of 10X bead volume of 1X PBS. Thrombin was used to cleave the ABC protein from the GST. The thrombin was added to the column and incubated at room temperature for 12-16 h. Following this incubation the elution containing the ABC protein was collected while the GST remained bound to the matrix. The GST was eluted by the addition of glutathione elution buffer. Both the ABC protein elution and GST elution were analyzed on an SDS gel.

2.3.6.4. Production of polyclonal antibodies

Antibodies against the SL0525 protein (cleaved from the GST) were raised in a female New Zealand white rabbit. At least 15min before the process of immunization and blood collection, the rabbit was sedated with 1mg/kg of acepromazine. For each immunization, two injection sites on the back of the rabbit (close to the shoulder blades) was used. Hair was clipped at the sites of subcutaneous injection and cleaned using soap

and water followed by alcohol swabs. Sterile syringes and needles (27 gauge) were used to minimize microbial contamination. Before immunization began 10ml of blood was drawn from the marginal vein of the rabbit ear to prepare about 5ml of serum, which represented the "preimmune" serum, utilized as a negative control. Immunizations consisted of an initial injection of 200μg of antigen in PBS (250μl) mixed with an equal volume of Freund's complete adjuvant. This was followed by repeated booster injections every 3 weeks, where the same amount of antigen was mixed with Freund's incomplete adjuvant. Booster injections were all given subcutaneously for a total of five times. Small volumes were used for each injection (250μl) to minimize inflammatory reactions. Before every immunization, blood samples were taken from the marginal vein of the rabbit ear, centrifuged, and the sera frozen. After a good reactivity to the SL0525 protein in a western blot was shown, the rabbit was bled out to yield a large volume of polyclonal antibody.

2.3.7. Western blot analysis

Both rat and sea lice tissues were weighed and placed in 4X volume of ice cold Tris/KCl buffer containing a general protease inhibitor. Tissues were placed on ice and homogenized using 30 second pulses with a hand held homogenizer. The tissue homogenate was centrifuged at 300 xg for 20 min at 4°C. The supernatant was collected and centrifuged at 100,000 xg for 30 min at 4°C. The pellet containing the membrane fraction was resuspended in 250μl of Tris/KCl buffer and stored at -20°C until required.

Two monoclonal P-gp specific commercially available antibodies, C219 and JSB-1 (Abcam, Cambridge, MA), were used for the detection of P-gp. The JSB-1 antibody is

specific to MDR1 and unlike C219 shows no cross-reactivity to MDR3. JSB-1 has, however, shown cross-reactivity with pyruvate carboxylase (Rao *et al.*, 1995). The C219 antibody shows a greater cross-species reactivity, but in human protein samples has been demonstrated to cross-react with other P-gp isoforms as well as with a 200kDa protein which migrates to the same position as myosin and with C-erb B2 (a tyrosine kinase receptor) which is 37kDa in size (Liu *et al.*, 1997).

Protein was quantitated using the Bio-Rad protein assay (Bio-Rad, Mississauga, ON), which is based on the Bradford dye-binding procedure (Bradford, 1976). An aliquot of protein was added to an equal volume of 2X loading buffer. Samples were then boiled for 3 min and proteins separated by electrophoresis on a 7.5% SDS-polyacrylamide gel. Electrophoresis was conducted for 4 h at 75 mA. Following electrophoresis, the samples were transferred at 25 V for 1 h onto a nitrocellulose membrane. Nonspecific binding sites were blocked for 1 h with 5% nonfat dry milk in PBS after the transfer. Primary antibodies C219 and JSB-1 were diluted 1:50 and the SL0525 polyclonal antibody was diluted 1:5000 in antibody dilution buffer (Phosphate buffered saline Tween 20 [PBST] (0.1%) containing 5% nonfat dry milk). The nitrocellulose membrane was incubated with C219 or JSB-1 overnight or SL0525 for 2 h at room temperature. The membrane was washed with PBST (0.1%) (3 x 15 min), and incubated with a horseradish peroxidase-linked secondary anti-mouse (C219 or JSB-1) or anti-rabbit (SL0525) antibody (Abcam) diluted 1:5000 and 1:16,000, respectively, for 1 h followed by rinsing with PBST (0.1%) (3 x 15 min). The membrane was exposed to Amersham ECL detection agent according to the manufacturer's instructions and exposed to an x-ray film for visualization.

2.4. Results

2.4.1. RT-PCR and EST library products

Using conventional PCR the sea lice cDNA failed to generated a product using the Xu primers although the control *D. melanogaster* and *H. contortus* cDNA both produced PCR products of the expected size (Fig. 2.2). After touchdown PCR using sea lice cDNA, the Drogemuller primers generated three PCR products of approximately 220bp, 410bp and 650bp, whereas the Xu primers generated a single 410bp PCR product (Fig. 2.2). The control *D. melanogaster* and *H. contortus* cDNA both produced PCR products of the same expected size using the Drogemuller and Xu primers. Using the additional primer in combination with the published primers failed to generate PCR products for any of the cDNA templates. Only the product generated using the Xu primers generated a sequence whose open reading frame showed similarity to P-gp. For convenience this product will be referred to as SL-Pgp1 during the remainder of this thesis. After searching two sea lice EST databases for the conserved P-gp motifs, only a single sequence (SL0525) was identified. This clone was sequenced using T3 and T7 primers generating a partial sequence of 717bp. The encoding nucleotide sequences for both SL0525 and SL-Pgp1 genes are shown in figure 2.3.

3` RACE extended all sequences to the 3` end, which were identified by the codon region encoding a stop signal. The use of 5` RACE, blunt end cloning, splice leader RT-PCR, and degenerate primer RT-PCR did not extend the genes encoding for either SL0525 or SL-Pgp1.

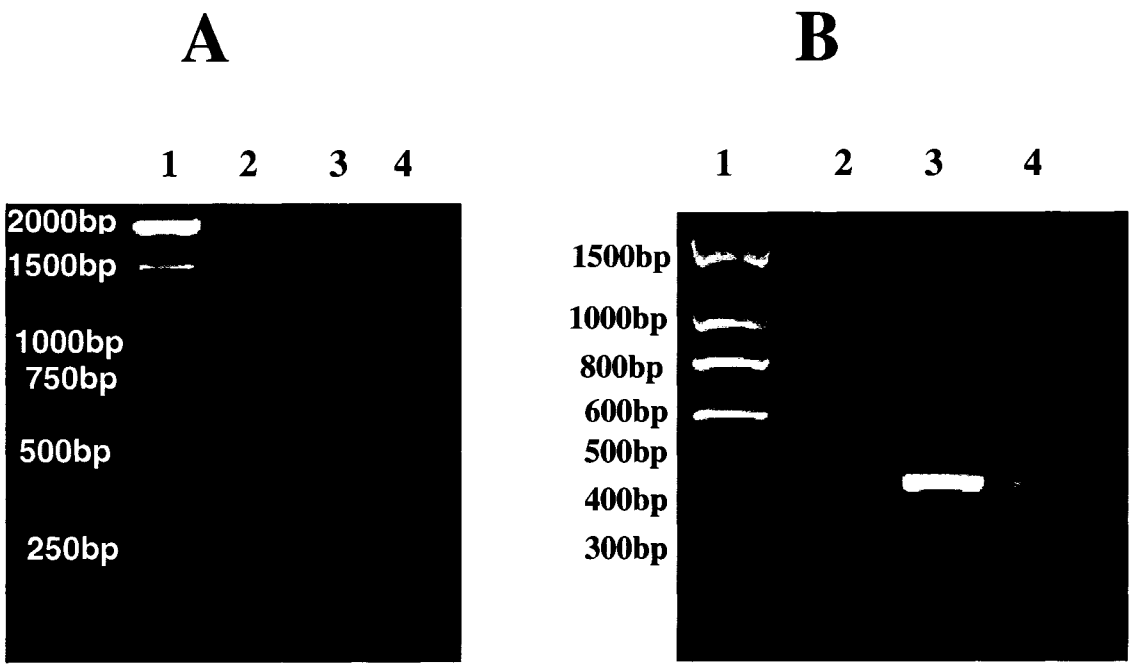

Figure 2.2. 1% agarose gel of PCR products using degenerate primers published by Xu *et al.* (1998)
(A) conventional RT-PCR. Lane 1 DNA molecular weight markers, Lane 2 *D. melanogaster*, Lane 3 *H. contortus*, Lane 4 *L. salmonis*; (B) touchdown RT-PCR . Lane 1 DNA molecular weight markers, Lane 2 *L. salmonis*. Lane 3 *D. melanogaster*, Lane 4 *H. contortus.*

SL-Pgp1

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....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        10         20         30         40         50         60         70         80         90        100        110
ACGGTAGCGT TGGTGGGTAC ATCTGGAGGT GGGAAGACTA CAATTGCAGC ACTCCTTGAG AGATTTTATG ACATCAATGG TGGTGGATCA CTCGAAATTG ATGGAATTAA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       120        130        140        150        160        170        180        190        200        210        220
TATTCGAGAT TTGGATCCAA GCTGGCTTAG AGGCTCTGCA ATTGGGTATA TAAATCAAGA GCCCGTTCTG TTTGCTACTT CAGTCATTGA GAATATTCGG TATGGCAGAC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       230        240        250        260        270        280        290        300        310        320        330
CTAATGCGAC AGATAATGAG GTATACGAAG CAGCGAAGGC AGCTCACGTT GATGACTTTG TTCGAACATT TCCTGACGGG TATTCTACAA TATTAGGTGA GCGTGGCGTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       340        350        360        370        380        390        400        410        420        430        440
ACCGTATCTG GGGGTCAAAA GCAGCGTATC GCTATTGCTC GTGCTCTTTT GAAGAATCCA CCCATTTTGA TTTTAGACGA AGCCACCTCC GCCCTCGATG CAGAGTCAGA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       450        460        470        480        490        500        510        520        530        540        550
GCGGATTGTT CAAGAGGCTC TTGATAAACT CTCCAAAGGT CGCACCTCCC TTGTTATCGC TCATAGACTA TCCACCATTA AAAATGCTGA CGTTATAGCA GTCATAGATA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....| ....|....| ....|
       560        570        580        590        600        610        620        630        640        650        660
AAGGTGTCAT GGCCGAGATA GGGACTCATG CTGCTCTTAA GCGAAAGGGT GGCATATATT CAAGACTTAT TGAGCAGCAG GAACTCAGAG AGTAAAAAAA AAAAAGAAAA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|
       670        680        690        700        710        720        730

ATTGTGATTC GTTAAACAAT ATACTATTTG CACCTTCGTT AATAAATGTT GTTCAAAAGA AAAAAAAAAA AAAA
```

SL0525

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....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        10         20         30         40         50         60         70         80         90        100        110
GAAACGTCGC ATTTACAGCC TCCATTTTTG GGGCTATATA ATGTTTCTTT CAAGTATGAA TCGCAAAATC CTTTATTTAA AAGTGTAGAC TTTGGTATAG ATATGGAGTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       120        130        140        150        160        170        180        190        200        210        220
AAGAATAGCT ATTGTTGGCC CCAATGGTGT TGGGAAATCA ACGTTTCTTA AGCTTTTGAT GGGAGATTTG GAACCAACAA AGGGGGAAAT GAGAAAGAAT GCCCGTTTAA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       230        240        250        260        270        280        290        300        310        320        330
AGATTGGACG CTTTGATCAA CACTCTGGTG AACATTTGAC GGCTGACGAG TCTCCAACTG AATATATTAT GCGTTTATTT AATTTACCTG TCGAAAAGGC ACGTAAGCAA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       340        350        360        370        380        390        400        410        420        430        440
TTAGGTTCCT TTGGTCTTCA ATCCCATGCA CATACCATCA AAATGAAGGA TCTCTCTGGT GGTCAAAAGT CCCGCGTTGC TCTTGCAGAG CTAACTCTAT CTGCTCCGGA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       450        460        470        480        490        500        510        520        530        540        550
TGTGGTCATT CTGGACGAAC CTACTAATAA TTTGGATATC GAGTCCATTG ACGCTTTGGG GGATGCCATT CGGGAGTATA AAGGTGGTGT CATCATCGTT ACTCACGACG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       560        570        580        590        600        610        620        630        640        650        660
AACGCCTGAT TCGTGACACG GAATGTCAAT TGTGGGTCGT TGAGGAACAA ACAATTAATG AAATCGAGGG TGACTTTGAC GACTATAGAA AGGAAGTTTT GGACTCACTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....|....| ....|....| ....|....| ....|....| ....|....|....|
       670        680        690        700        710        720        730        740        750        760
GGGGAAGAAG TGAACAATCC CAGTCTTATC GCCAATCAAG CTGTTGTTCA ATCTTAAACA AATTTTAATC TAACATTAAA TCCGAAAAAA AAAAAAAAA
```

Figure 2.3. Nucleotide sequences for sea lice SL-Pgp1 and SL0525 genes (Genbank # EF093796 and DQ458787). UTR's are in bold and underlined. Both genes were commercially sequenced by ACGT (Toronto, ON).

2.4.2. Sequence analysis

The alignments of SL-Pgp1 with invertebrate P-gp sequences showed this sequence to have a relatively high level of homology (~72%), ClustalW aligment shown in figure 2.4. The BLAST result matched this sequence to multi-drug transporters in other species (Appendix A). The alignment of the SL0525 with invertebrate P-gps showed less similarity (Fig. 2.4). The highest homology was seen within the conserved regions LSGGQ, GPNGVGKS, and DVVILDE which correspond to the signature and Walker A and B motifs, but the sequence showed less overall homology (~35%) than that seen with the SL-Pgp1 sequence (~75%). BLAST analysis identified SL0525 as an ATP binding cassette (ABC) protein. The BLAST result showed this amino acid sequence to have the highest homology to ABC protein subfamily F, with 65% homology to both mouse and rat GCN20 proteins (Appendix A).

2.4.3. Screening of the sea lice cDNA library

The sea lice cDNA library was constructed from size fractionated cDNA generated from high quality sea lice mRNA (Fig. 2.5 and 2.6). After library construction, plaques were picked at random and checked for the presence of a gene insert. This screen showed 80% of the plaques contained an insert (Fig. 2.7). Forty membranes each containing approximately 1500 plaques were screened using both the SL0525 polyclonal antibody and a DIG labeled SL0525 DNA probe to identify clones containing the SL0525 gene. The SL0525 pAb was absorbed with XL-Blue bacterial proteins which successfully

reduced cross reactivity with bacterial proteins (Fig. 2.8). The antibody screening identified a number of false positive plaques that after sequencing had no identity to the SL0525 gene. The production of a DIG probe was confirmed by gel electrophoresis with the PCR product being larger than the control unlabeled product due to the additional DIG tags (Fig. 2.9). The DIG screening again identified a single plaque that after sequencing showed only a 40% homology to the SL0525 nucleotide sequence.

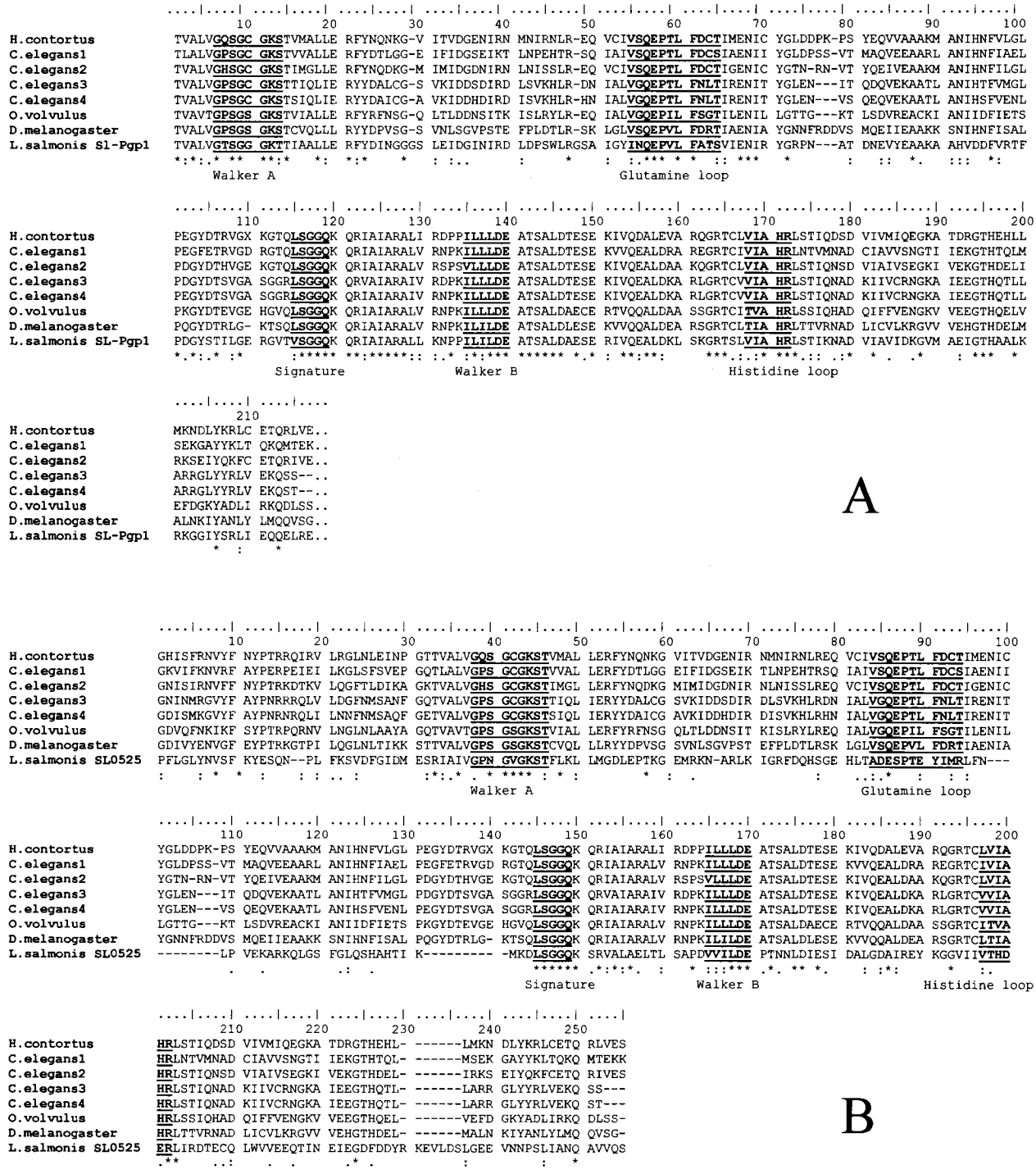

Figure 2.4. Amino acid alignment of both *L. salmonis* SL-Pgp1 (A) and SL0525 (B) proteins with other P-glycoproteins. Sequences aligned are: *L. salmonis* (accession number DQ458787 and EF093796), *H. contortus* P-GP-A (accession number AF003908), *O. volvulus* P-GP-1 (accession number AF083642), and the four P-gp iosforms of *C. elegans* P-gp-1 – P-gp-4 (accession numbers NM_070012, NM_059306, NM_077500, NM_077501). Conserved regions are indicated by (*), conserved substitutions by (:), and semi conserved substitutions by (.). Conserved motifs Walker A and B, signature sequence, and glutamine and histidine loops are in bold and underlined.

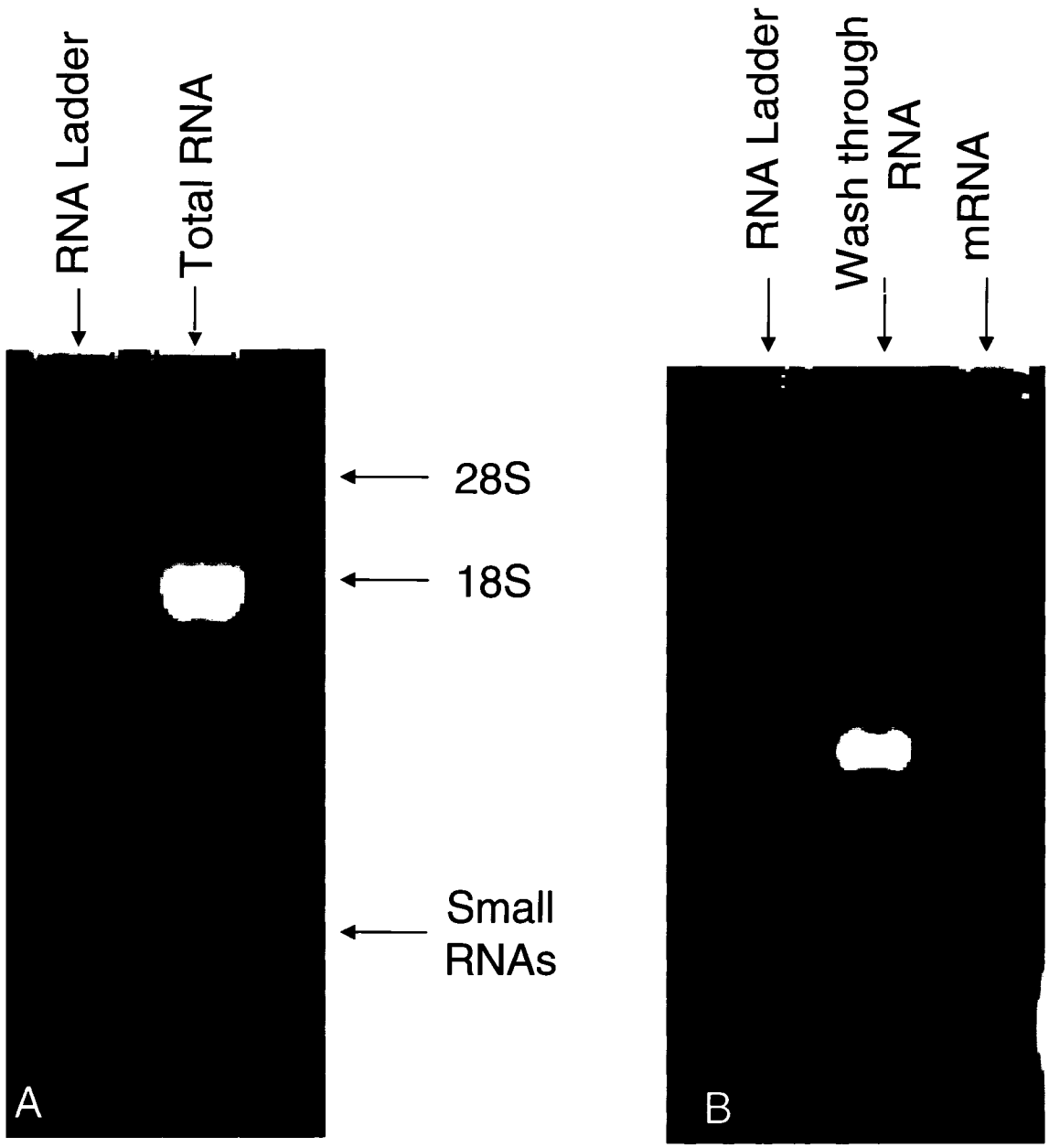

Figure 2.5. RNA analysis on a 1% agarose gel stained using ethidium bromide. A: high quality total RNA isolated from 10 pooled lice; B: mRNA purified from total RNA using a Poly (A) mRNA Isolation kit. The absence of any signal from the mRNA was expected due to the low concentration. It is clear that 28S and 18S rRNA's did not cross over into the eluted mRNA fraction.

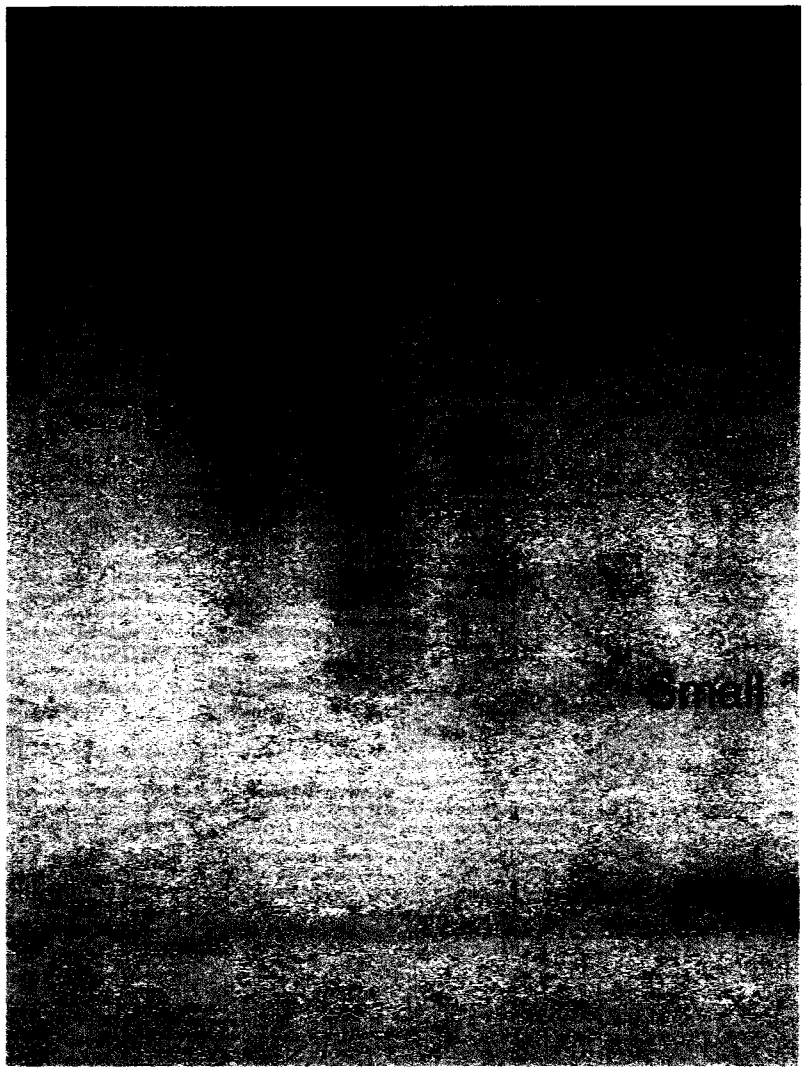

Figure 2.6. Size fractionation analysis of ^{32}P labeled cDNA fractions ran on a nondentaturing acrylamide gel and detected on an x-ray film after 24 hours exposure at -80°C.

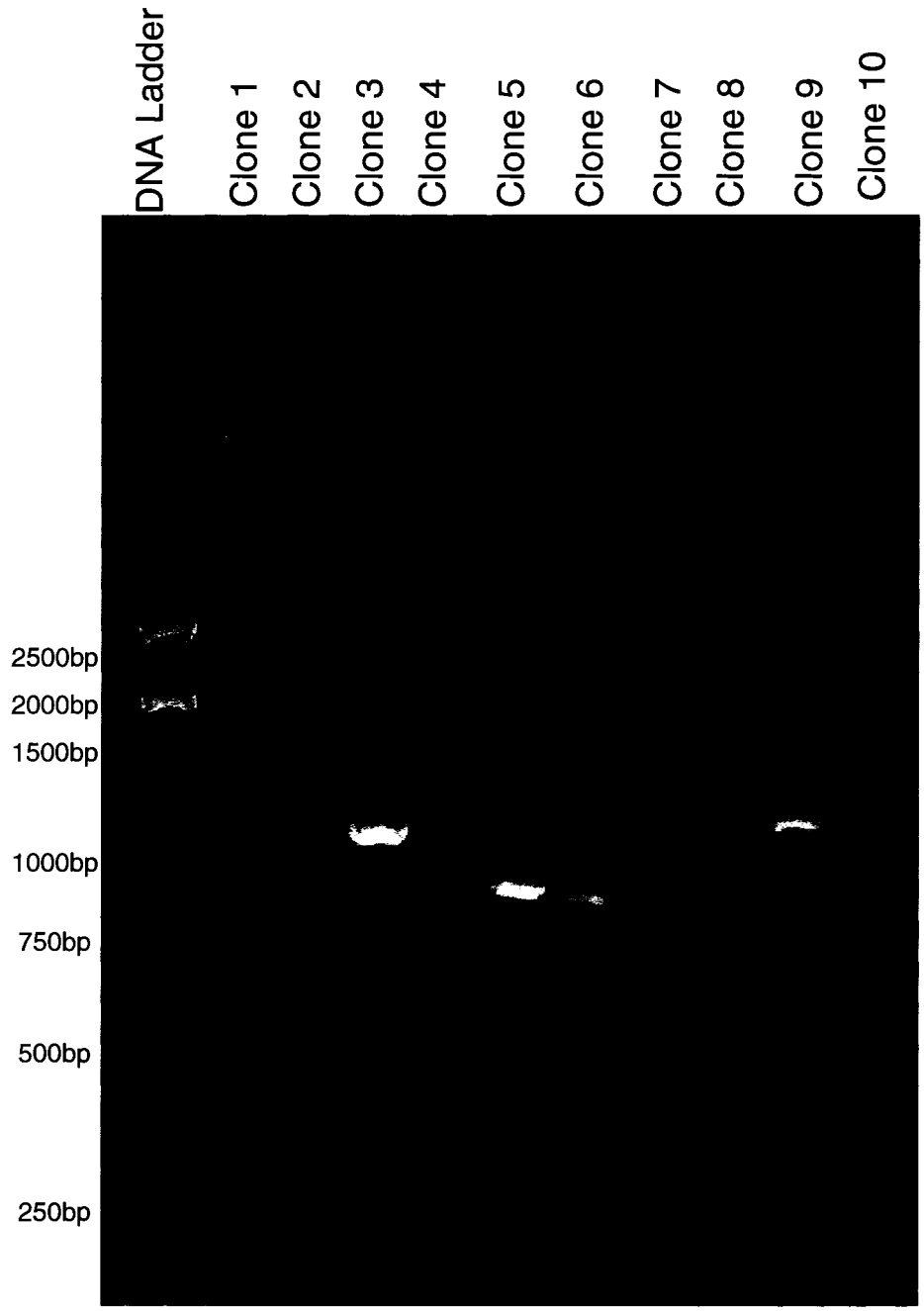

Figure 2.7. Clones screened for the presence of DNA inserts using T7 and T3 primers in PCR. Samples run on a 1% agarose gel stained with ethidium bromide.

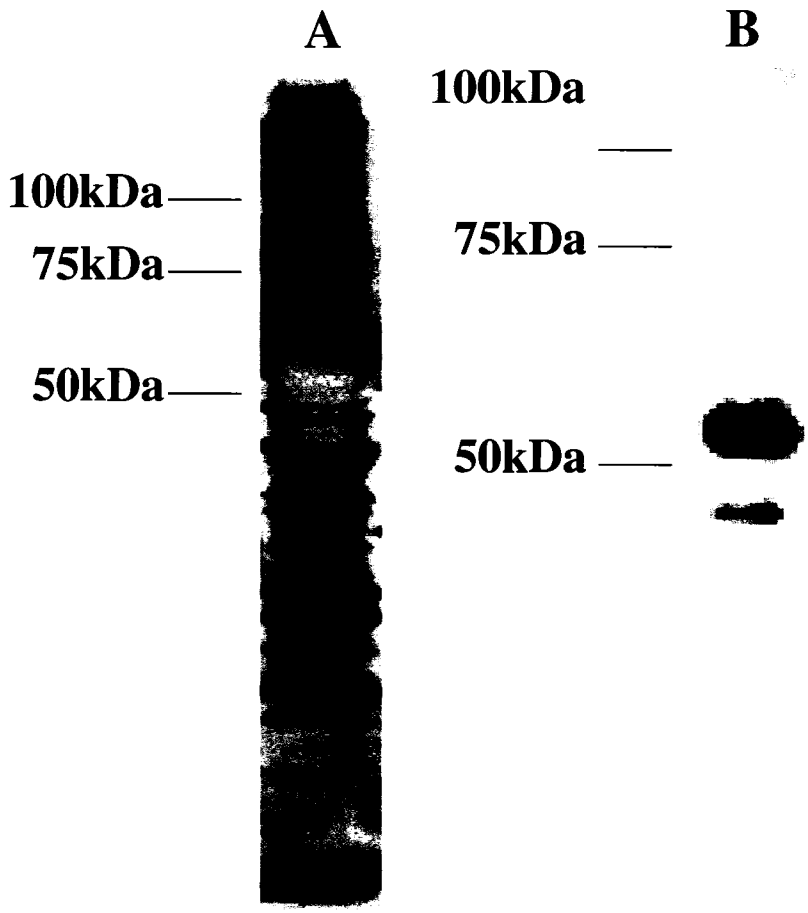

Figure 2.8. Western blot analysis showing reduction of bacterial protein binding of the absorbed SL0525 polyclonal antibody. A: Immunostaining of *E. coli* BL-21 lysate containing the SL0525 fusion protein using the SL0525 polyclonal antibody. B: Immunostaining of *E. coli* BL-21 lysate containing the SL0525 fusion protein using the absorbed SL0525 polyclonal antibody

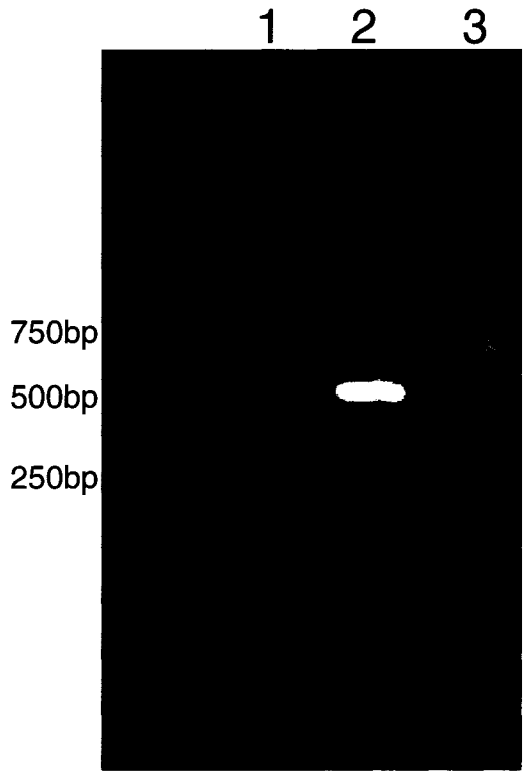

Figure 2.9. 1% agarose gel confirming the DIG labeling of the SL0525 PCR product identified by the larger molecular size than its unlabelled counterpart. Lane 1 DIG labeled SL0525 PCR product; Lane 2 Unlabeled SL0525 PCR product; Lane 3 Control DIG PCR product contained in Roche kit.

2.4.4. Production of SL0525 polyclonal antibody

In order to obtain a specific antibody for use to detect the corresponding protein in sea lice preparations, it was decided to prepare a purified SL0525 protein and use it to immunize a rabbit. The 717bp SL0525 DNA fragment was subcloned into a pGEX-4T-1 vector and transformed into *E. coli*. The DNA insert was confirmed by restriction digestion using BamHI and XhoI (Fig. 2.10) and by DNA sequencing.

The SL0525 GST-fusion protein was successfully expressed by inducing the tac promoter of the pGEX vector with IPTG. Cells of BL21 *E. coli* transformed with pGEX-4T-1 vector and induced with IPTG produced a protein of 26kDa (GST protein; Fig. 2.11, lane 1). *E. coli* transformed with pGEX-4T-1-SL0525 plasmid and induced with IPTG produced a protein of around 53kDa (GST–SL0525 fusion protein; Fig. 2.11, lanes 2 and 3). Purification using a glutathione sepharose column and thrombin cleavage yielded uncontaminated SL0525 protein (Fig. 2.12).

Western blotting shows that the SL0525 protein could be specifically detected by the polyclonal antibody against this protein, and there was also no cross-reactivity with the GST portion of the fusion protein indicating that a highly efficient protein purification procedure was performed for the SL0525 protein (Fig. 2.13). Pre-immunized rabbit serum was applied as a negative control and did not result in any detectable precipitation. Incidently, neither C219 nor JSB-1 showed cross-reactivity to this partial SL0525 protein, suggesting the epitopes recognized by either antibody are not present within this portion of the protein. Without the full SL0525 protein it is difficult to predict whether the epitopes recognize by these antibodies are absent in the full protein.

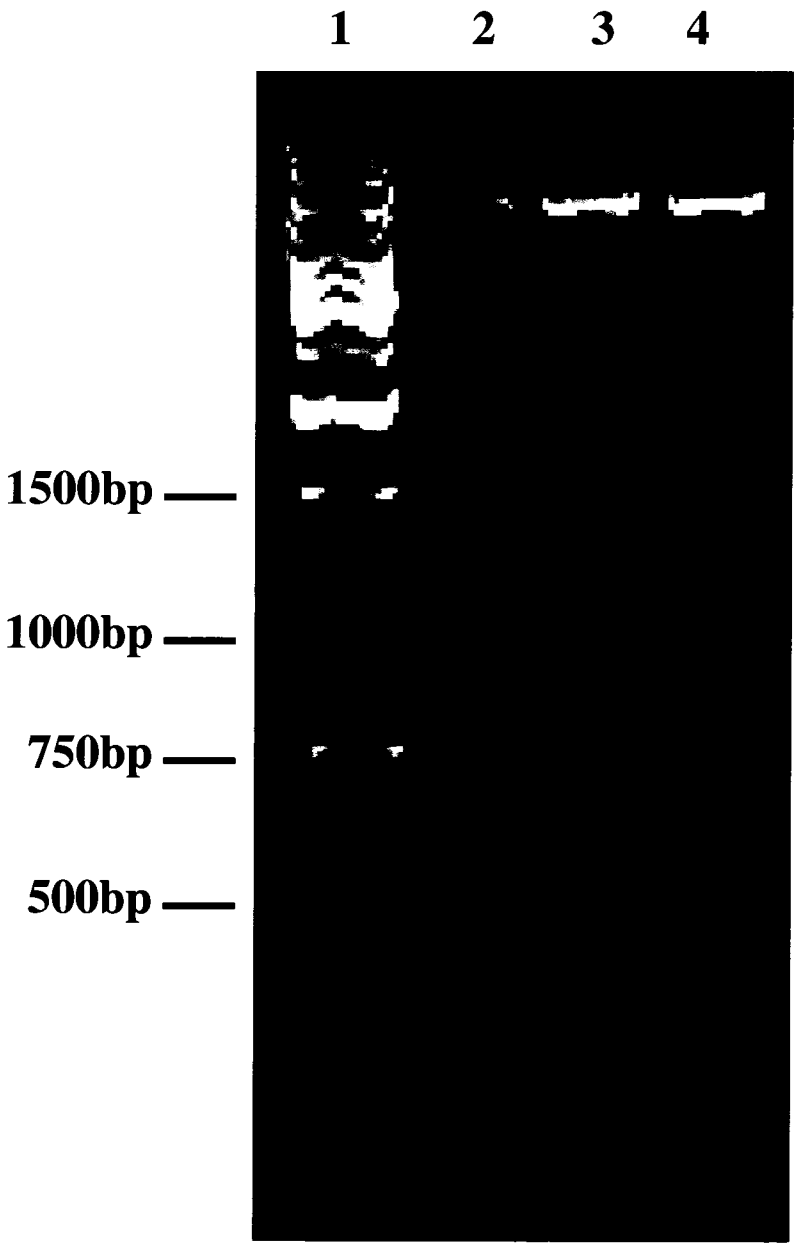

Figure 2.10. 1% agarose gel showing the identification of recombinant plasmid pGEX-4T-1-SL0525. Lane 1, Molecular weight marker; Lane 2 and 3, pGEX-4T-1-SL0525/XhoI and BamHI digest; Lane 4, pGEX-4T-1-no insert/XhoI and BamHI digest.

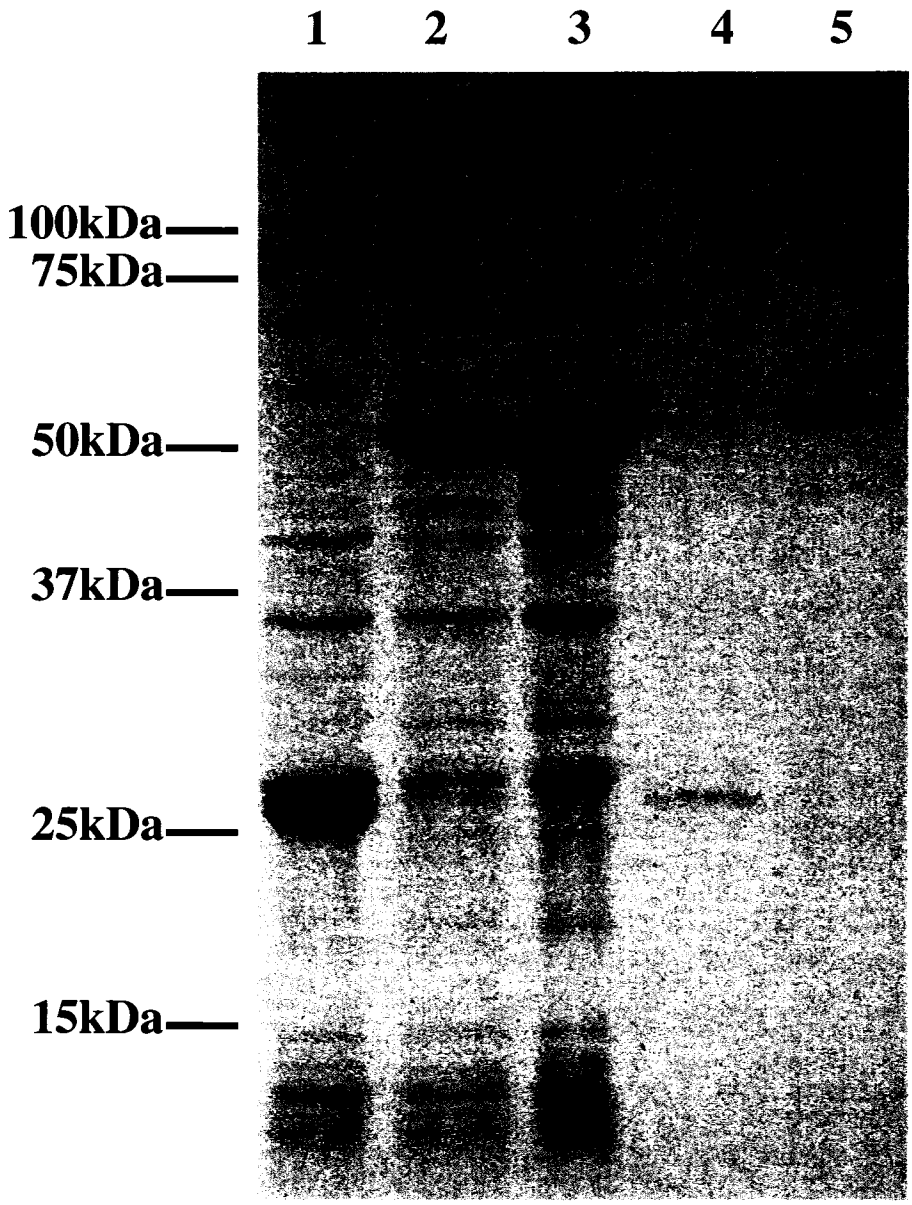

Figure 2.11. Solubilization of GST fusion protein and purification using glutathione beads run on a 12% SDS gel stained with Coomassie Blue dye; Lane 1, IPTG Induced control GST protein cell lysate; Lanes 2 and 3, IPTG Induced GST fusion protein cell lysate; Lane 4, Control GST protein elution after purification with glutathione beads; Lane 5, GST fusion protein elution after purification with glutathione beads.

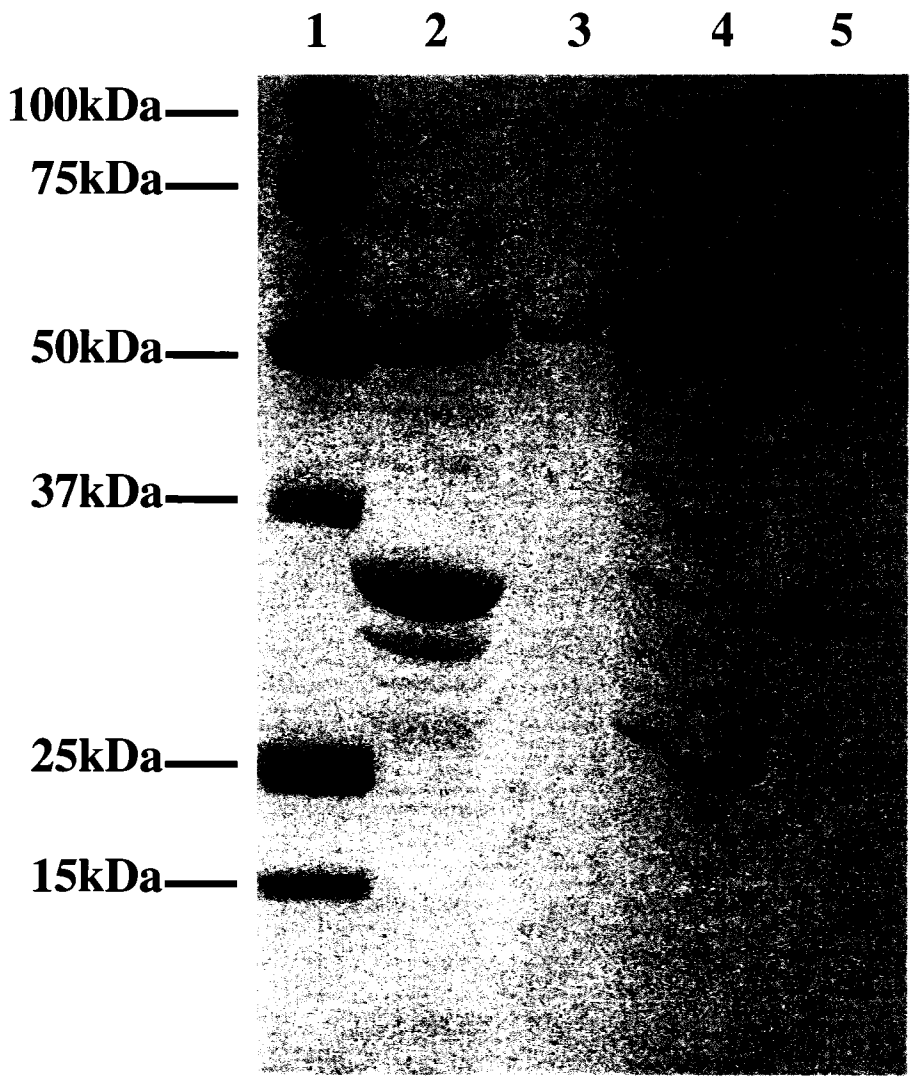

Figure 2.12. Purification of SL0525 protein using thrombin run on a 12% SDS gel stained with Coomassie Blue dye. Lane 1, Protein Ladder; Lane 2, IPTG Induced GST fusion protein cell lysate; Lane 3, GST fusion protein; Lane 4, Bacterial protein elution with GST fusion protein removed; Lane 5, Thrombin cleaved SL0525 protein

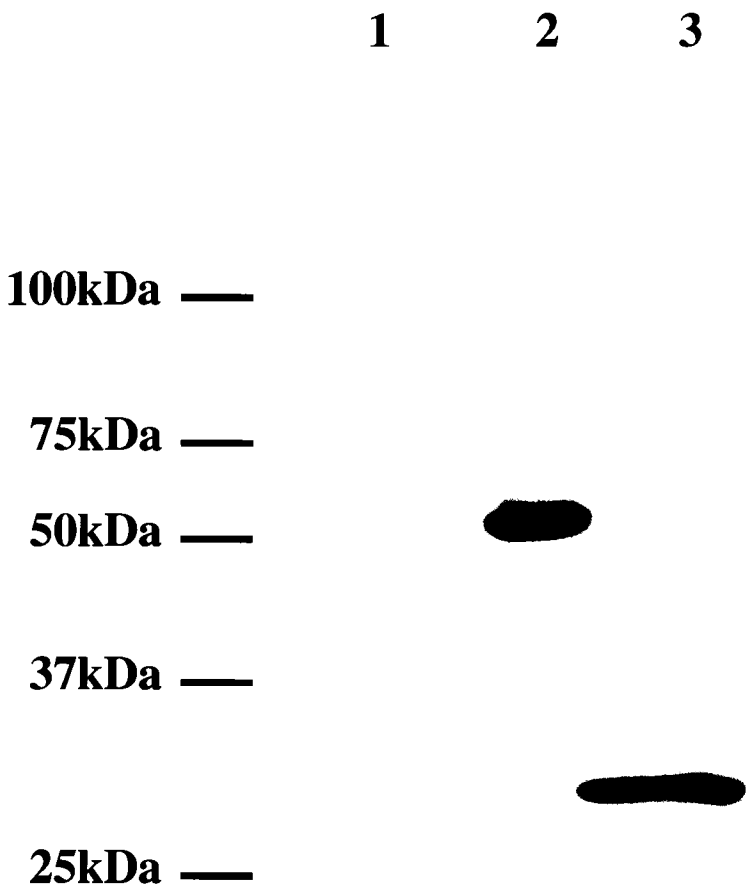

Figure 2.13. Specificity analysis of anti-GST-SL0525 antiserum using Western blotting. Lane 1, GST protein; Lane 2, GST-SL0525 protein; Lane 3, Thrombin cleaved SL0525 protein.

2.4.5. Protein detection using Western blot analysis

Using either of the C219, JSB-1 or SL0525 antibodies each detected a single band of 160kDa in the sea lice protein sample (Fig. 2.14), which is an appropriate molecular mass for P-gp. In the control rat liver protein sample, a single band of 170kDa was detected with C219 and JSB-1; this is the molecular mass expected for P-gp in this animal. SL0525 did not react with any rat protein. The C219 showed cross-reactivity to a smaller unknown protein of ~70kDa in the rat liver presumably possessing similar epitope homology to the C219 P-gp binding site. The JSB-1 antibody showed weak immunoreactivity to a smaller ~70kDa unknown sea lice protein. Pre-immune serum was used as a negative control and showed no reactivity to any protein.

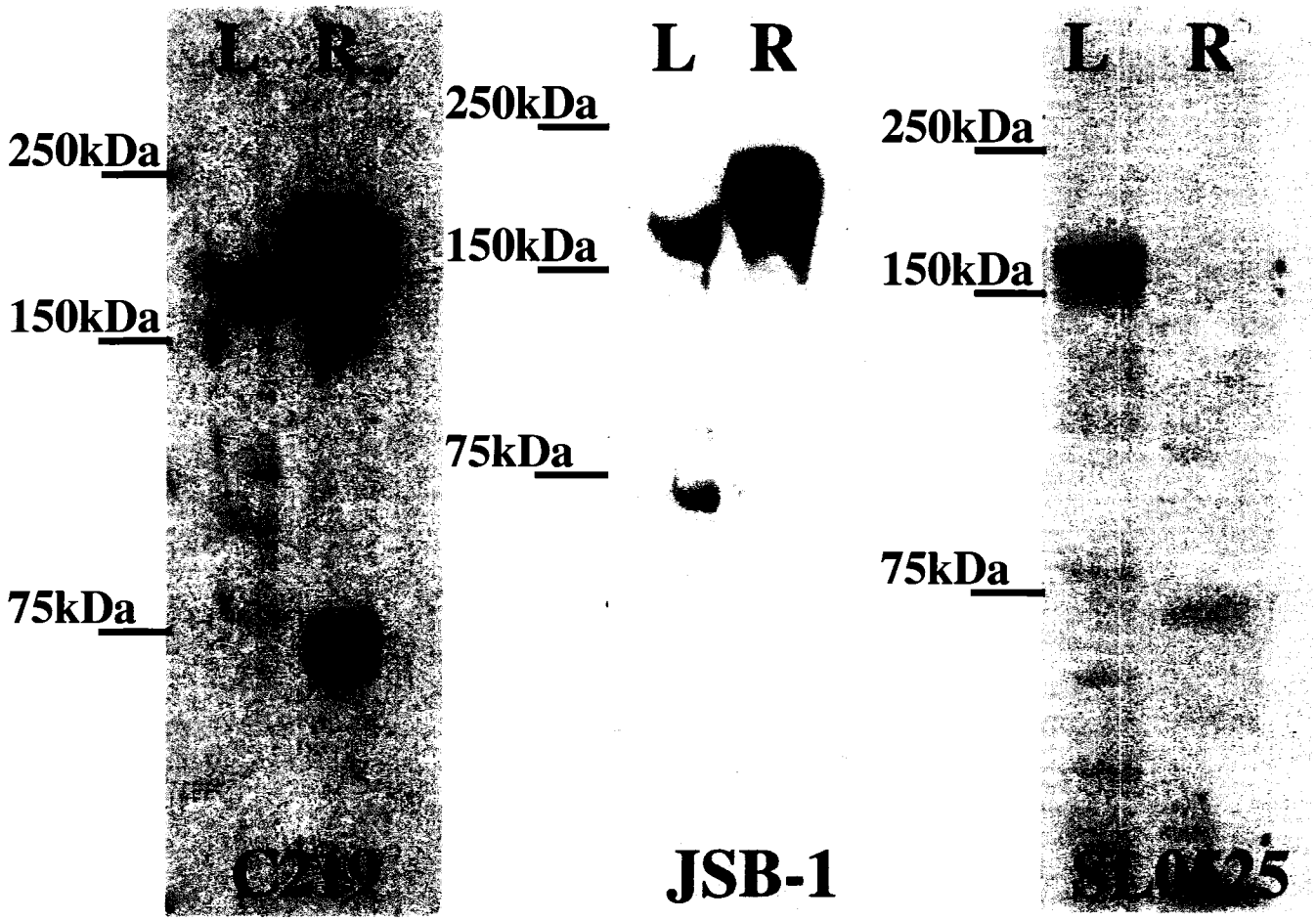

Figure 2.14. Immunoreactivity in both sea lice and rat liver using C219, JSB-1. and comparison to the protein detected by the SL0525 antibody (Western blot analysis).
L: 50µg adult sea lice membrane protein; R: 50µg rat liver membrane protein.

2.5. Discussion

Macrocyclic lactones are good substrates for P-gp transporters, with the avermectins being more potent substrates than the milbemycins (Pouliot *et al.,* 1997; Griffin *et al.,* 2005). Mammals deficient in P-glycoprotein display hypersensitivity to ivermectin (Schinkel *et al.,* 1994; Roulet *et al.,* 2003). Early invertebrate research found alterations to P-glycoprotein and increased mRNA levels in ivermectin resistant *H. contortus* (Xu *et al.,* 1998). Selection at a P-glycoprotein allele following ivermectin treatment has been observed in *H. contortus* (Blackhall *et al.,* 1998), and more recently *O. volvulus* (Ardelli *et al.*, 2006). To investigate potential mechanisms of emamectin benzoate resistance in sea lice, an important preliminary step is to identify and characterize candidate resistance genes. In this study, we screened two sea lice EST library databases, a sea lice cDNA library, and also used degenerate primers in RT-PCR to identify potentially relevant ABC transporter genes.

2.5.1. Gene Identification

In this study partial sequences of two ABC transporter genes, SL0525 and SL-Pgp1, were identified in *L. salmonis*. The SL0525 gene product was isolated from a sea lice EST database (NRC, Halifax). After a lengthy optimization process, the SL-Pgp1 gene sequence was generated by touchdown RT-PCR using degenerate primers previously used by Xu *et al.* (1998). 3` RACE successfully extended these genes, however, to obtain full length sequences of these genes, it is estimated that 5` extension of 4kb is required for each gene. The 5` RACE kit has only previously been successful in extending sequences of a maximum of 2kb (personal communication Roche). 5`

extension of these genes was attempted and modification to the original protocol involving numerous re-amplifications, different annealing temperatures, and the use of different high fidelity Taq Polymerases did not generate a detectable product. The failure to extend these two sequences therefore may reflect the limitations of the RACE kit being used.

After cDNA library construction, the library was validated for the presence of gene inserts in the expression vectors of the phage. This screen revealed that 80% of the selected phage contained a gene insert.

The screening of the sea lice cDNA library, which amounted to over 60,000 clones, failed to identify a positive clone. A previous study performed on a normalized *Sarcoptes scabiei* cDNA library only identified a single P-gp clone from the resulting EST database and PCR screen (Mounsey *et al.,* 2006). The sea lice cDNA library used in this study was not normalized and therefore the library did not enrich the population of rare genes. It is likely that both SL0525 and SL-Pgp1 genes are expressed in relatively low amounts and, therefore, due to the lack of normalization, the screening failed to identify any positive plaques. For effective library screening it is therefore important to reduce the number of commonly occurring genes in the cDNA pool, thus increasing the potential for identifying a clone of interest. This is also true for EST library construction, as both libraries screened in this study were also not normalized and resulted in only a single ABC transporter gene (SL0525) being identified.

Splice leaders are commonly seen in nematode and trypanosomes and can be described as an RNA-processing reaction called *trans* splicing, which results in the addition of a small, conserved (22- to 41-nucleotide) leader exon-like sequence (splice

leader) onto the 5' ends of some or all mRNAs (Ross *et al.,* 1995). Primers based on these regions were used by Xu *et al.* (1998), to extend to the 5` end of the P-gp sequence in *H. contortus*. Using two primers based on the two most common splice leaders in combination with gene specific SL0525 or SL-Pgp1 reverse primers in RT-PCR failed to generate a product. This either reflects a lack of *trans* splicing or variation in splice leader sequences in sea lice.

Invertebrate P-gp sequences show an average overall similarity of approximately 65%. BLAST results of the SL-Pgp1 sequence show this gene to have approximately 72% homology with other P-gps, the highest identity being to *D. melanogaster* P-gp (77%). From the sequence information available, a BLAST search showed SL-Pgp1 had a high level of similarity to other P-gps (~73%) whereas the SL0525 had a lower level of overall similarity (~35%). Previous studies identified the P-gps in both *H. contortus* and *Cylicocyclus spp.* having similarities ranging from 60 – 73% with other nematode P-gps (Xu *et al.,* 1998; Drogemuller *et al.,* 2004). It is therefore evident that the similarity seen with the SL0525 amino acid sequence is relatively low. It is, however, important to recognize that relating amino acid similarity to specific protein function cannot be made with complete confidence without further characterization.

A distinctive characteristic of ABC transporters is the presence of a nucleotide binding domain containing several conserved regions; Walker A (GXXGXGK[S/T]), Walker B (XXXXD[E/D]), Signature (LSGGQ), glutamine loop and histidine loop (Qian *et al.,* 2006). Both SL0525 and SL-Pgp1 amino acid sequences were aligned to the P-gp sequence used previously for primer design. The SL-Pgp1 sequence contains all of these conserved regions (Fig. 2.4). SL0525 sequence contains both Walker A /B and signature

motifs. In both loop regions of the SL0525 sequence, the conserved glutamine and histidine residues are substituted for glutamic acid (Fig. 2.4). The significance of substituting glutamine or histidine with a negatively charged amino acid is difficult to predict, although, as these are regions within the NBD they would not affect substrate specificity. The identification of these two genes is very significant for our investigation into potential multidrug transporters in *L. salmonis*. Future studies will investigate both the copy number and expression levels of these two genes and, importantly, determine if these genes up-regulate in response to emamectin benzoate exposure. This will offer insight into the potential involvement of these two genes in future resistance development to EMB in sea lice.

2.5.2. Protein Detection using Western Blot Analysis

This study demonstrates the efficient expression and purification of a sea lice ABC protein fragment, which was used to immunize a rabbit for the production of antiserum. This GST-fusion protein system has been applied widely in many fields of research. A major limitation of this system is the fact that many GST fusion proteins are partially or completely insoluble after lysis in nonionic detergents (Frangioni and Neel, 1993). During this study we used a method which utilizes the sodium salt of the alkyl anionic detergent N-laurylsarcosine previously demonstrated to effectively solubilize GST fusion proteins while still maintaining protein activity (Frangioni and Neel, 1993). Because the GST handle was cleaved off the fusion protein using thrombin, the resulting polyclonal antibody was only directed against epitopes of the SL0525 protein and not the GST portion of the fusion protein. This specificity was confirmed by the use of Western blot analysis (Fig. 2.13). The lack of cross-reactivity to GST also meant that its

application in either Western blot analysis or immunohistochemistry would result in the specific binding to SL0525 epitopes without the uncertainty of cross-reactivity to GST proteins. This method represents an efficient method for the production of a protein specific antibody and would be advantageous in immunological studies of invertebrate species where commercially available specific antibodies are very limited.

Both C219 and JSB-1 antibodies have been used extensively in the analysis of P-glycoprotein expression in various organisms, including numerous invertebrate species such as *H. contortus*, *Corbicula fluminea*, *Mytilus edulis*, and *Pandalus borealis* (Minier *et al.,* 1993; Lyons-Alcantara *et al.,* 2002; Kerboeuf *et al.,* 2003; Achard *et al.,* 2004). C219 recognizes an internal, highly conserved amino acid sequences corresponding to C-terminal and N-terminal regions of the NBD's, found in isoforms of P-gp (van den Elsen *et al.,* 1999). JSB-1 recognizes a highly conserved cytoplasmic epitope of the human P-gp (Scheper *et al.,* 1988). These antibodies can yield basic information concerning the occurrence and distribution of P-gp in an organism.

Immunoreactivity to these three antibodies was seen in the sea lice membrane protein fraction. The signal generated at 160kDa resembled P-gp with respect to the expected molecular mass (Fig 2.14). However, it is possible that within this 160kDa mass there are a number of proteins sharing epitope homology (possibly various isoforms of P-gp) that could have been detected by each of these antibodies. Further research will examine the localization of P-gp in sea lice using these antibodies on whole sea lice sections.

The SL0525 polyclonal antibody did not cross-react to any membrane protein in rat liver which demonstrated the specificity of the antibody. The original BLAST analysis

of the SL0525 sequence indicated it to belong to the ABC-F subfamily (Appendix A). However, the estimated size of the SL0525 full-length protein determined from the Western blot results suggest that this gene is too large to encode for a protein of the ABC protein subfamily F. These proteins do not contain transmembrane regions and thus containing only two fused nucleotide binding domains. These proteins are classified as "half transporters" and are only approximately 90kDa in size (Klein *et al.,* 1999).

In summary we have identified two partial gene sequences, encoding for sea lice ABC proteins. The SL0525 gene fragment was used to produce a specific polyclonal antibody. This antibody was demonstrated to detect 160kDa protein(s), the same size as that detected using C219 and JSB-1. These antibodies were further used to investigate the localization in sea lice (Chapter 3). It was hypothesized that the expression of P-gp would be localized primarily to the intestinal tract as the intestine is the initial absorption site for emamectin and thus a critical site for decreased drug absorption should P-gp be over-expressed. In addition to the sea lice, the expression of P-gp was examined in the Atlantic salmon host. Changes in P-gp expression may alter the pharmacokinetics of SLICE® subsequently lowering the actual concentration of drug to which the sea lice are exposed. Localizing the expression of the SL0525 protein in sea lice would also help gain a better understanding of its potential function.

In subsequent investigations we also apply the sequence information from these two genes by performing quantitative analysis to determine their relative levels of expression and possible changes in their transcription levels in response to EMB

exposure (Chapter 4). This study offered insight into the implications of P-gp over-expression and the potential for resistance development.

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CHAPTER 3. IMMUNOLOCALIZATION OF P-GLYCOPROTEIN IN SEA LICE (*LEPEOPHTHEIRUS SALMONIS*) AND ITS HOST THE ATLANTIC SALMON (*SALMO SALAR*)

3.1. Abstract

The continued dependence on emamectin benzoate for the control of sea lice *Lepeophtheirus salmonis* brings with it the risk of resistance development. The overexpression of the energy-dependent transporters, P-glycoprotein (P-gp), is suggested to be a major mechanism conferring resistance to avermectins in both nematodes and arthropods. The aim of this study was to examine the presence of P-gp in both sea lice and its Atlantic salmon host using monoclonal antibodies (C219 and JSB-1) and a new polyclonal antibody (SL0525) generated against a putative sea lice P-gp. C219 was the only antibody to localize P-gp in all three salmon tissues (intestine, kidney and liver). American lobster was used as a reference crustacean for *L. salmonis* immunostaining reactions and showed positive staining for P-gp in the hepatopancreatic and intestinal tissues with all three antibodies. The *L. salmonis* showed positive staining for P-gp in the intestinal epithelial lining with all antibodies. This report represents the first documented evidence for the expression of P-gp in *L. salmonis*, its Atlantic salmon host, and the American lobster. The expression of P-gp in sea lice demonstrates that sea lice have one of the mechanisms present whose over-expression has been documented to be responsible for resistance development in other invertebrate species.

3.2. Introduction

The salmon louse, *Lepeophtheirus salmonis*, is an ectoparasitic copepod of Atlantic salmon (*Salmo salar*). Control of infections has been largely through the use of chemotherapeutic drugs that are selectively toxic to the parasite (Pike & Wadsworth 1999). Emamectin benzoate, the active ingredient in SLICE®, is currently one of the most effective chemotherapeutants administered against sea lice infections (McHenery & Johnson 2000; Schering Plough Animal Health 2003) and represents the most commonly used compound to control sea lice infections in Atlantic Canada (Westcott *et al.,* 2004). SLICE® is safe for salmon when administered at a dose of 0.05mg kg^{-1} for a seven-day period and at this dose is highly efficacious (99%), killing all parasitic stages of sea lice, i.e. chalimus, pre-adults and adults including gravid females (Stone *et al.,* 2000). When SLICE® is administered to fish, it is readily absorbed in the gut due to its highly lipophilic nature and is distributed into areas including the skin and mucus (Kim-Kang *et al.,* 2004; Sevatdal *et al.,* 2005). When sea lice feed on treated fish, emamectin benzoate is absorbed through the intestinal tract and distributed into the tissues of the lice. Avermectins bind irreversibly to both γ-aminobutyric acid (GABA) – and glutamate-gated chloride channels causing an increased flow of chloride ions into nerve cells which results in paralysis and death of the lice.

P-glycoproteins (P-gps), members of the ATP-binding cassette (ABC) superfamily, are expressed constitutively in many tissues including the intestinal epithelia, blood brain barrier, bile canaliculi, and renal tubules in mammals (Cascorbi 2006). P-gps function as ATP-dependent transporters for a wide number of structurally diverse compounds including anticancer drugs, steroid hormones, cardiac drugs,

immunosuppressants, antimicrobial agents and other miscellaneous agents including the macrocyclic lactones (Cascorbi, 2006). Due to the wide variety of compounds recognized, P-gps play a major role in the absorption, distribution, metabolism, and excretion of many drugs. Different studies have demonstrated the presence of P-gps in marine vertebrates and invertebrates using immunological methods (Hemmer *et al.,* 1995; Lyons-Alcantara *et al.,* 2002). Studies directed at immunolocalization of P-gps in teleosts showed that the distribution resembled that seen in mammals (Hemmer *et al.,* 1995).

The over-expression of P-gp has been implicated in resistance development in numerous invertebrates including the trichostrongyle nematode *Haemonchus contortus* by preventing drug uptake from the intestine and distribution to target tissues (Blackhall *et al.,* 1998). P-gp expression has not previously been identified in sea lice and its detection could prove useful in understanding the potential for resistance development to SLICE®.

The aim of this study was to examine the presence of P-gp in both sea lice and their Atlantic salmon host. Two widely used monoclonal antibodies (mAb C219 and JSB-1) against conserved human P-gp epitopes, as well as a polyclonal antibody (pAb SL0525) generated in our lab against a putative sea lice P-gp were analyzed for their immunoreactivity to tissues fixed in different fixatives previously suggested as useful for P-gp localization in teleost tissues (Hemmer *et al.,* 1998).

3.3. Materials and Methods

3.3.1. Antibodies used

The mAbs C219 and JSB-1 were purchased from Abcam (Cambridge, MA). C219 recognizes an internal, highly conserved amino acid sequence VQEALD and VQAALD, within the N-terminal domain of human *MDR1* and *MDR2* isoforms, respectfully, and VVQEALD in the C-terminal domains of both MDR isoforms. JSB-1 recognizes a highly conserved cytoplasmic epitope specific to the human *MDR1*. pAb SL0525 was generated in our lab against a sea louse ABC transporter purified from a GST-fusion protein expressed in *Escherichia coli*. The gene used to express this protein was isolated from a sea lice expressed sequence tag (EST) data base (provided by Dr Neil Ross, NRC-IMB, Halifax, NS).

3.3.2. Tissue preparation

Sea lice were obtained from infected farmed Atlantic salmon (*Salmo salar*) from the Bay of Fundy, NB, Canada. After removal from the salmon the sea lice were immediately placed in a fixative solution. The Atlantic salmon and lobsters (*Homarus americanus*) used were donated by colleagues at the Atlantic Veterinary College. Lobsters and sea lice are both crustaceans and so lobsters served as a reference organism during this study. Animals were euthanized according to Canadian Council on Animal Care guidelines, fish were placed in a bath containing a lethal does of buffered tricaine methanesulfonate (TMS) at a concentration of 500mg l^{-1}; lobsters were injected with a lethal concentration of KCl (100mg g^{-1}). Intestine (lobster and salmon), hepatopancreas (lobster), kidney (salmon), and liver (salmon) were rapidly removed after euthanization

and immersed in fixative. Tissues were subsequently cut into smaller pieces of 50 mm^2 to ensure optimal fixation. For paraffin embedding, tissues were placed in one of each of the following fixatives for 72 h: 10% buffered neutral formalin; Bouin's fixative: saturated aqueous solution of picric acid (75 ml), 37% formalin (25 ml), and glacial acetic acid (5 ml); Dietrich's fixative: 37% formalin (39 ml), glacial acetic acid (6 ml), 95% ethanol (90 ml), and distilled water (180 ml). Specimens fixed in Bouin's were rinsed for 4 h in running tap water. All tissues were then dehydrated in EtOH, cleared in xylene, and embedded in paraffin. Tissues were sectioned at 5 μm, placed on positively charged slides, and either air dried overnight or oven dried at 56°C for 3 h.

Sea lice were also placed in a variety of cryoprotecting solutions for frozen tissue sectioning. The solutions used included: glycerol/phosphate solution: $NaH_2PO_4 \cdot H_2O$ (9.37g), NaOH pellets (2.14g), thimerosal (0.05g), ddH_2O (450ml), glycerol (50ml); sucrose/phosphate solution: $NaH_2PO_4 \cdot H_2O$ (9.37g), NaOH pellets (2.14g), sucrose final concentration 10%, 20%, and 30% (50g, 100g, 150g, respectively), thimerosal (0.05g), ddH_2O (500ml). Sea lice were embedded in Shandon Cryomatix™ (Fisher, Ottawa, ON) on a freezing stage placed on a pool of liquid nitrogen. Blocks of frozen tissue in Cryomatrix were cut into 25-40μm sections on a cryo-microtome at -20°C and placed on positively charged slides.

3.3.3. Immunohistochemistry procedure

Tissue sections were deparaffinized and rehydrated. To optimize antigenicity some sections fixed in formalin were incubated for 10 min in a sodium citrate solution buffer (10mM sodium citrate, 0.005% Tween 20, pH 6.0) pre-heated to 95°C. The buffer

was allowed to cool on ice for 5 min before further processing of the sections. All sections were then pre-treated for 10 min with 1% H_2O_2 in water, and rinsed in phosphate buffered saline (PBS) (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 per liter).

Atlantic salmon and lobster tissue sections were blocked with 5% normal horse serum (NHS) for 20 min. Sea lice tissue sections were not incubated in NHS before addition of the primary antibody. All sections were then incubated overnight at 4°C in a moist chamber with one of the following primary antibodies: C219 (1:40), JSB-1 (1:50), or SL0525 (1:16,000). The sections were washed with PBS and primary antibody was detected using either the mouse IgG (C219 or JSB-1) or rabbit IgG (SL0525) Vectastain®Elite ABC Kits (Vector Laboratories, Burlington, ON) according to the manufacturer's instructions, with a minor modification: the secondary biotinylated antibody was used at a dilution of 1:100 in PBS. All sections were washed with PBS and the resulting complexes of immobilized antibody/streptavidin conjugated to peroxidase were detected by the addition of 300µl of 3,3`-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 for 2 min. Slides were rinsed with tap water, dehydrated to xylene and mounted with Permount® (Fisher). Negative controls included serial sections of tissue to which either (1) no primary antibody was added; (2) rabbit IgG directed toward an antigen known not to be present in any of the tissue sections had been applied at the same concentration of SL0525 or (3) use of SL0525 after absorption with the antigen it was originally raised against. These controls were processed in parallel with test sections.

3.4. Results

3.4.1. Tissue processing and P-glycoprotein antibody immunostaining characteristics

Of the three fixatives, only the formalin-fixed tissues exhibited staining reactions in sea lice, Atlantic salmon, and the American lobster. Antigen retrieval using a sodium citrate buffer enhanced the signal intensity of all antibodies, although this process was not essential for a staining reaction. Air drying the tissue sections rather than heat drying was essential for a staining reaction since there was no staining of heat dried tissues.

Cryoprotection of sea lice for frozen tissue sectioning using any of the solutions did not sufficiently preserve the integrity of the sea lice for successful staining.

3.4.2. Immunolocalization of P-glycoprotein in sea lice

Although crustaceans, sea lice are copepods and therefore do not possess an hepatopancreas to compare to the staining seen in the lobster tissue. Organ selection in sea lice was not an issue due to the ability to use whole body sections containing numerous organs. The primary interest was to determine if the intestinal tract expressed P-gp; other organs in the plane of section were also examined. All antibodies showed immunoreactivity in the intestinal tract of the sea lice although the signal gained using C219 was far weaker and less defined than that gained using JSB-1 or SL0525 (Fig. 3.1). The oogonia within the paired ovaries of the sea lice stained positive for cytoplasmic P-gp expression only with C219 (Fig. 3.1E). The pre-immune serum control did not generate any specific signal (Appendix B).

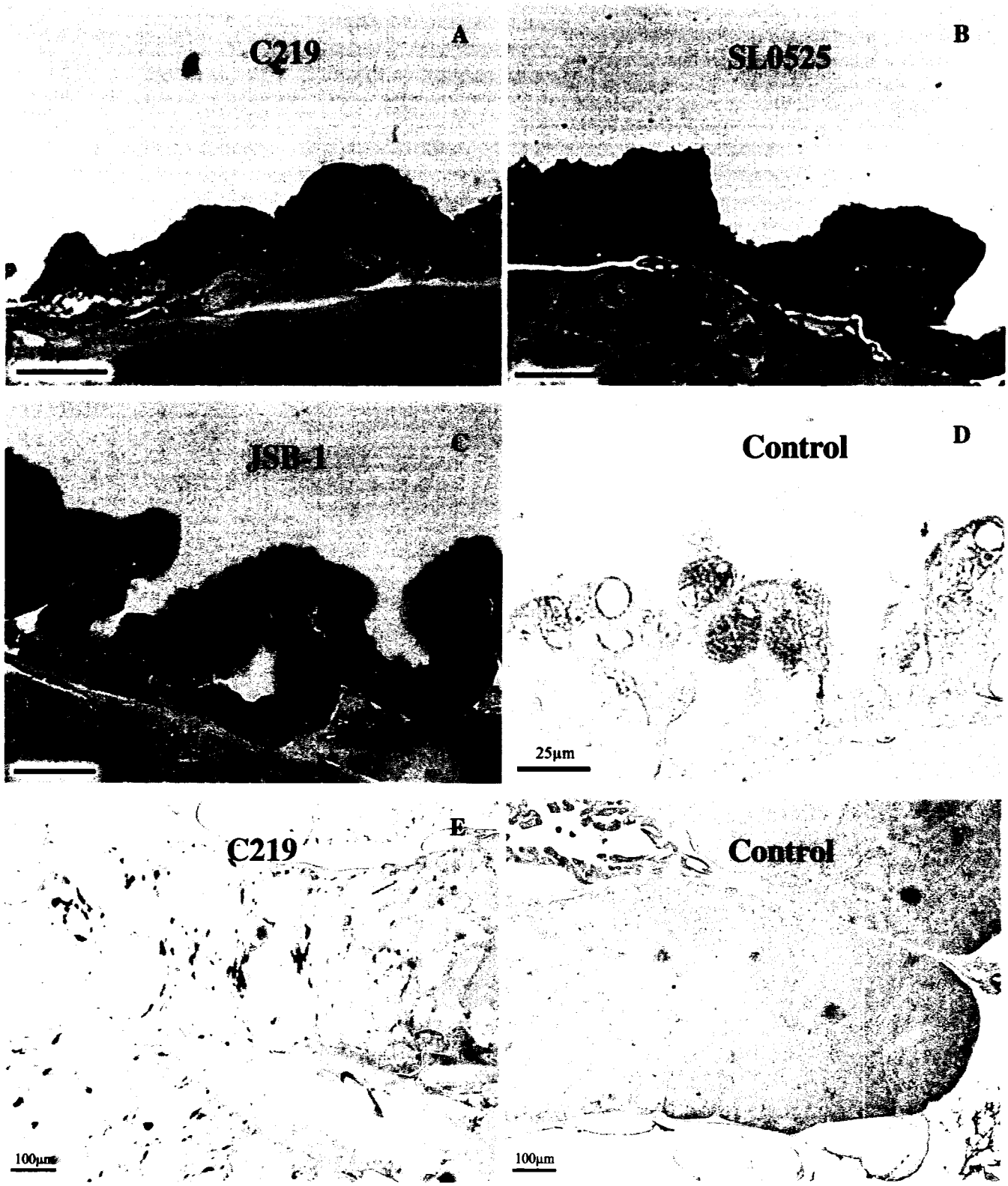

Figure 3.1. Immunolocalization of P-gp in *Lepeophtheirus salmonis* intestine and ovary. Immunoreaction of epithelial surface with (A) C219 (B) SL0525 (C) JSB-1, (D) represents intestine where primary antibody was omitted from the protocol, (E) C219 reacts with the oogonia, (F) C219 was omitted from the protocol.

3.4.3. Immunolocalization of P-glycoprotein in lobster tissues

All antibodies showed specific staining of the luminal surface of the intestinal epithelium, although the signal intensities varied between antibodies. Both JSB-1 and SL0525 generated a stronger immunostaining reaction than C219 (Fig. 3.2). The hepatopancreas showed immunohistochemical staining for P-gp within the F-cells (identified on the basis of their morphological characteristics as discussed by Sousa *et al.*, 2005) and along the luminal surface of the tubules (Fig. 3.3). SL0525 showed a stronger staining of the luminal surface than either the C219 or JSB-1. F-cells were intensely stained with all three C219, JSB-1, and SL0525 antibodies (Fig. 3.3A, B, and C). The pre-immune serum control did not generate any specific signal (Appendix B).

3.4.4. Immunolocalization of P-glycoprotein in Atlantic salmon tissues

Immunohistochemical staining with C219 in the salmon liver was localized to the bile canaliculi (Fig. 3.4A). Neither JSB-1 nor SL0525 showed any staining of the canalicular surfaces of the hepatocytes. C219 was also the only antibody to generate a signal in some of the renal tubules (Fig. 3.4B). Both C219 and JSB-1 specifically stained the luminal surface of intestinal epithelia with C219 showing a far greater intensity of stain reaction than that generated by JSB-1 (compare Fig. 3.4C and 3.4D). The polyclonal antibody SL0525 again demonstrated no immunoreactivity in these salmon tissues. All tissues where primary antibody was omitted from the protocol showed no staining reaction. The pre-immune serum control did not react to any proteins (Appendix B).

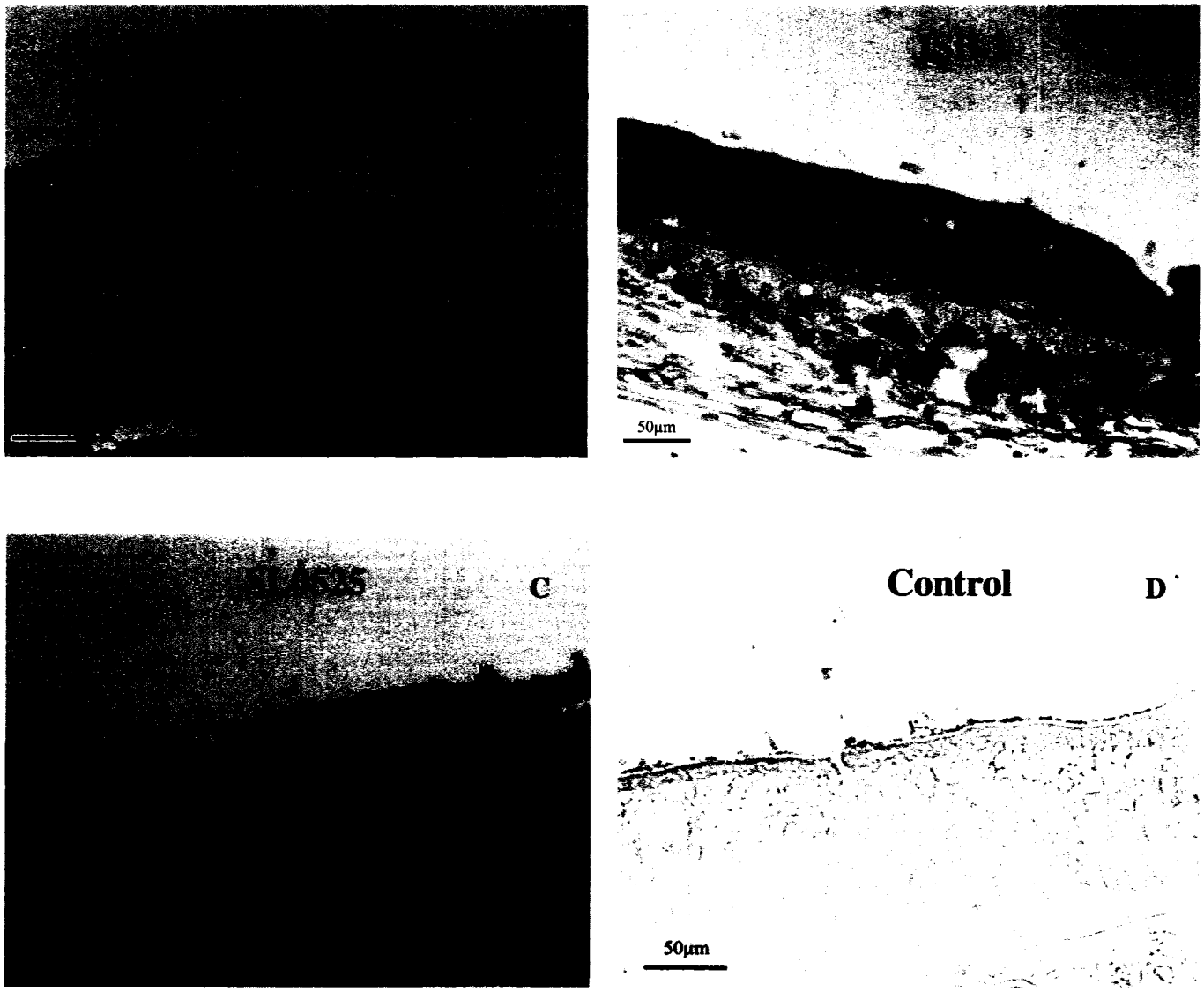

Figure 3.2. Immunolocalization of P-gp in *Homarus americanus* intestinal tissue with (A) C219 (B) JSB-1 (C) SL0525, (D) represents a control where primary antibody was omitted from the protocol.

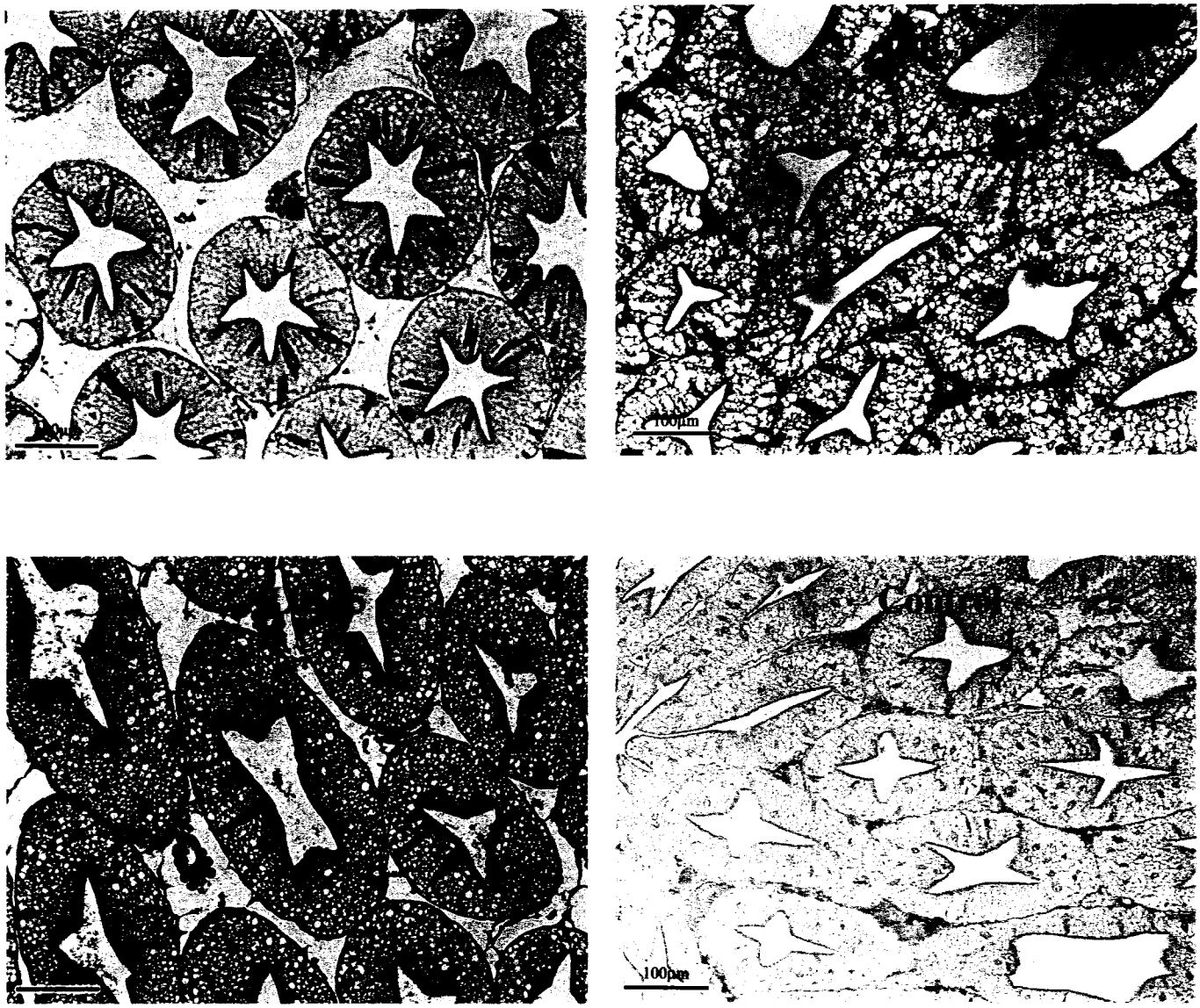

Figure 3.3. Immunolocalization of P-gp in *Homarus americanus* hepatopancreatic tubules. (A) C219 reacts strongly with F cells and weakly with luminal epithelial surface, (B) JSB-1 reacts strongly in F cells and weakly with luminal epithelial surface, (C) SL0525 reacts strongly with F cells and luminal surface, (D) represents a control where primary antibody was omitted from the protocol.

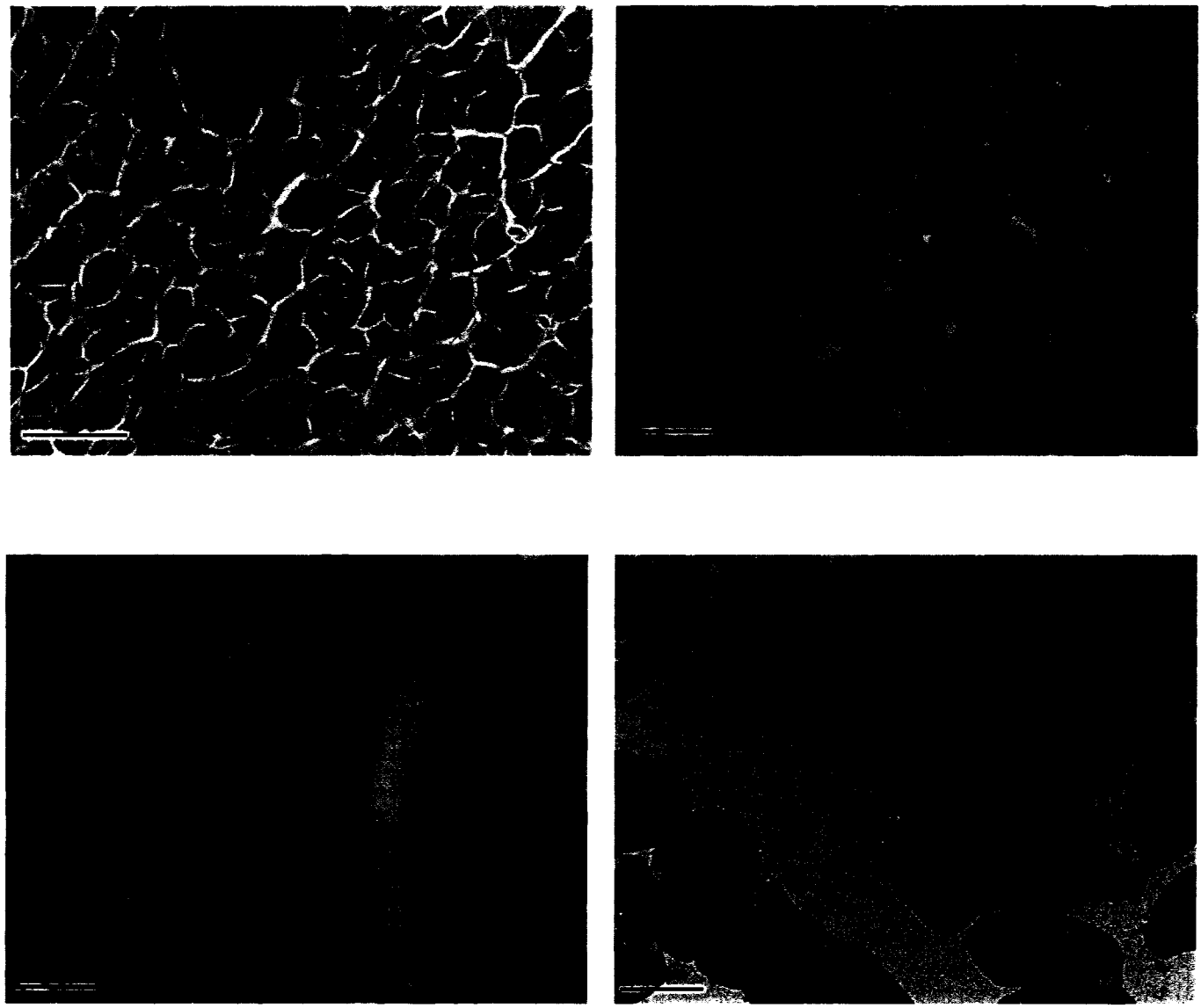

Figure 3.4. Immunoreactivity of C219 and JSB-1 against P-gp in *Salmo salar*. (A) C219 reacts with the canalicular surfaces of the hepatocytes, (B) C219 reacts with the luminal surface of many renal tubules, (C) C219 reacts strongly with the surface of intestinal epithelia, (D) JSB-1 reacts weakly with the intestinal epithelium.

The results of formalin-fixed sea lice, lobster, and Atlantic salmon tissues immunostained with C219, JSB-1 and SL0525 are summarized in Table 3.1.

Table 3.1. Summary of immunoreactivity of antibodies C219, JSB-1 and SL0525 against sea lice, lobster, and Atlantic salmon tissues.

Tissue	mAb C219	mAb JSB-1	pAb SL0525
Sea Lice intestine	*	***	***
Lobster intestine	**	***	***
Salmon intestine	***	*	-
Sea Lice Ovary	***	-	-
Lobster hepatopancreatic:			
F-cells	***	***	*
Luminal surface	*	**	***
Salmon liver	***	-	-
Salmon kidney	***	-	-

– negative reaction, * slight reaction, ** moderate reaction, *** strong reaction

3.5. Discussion

This study shows the development and optimization of methods for the immunolocalization of P-gp in sea lice and its Atlantic salmon host. American lobster was used as a control in the development of these methods due to the taxonomical closeness to sea lice. The two commercial antibodies (C219 and JSB-1) used in this study are directed toward different epitopes of human P-gp. It is possible that different P-gp isoforms could possess epitopes homologous to those recognized by any of these antibodies and therefore be immunoreactive. The nature of the pAb means it is directed toward numerous epitopes present in the SL0525 protein offering more chance of possible cross-reactivity to other proteins, although no indication of such cross-reactivity was seen. The use of multiple anti-P-gp antibodies with differing antigenic determinants reduces the likelihood of false positive results and allows determination of P-gp isotopes.

3.5.1. Tissue processing

Bouin's and Dietrich's have been used as fixatives for the immunodetection of P-gp in both rainbow trout (*Oncorhynchus mykiss*) and sheepshead minnow (*Cyprinodon variegates*) (Hemmer *et al.*, 1998; Sturm *et al.*, 2001). Using these fixatives in our laboratory did not generate a specific reaction for P-gp, which could be attributed to either the uniqueness of the specific species being used or the conditions used differing in some aspect. The effectiveness of formalin, a fixative which is commonly used in the clinical setting for the majority of mammalian P-gp studies was not evaluated by Hemmer *et al.* (1998) or Sturm *et al.* (2001). It was evident during this study that formalin fixation does not create the problem of cross-linking the P-gp epitope, with

staining being seen without antigen retrieval. Heat retrieval did, however, maximize the signal intensity.

Numerous groups have successfully used frozen tissue sections for immunodetection of P-gp (Georges *et al.,* 1990; Volk *et al.,* 2005); no previous studies have been conducted on crustaceans. The use of a variety of cryoprotection solutions were unable to maintain and preserve the tissue well enough to allow for cryosectioning. Penetration of the solution into the sea lice was considered as a possible factor for the ineffectiveness of these solutions. This was investigated further by piercing the sea lice exoskeleton several times using a 22 gauge needle before submerging in the solutions, but this still did not improve the cryoprotection of the internal organs. Thus sectioning was not possible following the pre-treatments described.

3.5.2. Immunolocalization of P-glycoprotein

Both sea lice and lobsters showed many similarities in the staining pattern and signal intensity generated for each of the antibodies used. Both showed immunoreactivity on the epithelial lining of the intestinal tract with the greatest intensity being seen with JSB-1 and SL0525. C219 only generated a weak signal suggesting that epitope presentation in the intestinal tract does not favor the binding of this antibody. Alternatively, the lower signal intensity in the intestinal tract seen with C219 could represent detection of a P-gp isotope in low abundance.

The staining pattern seen with all antibodies strongly suggest P-gp detection. The localization of P-gp in the intestinal tract of the sea lice was the primary interest of this study as the intestine is the initial absorption site for emamectin and thus a critical site for

decreased drug absorption should P-gp be over-expressed. Smith and Prichard (2002) also localized P-gp primarily in the intestinal tract of *H. contortus* and suggested over-expression as a potential mechanism of ivermectin resistance. Other staining was observed in the oogonia of the sea lice using C219; staining has previously been documented in ovarian tissue of shrimp, *Pandalus borealis* (Lyons-Alcantara *et al.,* 2002). This staining pattern was not seen with the other antibodies, and the cytoplasmic distribution of the signal seems to suggest that it could be cross-reactivity with a different protein than P-gp. The staining pattern generated in sea lice using the SL0525 appears to mimic that seen with JSB-1. Western blot analysis using SL0525 has shown this antibody to detect the same size 160kDa protein(s) in sea lice membrane fractions as that detected by both C219 and JSB-1. This data thus adds further to the evidence that this pAb is generated against sea lice P-gp, although this assumption requires further characterization of the SL0525 protein.

The hepatopancreas is a primitive form of digestive gland found in marine arthropods that provides the functions which in mammals are provided separately by the liver and pancreas. SL0525 generated a strong signal on the luminal surface of hepatopancreatic tubules, with only a weak signal being generated by C219 and JSB-1. The presence of P-gp on the luminal surface will function to eliminate various compounds from the organism (as in the liver and kidneys of the salmon). The F-cells of the hepatopancreas also showed strong immunoreactivity, although the cytoplasmic distribution suggests that this reaction is not P-gp specific. These cells are believed to be involved in the synthesis and secretion of digestive enzymes (Al-Mohanna *et al.,* 1985;

Sousa *et al.,* 2005). No comparison could be made with sea lice as they do not possess an hepatopancreas.

The salmon tissues showed expression of P-gp in all three tissues (liver, kidney, and intestine) investigated. The staining patterns generated by C219 compare well to those seen in other vertebrate species. C219 was the only antibody able to specifically detect P-gp in all three tissues with JSB-1 only generating a weak signal in the intestinal tissue. This suggests that P-gp in Atlantic salmon contains the highly conserved amino acid sequence VQEALD and/or VQAALD, whereas the epitopes recognized by JSB-1 and SL0525 are either not as well conserved or masked during histological processing. Based on our findings C219 should be chosen for any further immunohistological studies of P-gp expression in formalin fixed Atlantic salmon tissues.

SLICE®, containing emamectin benzoate as the active drug, is administered to salmon as an in-feed treatment. Once ingested by the salmon it is readily absorbed in the gut and distributed to the skin, mucus and blood which are fed upon by the sea lice (Sevatdal *et al.,* 2005). The expression levels of P-gp at the host and parasite level may therefore have an impact on the efficacy of SLICE®. Over-expression of P-gp in the salmon intestinal tract can both reduce drug uptake as well as allow for greater metabolism by the cytochrome P450 (CYP450) enzyme system via increased enterohepatic recirculation. In addition, increased P-gp levels in both the kidney and liver could increase drug elimination rates. Therefore increased P-gp expression in the salmon intestine, liver and kidney can subsequently alter the pharmacokinetics of SLICE® reducing the amount of drug present in the salmon mucus, skin and blood thus lowering the actual concentration of drug to which the sea lice are exposed. Over-expression of P-

gp is an important mechanism of resistance to avermectin compounds seen in numerous nematodes (Xu *et al.,* 1998; Ardelli *et al.,* 2006). Over-expression of P-gp in the sea lice intestinal tract could provide sea lice with a mechanism to actively limit the entry of many compounds, including emamectin, resulting in drug resistance. Due to the broad spectrum of drugs recognized by P-gp, over-expression of this protein will result in resistance to multiple drug groups and therefore resistance will not be limited to avermectins.

This report represents the first documented evidence for the expression of P-gp in the sea lice parasite, its Atlantic salmon host, and the American lobster. The expression of P-gp in sea lice does not imply that they are resistant to emamectin benzoate, but demonstrates that sea lice have one of the mechanisms present whose over-expression has been documented to be responsible for resistance development in other invertebrate species. The ability to analyze P-gp expression at both the host and parasite level allows treatment failure related to drug resistance to be investigated more comprehensively and possible treatment alternatives assessed. The methods developed in this study offer the potential as novel tools for the future monitoring of EMB resistance development in Atlantic salmon farms.

3.6. References

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CHAPTER 4. INDUCTION OF mRNA OF TWO PUTATIVE P-GLYCOPROTEIN GENES IN SEA LICE (*LEPEOPHTHERIUS SALMONIS*) IN RESPONSE TO EMAMCTIN BENZOATE EXPOSURE

Chapter accepted for publication:

Tribble ND, Burka JF, Kibenge FSB. (2007) Evidence for changes in the transcription levels of two putative P-glycoprotein genes in sea lice (*Lepeophtheirus salmonis*) in response to emamectin benzoate exposure. *Molecular and Biochemical Parasitology* doi:10.1016/j.molbiopara.2007.02.002

4.1. Abstract

Over-expression of P-glycoproteins (P-gps) is assumed to be a principal mechanism of resistance of nematodes and arthropods to macrocyclic lactones. Quantitative RT-PCR (Q-RT-PCR) was used to demonstrate changes in mRNA levels of two putative P-glycoprotein genes, designated here as SL0525 and SL-Pgp1, in sea lice (*Lepeophtheirus salmonis*) following exposure to emamectin benzoate (EMB). Pre-adult *L. salmonis* were challenged in an EMB bioassay for 24h and gene expression was studied from lice surviving EMB concentrations of 0, 10, and 30ppb. Gene expression was measured using Q-RT-PCR with elongation factor 1 (eEF1α) as an internal reference gene. One way analysis of variance followed by Bonferroni adjustments for multiple comparisons show that both target genes, SL0525 and SL-Pgp1, had significantly increased levels of expression with exposure to 10ppb EMB compared to the zero exposure group ($p = 0.009$ and $p = 0.015$, respectively). However, the group exposed to 30ppb showed no significant differences in gene expression. Comparative Ct analysis showed mRNA levels for SL0525 and SL-Pgp1 increased over 5 fold at 10ppb EMB. The results demonstrate that the upregulation of these target genes may offer protection in lice that are exposed to EMB. Our optimized Q-RT-PCR can be used to determine if over-expression of these genes could form the basis for development of resistance in sea lice and thus allow suitable alternative chemotherapeutic options to be assessed.

4.2. Introduction

Sea lice (*Lepeophtheirus salmonis*) are marine ectoparasitic copepods of salmonids affecting Atlantic salmon farms in many areas of the world (Johnson *et al.,* 2004). Presently the use of good husbandry practices, assisted by the use of chemicals and drugs to treat salmon helps to prevent sea lice outbreaks (Grant, 2002; Wall, 2005). Emamectin benzoate (EMB), a macrocyclic lactone, is one of the most effective drugs administered against *L. salmonis* infestation. EMB has a high efficacy against all parasitic stages of *L. salmonis*. Due to this high efficacy comes the consequence of overuse and limited rotation of other compounds in Atlantic salmon aquaculture, as was highlighted in a survey conducted in the Bay of Fundy, NB, Canada (Westcott *et al.,* 2004). Resistance development is becoming a major concern with the continued reliance on EMB as the sole chemotherapeutic source of *L. salmonis* control.

Macrocyclic lactones (avermectins and milbemycins) have been used to treat for various parasites of humans, pets, and livestock (Vercruysse and Rew, 2002). Resistance to avermectin compounds has become increasingly documented, most commonly in nematodes (Le Jambre *et al.,* 2000; Kaplan, 2002; Ardelli *et al.,* 2006). Studies have indicated three major potential resistance mechanisms involved against the toxicity of macrocyclic lactones, including decreased penetration, increased oxidative metabolism, and altered target site (Clark *et al.,* 1995). Of these resistance mechanisms, several studies indicate the most prevalent in terms of avermectin resistance is the over-expression of multi-drug transporter P-glycoproteins (P-gps) (Xu *et al.,* 1998; Molento and Prichard, 2001). Macrocyclic lactones are good substrates for P-glycoprotein transporters, with the avermectins being more potent substrates than the milbemycins

(Pouliot *et al.,* 1997; Griffin *et al.,* 2005), upregulation of these proteins would therefore serve to eliminate avermectins from the parasite, limiting the accumulation of a toxic concentration at the target receptors.

Increased P-gp activities and expression has been reported in various aquatic organisms following exposure to xenobiotics. P-gp expression in the field have been observed in populations of i) snails, ii) shrimp, iii) mussels, iv) oysters, and v) fish. Snails (*Monodonta turbinate*), collected from an unpolluted site were shown to accumulate 67% more ^{3}H-labeled vincristine in gill tissue than snails from a polluted site, therefore demonstrating less overall P-gp mediated efflux (Kurelec, 1995). Populations of grass shrimp (*Palaemonetes pugio*) exposed to increased amounts of urban and agricultural runoff in the field were shown to express higher levels of P-gp using Western blot analysis using the monoclonal antibody C494 (Fulton *et al.,* 1999). The level of P-gp expression has also been examined in oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis*), measured using protein dot blots with the monoclonal antibody C219. Again, there were higher levels at sites with organic pollutants (Minier *et al.,* 1993). Western blot and immunohistochemical studies of P-gp expression have also been conducted in the non-migratory fish species *Fundulus heteroclitus* which have a small home range and thus reflect xenobiotic exposure at the site where they were collected. Cooper *et al.* (1999) reported up to a three fold induction of hepatic P-gp in a population of *F. heteroclitus* exposed to PAH's in the field compared to fish from a reference site. Large individual variability was also observed in hepatic P-gp levels of these fish. These results suggest that individual fish may have variable abilities to respond to P-gp inducers.

The major advantage of using immunological analysis to determine changes in P-gp expression is that it measures the final protein produced apposed to measuring mRNA levels which assumes translation will occur for most or all of the transcripts. Immunological analysis does, however, have the disadvantage of lower sensitivity and specificity than quantitative RT-PCR. Commercially available antibodies used to detect P-gp are not 100% specific and are able to bind to other proteins with a homologous binding site. The available antibodies are directed toward human P-gp epitopes and thus differences in cross-reactivity with other species also limits the reliable of immunological analysis. The sensitivity of immunological analysis is also lower than that of quantitative RT-PCR and thus a small fold change in protein expression may not be detected using this method.

Northern blot analysis has determined that an ivermectin resistant phenotype is associated with P-gp over-expression in *H. contortus* (Xu *et al.,* 1998). However, no study to date has investigated the induction of P-gp in response to avermectin exposure, nor has quantitative RT-PCR (Q-RT-PCR) been used to analyze P-gp expression in response to any xenobiotic in a parasitic organism. Using Q-RT-PCR would allow for a sensitive method to detect increased mRNA levels of P-gp in response to EMB exposure. However, due to the absence of overt resistance to EMB in Atlantic salmon aquaculture at this time, no definite association can be established between P-gp over-expression and resistance to EMB.

Two putative sea lice P-gps genes were previously identified (Chapter 2). The SL0525 gene was isolated from a sea lice expressed sequence tag (EST) database (provided by Dr Neil Ross, NRC-IMB) and has similarity to multiple drug transporters

found in other species, particularly in the conserved Walker A and B motifs and the signature "LSGGQ" motif (Tip *el al.*, 2002). However, the overall homology of this protein sequence is only ~35% with other nematode and arthropod P-gps. This gene has been further characterized and has been demonstrated to encode a 160kDa protein localized on the epithelium of the intestinal tract of *L. salmonis*. The SL-Pgp1, amplified from sea lice total RNA using degenerate primers based on the conserved ATP-binding domains TVALVG and DEATSA in RT-PCR, has a higher overall level of homology to P-gp of other species (~73%).

The aim of this study was to establish if increased P-gp expression is correlated to *L. salmonis* exposure to EMB by using quantitative or real-time RT-PCR (Q-RT-PCR). Q-RT-PCR can be used to measure the mRNA levels of a regulated gene relative to a constitutively expressed gene as an internal reference. This study provides evidence for P-gp upregulation, thus offering insight into the potential for resistance development to EMB in sea lice.

4.3. Materials and methods

4.3.1. Sea Lice Collection

Gravid female *L. salmonis* were collected from Atlantic salmon farms in the Bay of Fundy, New Brunswick, Canada. Lice were carefully removed from the fish using forceps and placed in a glass container with seawater from the location of the farm being sampled. Once in the lab, egg strings were cultured at 10 to 12°C and copepods allowed to infect Atlantic salmon housed in the Atlantic Veterinary College aquatic animal facilities. Lice were allowed to develop to the pre-adult stage when they were removed

for use in the bioassay. A detailed description of laboratory development of sea lice is published elsewhere (Westcott *et al.* submitted).

4.3.2. Bioassays

All of the assays were performed using pre-adult lice due to the low differential in body size compared to adult lice. Lice were gently removed from the salmon using forceps and placed in a flask containing cold seawater. Bioassays were set up in covered glass Petri dishes. An EMB (PESTANAL®, Sigma-Aldrich) stock solution was prepared by dissolving 5 mg EMB in 50 ml of methanol. From this stock solution, a working solution was prepared by diluting 1:10 with seawater. EMB concentrations were prepared by diluting the working solution with seawater to make the following EMB concentrations: 0, 1, 3, 10, 30, 100, 300 ppb. Each concentration was set up in triplicate and 10 lice placed in each dish (n=210). Lice were randomly allocated into Petri dishes containing seawater (i.e. 0 ppb EMB). Following equal allocation of sea lice to each Petri dish, the seawater was replaced with 50 ml volumes of the different EMB dilutions. Dishes were subsequently incubated in a temperature controlled chamber at 10 to 12°C for a 24h period after which the sea lice were evaluated by dish as being live or dead by criteria similar to those already established (Sevatdal and Horsberg, 2003). For the purpose of this study moribund lice were classified as having survived treatment. The surviving lice were stored in RNAlater® (Sigma). Because all the conditions used were identical for each of the triplicate dishes, lice were analyzed as one pooled sample per drug group, rather than three individual groups per concentration.

The number of dead sea lice at the different concentrations of EMB was analyzed by a regression model with natural responsiveness (Finney, 1971). The model was used to estimate the LD_{50} "lethal dose" killing 50% of the lice adjusting for naturally occurring mortalities. The analysis was implemented in SAS 8.2 software (SAS Institute Inc, Cary, NC, USA). In this study we were interested in examining the EMB concentrations where the surviving sea lice after 24 hours indicate a possible selective advantage over those that had died. The hypothesis was that this selective advantage is due to increased transcription of SL0525 and/or SL-Pgp1 possibly providing protection against EMB toxicity.

4.3.3. RNA Isolations

Individual lice were removed from the RNAlater® and placed in sterile DNase/RNase-free 1.5ml microcentrifuge tubes containing 1ml Trizol. Sea lice were homogenized and incubated for 5 min at room temperature, 200µl of chloroform was then added and the sample shaken vigorously at room temperature for 3 min. After centrifugation at 12,000xg for 15 min, the upper colourless layer was transferred into a new 1.5ml tube ensuring absolutely none of the bottom pink layer containing the genomic DNA and proteins was removed. Isopropanol (500µl) was added to the supernatant and mixed vigorously. After 10 min incubation at room temperature the sample was centrifuged at 12,000xg for 10 min. The supernatant was carefully removed and the RNA pellet was washed using 75% ethanol in diethylpyrocarbonate (DEPC) treated water and centrifuged at 7,500xg for 5 min. The supernatant was removed and the pellet air dried before resuspending in 100µl DEPC treated water. Prior to storage at -80°C, RNA purity

was verified on an agarose gel and by spectrophotometry 260/280 ratios. In addition to confirming the purity of the RNA, potential DNA contamination was checked for by the use of non-RT PCR using specific primers for both target and reference genes. Any products generated would indicate the presence of DNA.

4.3.4. Target Gene Primer and Probe design

Two genes, SL0525 and SL-Pgp1, encoding putative drug transporters in *L. salmonis* were examined for changes in mRNA levels after exposure of the sea lice to EMB. A previously validated reference gene, translation eukaryotic elongation factor 1 (eEF1α), was used for relative quantitative analysis (Frost and Nilsen, 2003).

eEF1α (Frost and Nilsen, 2003), SL0525 (Gen Bank accession # DQ458787), and SL-Pgp1 (Gen Bank accession # EF093796) cDNA sequences were used to design primers and probes for each assay using the ProbeFinder online software (Roche). The primers and amplicons for each assay were confirmed not to be homologous to any other mRNA sequences in Genbank. The ProbeFinder software was not able to design suitable assays spanning intron/exon boundaries. The primer and probe locations within each cDNA sequence is show in figure 4.1. The primers were initially tested in conventional RT-PCR to confirm their specificity. The probes used were commercially supplied by Roche and contained locked nucleic acid (LNA) nucleotides representing the DNA analog. The LNA molecule offers greater thermal stability and the higher binding affinity of LNA oligonucleotides allows for the use of probes significantly shorter than the conventional Taqman probes (Ugozzoli *et al.,* 2004). The probes contain a reporter 6-carboxyfluorescein (FAM) and a quencher 6-carboxytetramethylrhodamine (TAMRA)

incorporated on the 5` and 3` end of the probe, respectively. Table 4.1 lists the primers and the corresponding universal probe library probes for each assay.

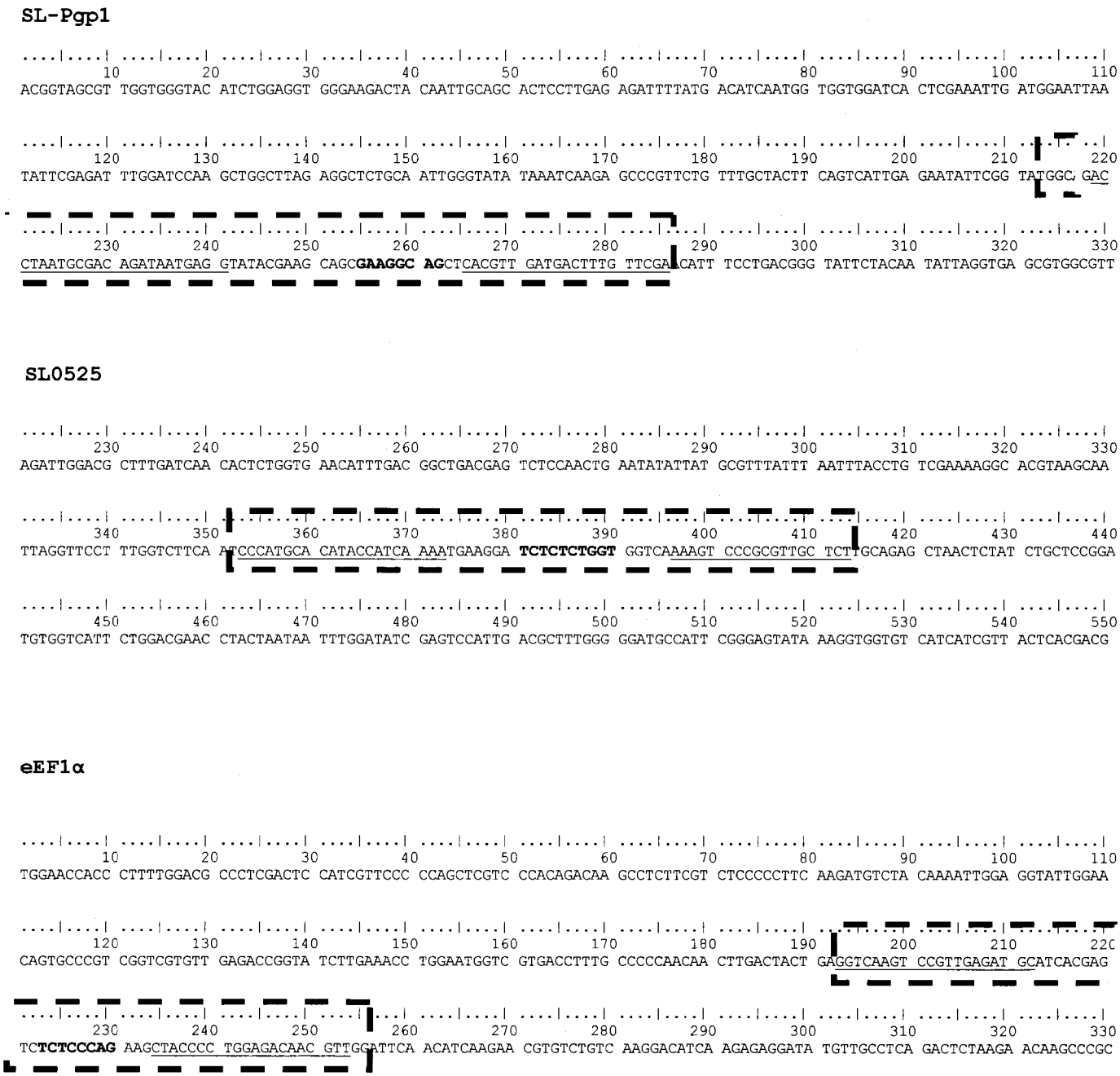

Figure 4.1. Primer and probe locations in target genes SL-Pgp1 and SL0525 and reference gene eEF1α. Amplicons are boxed, primer locations are underlined and probes are in bold.

Table 4.1. Primers and probes for the quantitative RT-PCR assays

Primers and LAN probes (FAM-NN-TAMARA)	
eEF1α (Frost and Nilsen, 2003)	Forward: GGTCAAGTCCGTTGAGATGC Reverse: AACGTTGTCTCCAGGGGTAG Probe: TCTCCCAG
SL0525 (Accession # DQ458787)	Forward: CCCATGCACATACCATCAAA Reverse: AGAGCAACGCGGGACTTT Probe: TCTCTGGT
SL-Pgp1 (Accession # EF093796)	Forward: ACCTAATGCGACAGATAATGAGG Reverse: TCGAACAAAGTCATCAACGTG Probe: GAAGGCAG

4.3.5. Q-RT-PCR and analysis

Q-RT-PCR was carried out using a one-step RT-PCR kit (Qiagen) on total RNA extracted from individual pre-adult sea lice, and was performed in triplicate under the same conditions for the eEF1α reference gene and two test genes. The total number of pre-adult lice used during this study was 25 (9 = 0ppb, 8 = 10ppb and, 8 = 30ppb). Water without RNA template was used as the negative control and was also performed in triplicate during each run. Q-RT-PCR were performed in 20µl volumes and contained 5µl water, 4µl 5X buffer, 1.6µl dNTP's (10mM), 0.8µl $MgCl_2$ (25mM), 0.5µl each primer (20µM), 0.4µl RNase inhibitor (20U/µl), 1µl enzyme mix, 0.2µl Probe (10µM), and 6µl template. Q-RT-PCR was performed using the LightCycler System (Roche) with reactions in glass capillaries. The optimized cycling conditions comprised of RT at 50°C for 30min, denaturation at 95°C for 15 min, 65 amplification cycles each at 94°C for 1 sec, 60°C for 30 sec, and 72°C for 1 sec. Each Q-RT-PCR run was analyzed using LightCycler software version 3.52, and the results were expressed in terms of the threshold cycle (Ct) value, the cycle number at which the fluorescence (measured at 530nm) rises above the background and enters into a log-linear phase. The Fit Points Method analysis algorithm was used to pick the Ct values.

4.3.6. Data analysis

Standard curves for all three genes were generated using the Ct values from serial dilutions of the RNA template from three pooled lice (Fig. 4.2). The slopes were examined for interactions to determine if the two target genes differed significantly from that of the eEF1α reference gene.

Relative mRNA levels of SL0525 and SL-Pgp, normalized to the eEF1α reference gene, were analyzed using the comparative Ct method to estimate differences in gene expression compared to the 0ppb exposure group (Yuan *et al.,* 2006). This method normalizes the average target gene Ct value to the average Ct value of the reference gene. The fold induction was calibrated to the zero exposure group and the resulting induction values were plotted as a bar graph.

MINITAB statistical software was used to determine if EMB exposure influenced levels of gene expression relative to the reference gene. The mean Ct value ratio of target gene to reference gene was used in a one way analysis of variance (ANOVA) followed by multiple comparisons using a Bonferroni adjustment.

4.4. Results

4.4.1. EMB bioassays

The lice survival and mortality figures are shown in Table 4.2. The groups exposed to 0ppb, 1ppb, and 3ppb EMB had a large number of lice surviving after 24 h. At an EMB concentration of 10ppb only 60% of the sea lice survived and at 30ppb the survival decreased further to only 26%. Concentrations higher than 30ppb resulted in 100% mortalities.

Statistical analysis showed the natural occurring mortality to be 2.8%. The LD_{50} was estimated at 15.1ppb EMB with a 95% confidence interval of 11.3 – 20.1ppb. Clearly lice surviving at both 10ppb and 30ppb suggest an increased tolerance to EMB and were subsequently used for further analysis.

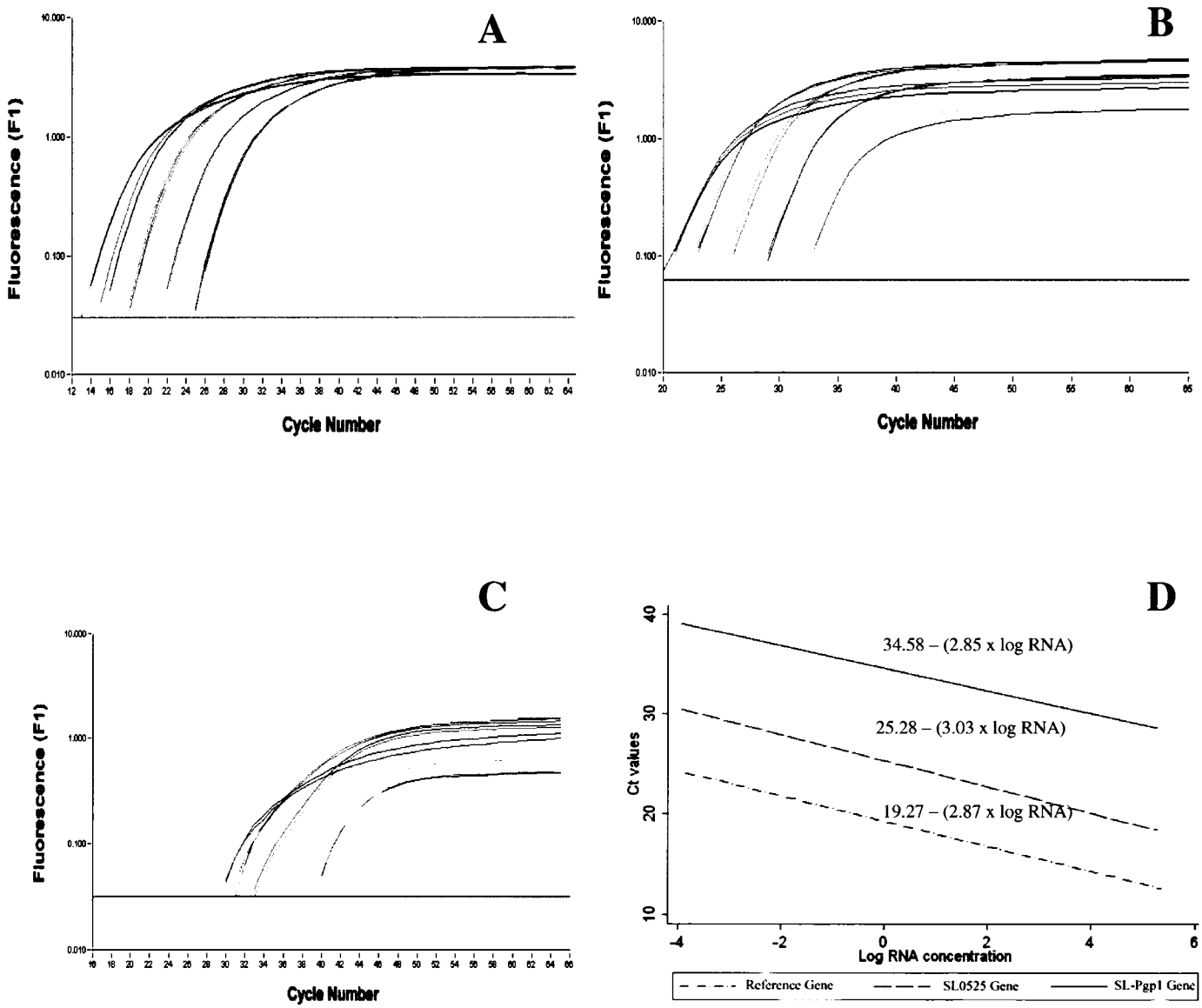

Figure 4.2. Amplification plots for the three *L. salmonis* genes; eEF1α (A), SL0525 (B), and SL-Pgp1 (C) obtained using the LightCycler. Serial dilutions were performed on sea lice total RNA of known concentration. Logarithmic plot (F1) of FAM fluorescence was measured at 530nm versus cycle number. The respective standard curves were obtained by plotting the log starting RNA concentration against their threshold cycle (Ct) values (D).

Table 4.2. Sea lice survival in emamectin benzoate (EMB) bioassays at 10 – 12°C after 24h exposure at various EMB concentrations each containing a total of 30 pre-adult sea lice.

EMB Concentration	Surviving Lice	Dead Lice
0ppb	29	1
1ppb	29	1
3ppb	29	1
10ppb	18	12
30ppb	8	22
100ppb	0	30
300ppb	0	30

4.4.2. Q-RT-PCR assay efficiencies

The specificity of each of the primers was confirmed using conventional RT-PCR (Figure 4. 3).

Serial dilutions were performed on sea lice total RNA of known concentration and each gene was demonstrated to be quantitative using standard curves. The slopes of the target gene standard curves were checked against the reference gene for interactions. The interactions were non-significant between either the SL0525 or SL-Pgp1 and the eEF1α ($p=0.43$ and $p=0.2$ respectively); thus the assay efficiencies can be considered the same. The amplification and standard curves for each assay are shown in Figure 4.2.

Figure 4.4 shows the typical amplification plot generated for each of the three genes in an individual pre-adult louse. From the Ct values it was evident that the relative levels of expression of the genes were eEF1α>SL0525>SL-Pgp1.

4.4.3. Q-RT-PCR analysis

ANOVA followed by Bonferroni adjustment was used to analyze SL0525 and SL-Pgp1 gene expression. Lice at 10ppb EMB exposure showed a 9.4% (SE=2.8 P=0.009) increase in mRNA levels of the SL0525 target gene compared to the zero exposure group. The SL-Pgp target gene showed an increase in mRNA levels of 8% (SE=2.6 P=0.015). In the 30ppb EMB exposure group the SL0525 mRNA showed a 4.2% (SE=2.8 P=0.44) increase and the SL-Pgp showed a 4.9% (SE=2.6 P=0.21) increase in mRNA levels. There was no significant difference between the mRNA levels of the target genes between 10ppb and 30ppb EMB concentrations.

Comparative Ct analysis showed that at 10ppb EMB exposure the expression of both SL0525 and SL-Pgp1 genes increased over 5 fold (5.6 ± 3.3 and 5.3 ± 2.4, respectively) relative to the zero exposure group (Fig. 3). The level of expression was reduced in both genes at the 30ppb exposure to marginally lower than 5 fold for SL-Pgp1 (4.9 ± 2.0) and 2 fold for SL0525 (2.0 ± 0.7) compared to the zero exposure group.

The negative water controls did not show amplification in any of the reactions during this study.

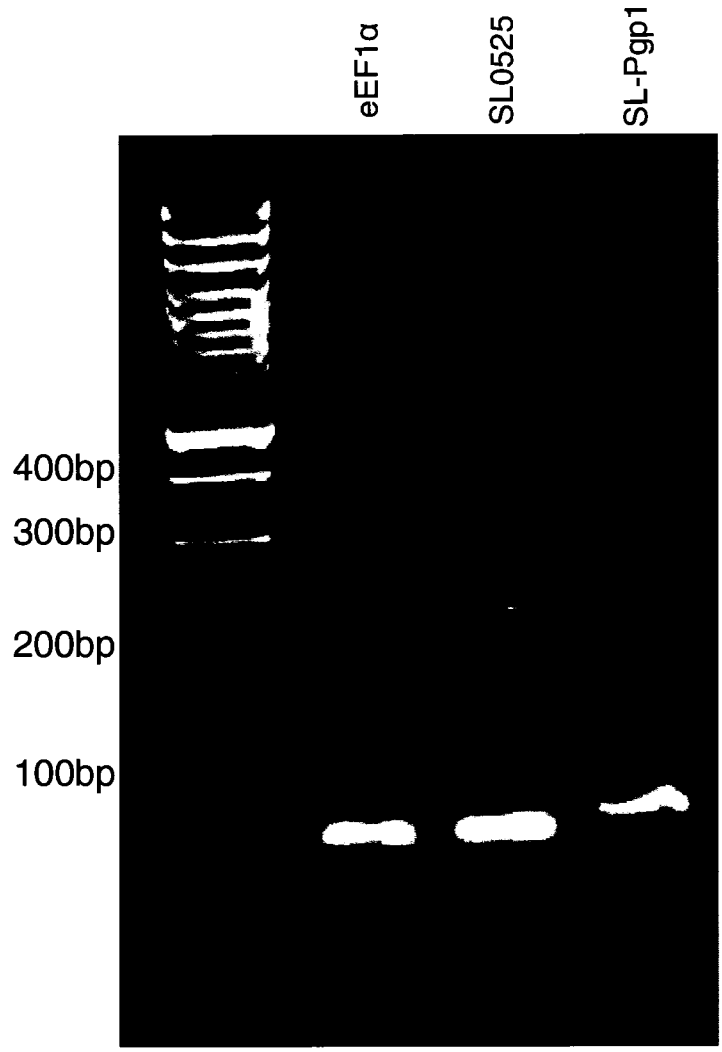

Figure 4.3. Analysis of primer specificity for eEF1α, SL0525, and SL-Pgp1 genes in a 2% agarose gel.

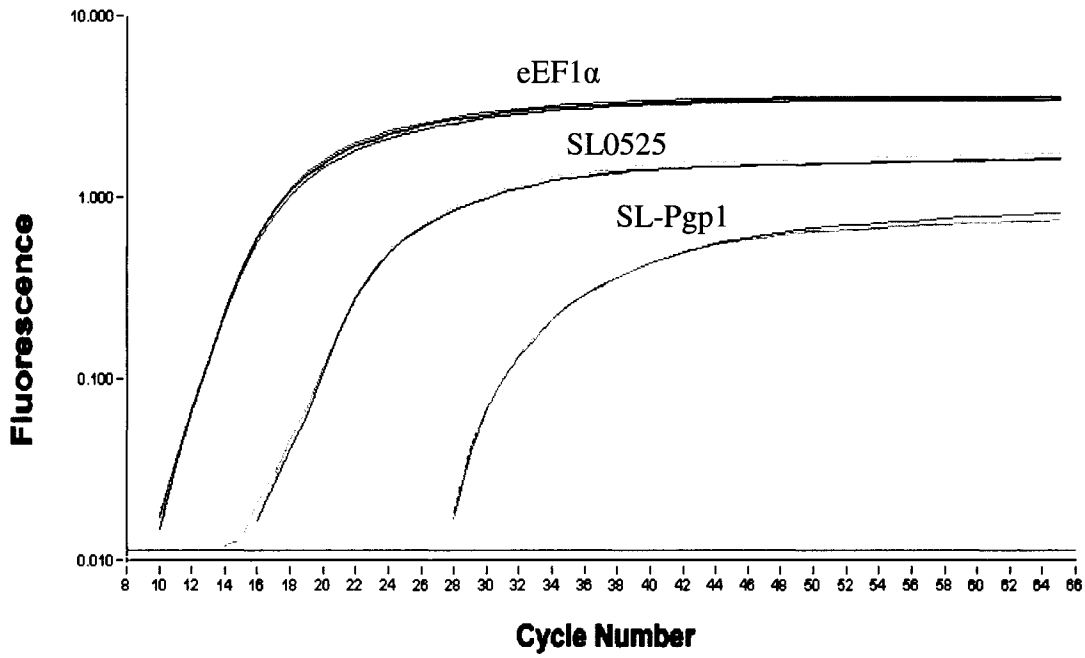

Figure 4.4. Amplification plots of the three genes using total RNA from a single pre-adult sea louse and gene-specific probes. The relative levels of expression of the genes are eEF1α>SL0525>SL-Pgp1. Logarithmic plot of FAM fluorescence measured at 530nm is plotted against cycle number.

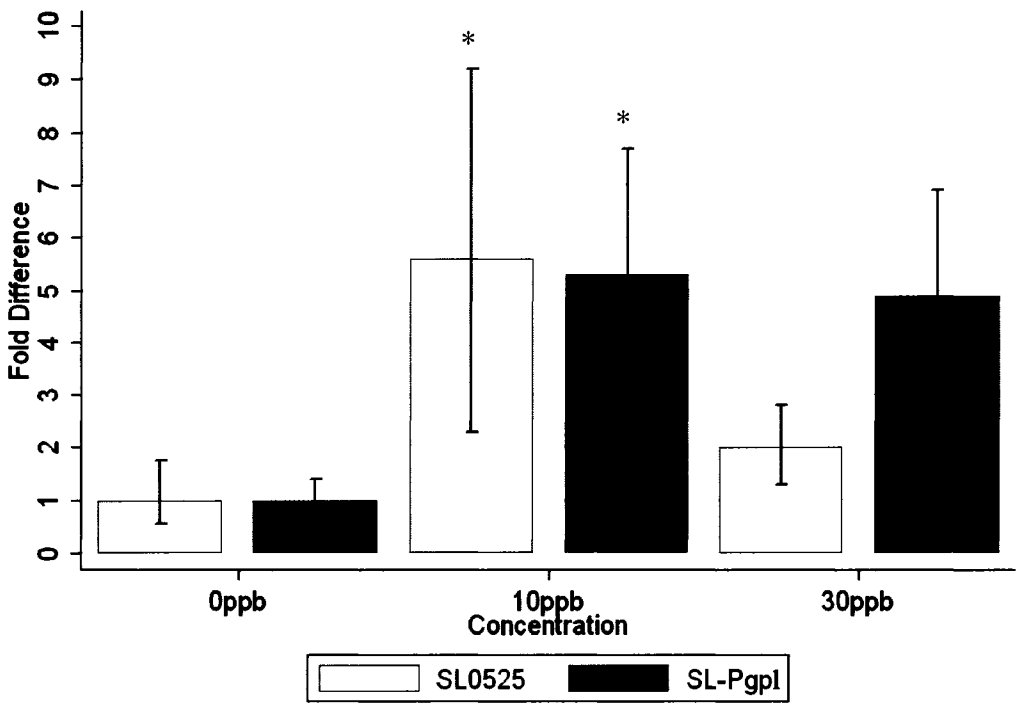

Figure 4.5. Changes in mRNA levels of both SL0525 and SL-Pgp1 genes, normalized to eEF1α, in pre-adult sea lice exposed to 10ppb and 30ppb emamectin benzoate in relation to a 0ppb exposure group (0ppb n=9, 10ppb n=8, 30ppb n=8). Bars represent mean ± S.E. from each exposure group. * Indicates statistical significance using one way ANOVA followed by Bonferroni adjustment compared to zero exposure.

4.5. Discussion

A major obstacle for many drug therapies is the over-expression of P-gps which make cells and organisms resistant to a broad spectrum of drugs, a phenomenon that has been documented in numerous bacteria, protozoa, invertebrates, and mammals. P-gp plays a major role in reducing the efficacy of macrocyclic lactones in trichonstrongylid nematode (Blackhall *et al.,* 1998; Xu *et al.,* 1998; Kerboeuf *et al.,* 2003) by decreasing uptake of the drug, thus reducing the concentration at the receptor site. Macrocyclic lactones are good substrates for P-gp transporters, with the avermectins being more potent substrates than the milbemycins (Pouliot *et al.,* 1997; Griffin *et al.,* 2005). Inhibitors such as verapamil have been demonstrated to enhance the activity of avermectins (Molento and Prichard, 2001).

The present study has demonstrated that two target genes (SL0525 and SL-Pgp1), encoding for putative drug transporters, are upregulated in sea lice, a parasitic crustacean, surviving exposure to low EMB concentrations. This complements our earlier findings by immunolocalization using specific antibodies, that P-gps are localized on the epithelium of the intestinal tract of *L. salmonis* (Chapter 3). Upregulation or over-expression of P-gp would serve to limit the absorption of various compounds including EMB ingested by the louse.

This report is the first to document that putative P-gps in *L. salmonis* have increased levels of mRNA in response to a xenobiotic, in this case EMB. A statistically significant increase in gene expression of both SL0525 and SL-Pgp1 genes was seen in the 10ppb exposure group (over 5 fold increase relative to 0ppb), but no significance in either gene was seen with 30ppb EMB exposure. The reason for lower mRNA levels at

the 30ppb exposure level is unknown. Differentiation of moribund and live sea lice is difficult and it is possible that sea lice surviving in the 30ppb group were moribund; lower mRNA levels are attributed to the lice struggling to survive at this higher concentration. The high energy costs required to sustain high levels of P-gp pumping has also been suggested to decrease the overall fitness of an organism (Renfro *et al.,* 1993; Epel *et al.,* 2006). Although an increase in target gene mRNA was seen within each EMB exposure group, there was a large range in the estimated fold increase. This range could be a factor of genetic predisposition with some lice responding with greater magnitude than others. It could also represent the general health status of the lice with more healthy lice expressing higher mRNA levels of the target genes. Further research using sea lice collected from the field will help in assessing the level of variation within a larger wild population.

An exposure time of 24 hours was chosen to ensure low numbers of natural mortalities and to give enough time for increased transcription to occur in response to EMB. Westcott *et al.* (2006) demonstrated low naturally occurring mortalities of sea lice after 24 hours in identical EMB bioassays. The LD_{50} value in this study, adjusted for natural mortalities (2.8%), compare well to Westcott *et al.* (2006). A previous study showed *mdr1* transcript induction could be detected as early as 7 hours after exposure to the potent inducer doxorubicin *in-vitro* using rat liver epithelial cells (Fardel *et al.,* 1997).

This study is the first to examine expression levels of P-gp in a parasitic organism in response to drug exposure using Q-RT-PCR. Q-RT-PCR is a powerful technique for profiling gene expression and is currently one of the most frequently used methods in biological studies of differentially expressed genes. Successful Q-RT-PCR comparative

quantification of target genes requires the use of an appropriate reference gene that has a constant and non-regulated transcription level within the sea lice. The use of eEF1α as a suitable reference gene for Q-RT-PCR with sea lice was previously validated by Frost and Nilsen (2003). When using the comparative Ct method for analysis it is also important that the target genes and endogenous control have similar or relatively equivalent RT-PCR efficiencies. Examining the slopes of the log RNA concentration standard curves for any significant interactions demonstrated that these reactions were relatively equivalent. Low variation between reactions is a particular quality of using a probe based reaction.

To date Q-RT-PCR studies in aquatic organisms have primarily only focused on immune response to infectious agents (viral and bacterial) (Holland *et al.,* 2003; Bridle *et al.,* 2006; Morrison *et al.,* 2006; Hauton *et al.,* 2007). Previous studies examining changes in P-gp expression in invertebrates have used methods that are far less sensitive than Q-RT-PCR, such as Northern blot, semi-quantitative PCR and RNase protection analysis (Lincke *et al.,* 1993; Xu *et al.,* 1998; Huang and Prichard, 1999). It is evident from this study that the use of Q-RT-PCR could be a useful tool for examining P-gp expression not only in sea lice but also in other parasites.

Over the past two decades much work has been directed at understanding P-glycoprotein expression and its regulation but it is still poorly understood. Evidence indicates that expression and activity of P-gp can be controlled either pre- or post-transcriptionally by numerous environmental stimulants (reviewed comprehensively by Sukhai and Piquette-Miller, 2000). Modulations in protein stability, plasma membrane incorporation, mRNA stability and processing, gene transcription and gene amplification

have each been reported for P-gp. Of these, changes that occur at the level of mRNA are most frequently observed in increased expression of P-gp (Perez *et al.,* 1998; Sukhai and Piquette-Miller, 2000). The use of mRNA quantification to predict changes in actual protein levels is of course an assumption that is made during Q-RT-PCR studies where Western blot analysis is not performed in parallel. The dry weight of pre-adult sea lice (approximately 5mg), of which the majority is exoskeleton, means that obtaining enough membrane protein is a limitation in performing Western blot analysis in parallel to Q-RT-PCR, especially when the lice are being collected from the field rather than a lab culture population. Studies conducted by our research group show that for a sufficient amount of protein to be isolated for use in such a study it would require the pooling of approximately 10 lice rather than the use of a single louse when applying Q-RT-PCR. A larger study would therefore be required where far greater numbers of pre-adult sea lice could be obtained and applied to increased replicate bioassays to obtain sufficient numbers.

EMB resistance development has not yet been reported in sea lice. Without a resistant sea lice model to examine, EMB bioassays are a useful tool that can be applied to investigate the effects of EMB on gene expression in surviving lice. The bioassays used in this study were developed previously by our research group and the lice survival rates are similar to those previously predicted (Westcott *et al.* submitted). High levels (100ppb – 300ppb) of EMB caused high levels of mortality, whereas lower levels (10ppb and 30ppb) resulted in 60% and 23% survival rates, respectively. Only lice surviving after the 24h period were used for Q-RT-PCR analysis due to the possible degradation of RNA in dead lice. In aquaculture, the maximum EMB concentration that a sea louse

would be exposed to has been estimated at slightly above 100ppb in the skin, mucus and blood of a recently treated fish (Sevatdal *et al.,* 2005). This concentration also appropriately resulted in 100% mortality in the bioassay. It should however be recognized that the EMB exposure in these assays are slightly different to the exposure of a louse on a treated salmon. Lice present on an EMB treated salmon are actively feeding on the mucus containing EMB whereas in the bioassay it is assumed that they are absorbing the EMB and possibly taking it up through their oral cavities. It could therefore be suggested that lice exposed to EMB on a treated fish may take up more of the drug than the equivalent concentration in our bioassay.

In the absence of EMB resistant sea lice, this study investigated the induction of both SL0525 and SL-Pgp1 genes after EMB exposure. If these genes are responsible for reducing EMB absorption in sea lice, it is a heritable constitutive over-expression rather than induction that will be important for development of a resistant phenotype. Over-expression is a term used when a protein has increased dramatically in the basal level of expression compared to that in an identical cell or organ. The potential mechanisms that have been proposed to lead to over-expression of the P-gp gene include changes at the level of the gene by amplification, duplication or mutation, or in the stability of mRNA (Stein, 1997). This study not only gives an insight into the potential risk of over-expression of these genes, but also offers a new tool for monitoring the expression of these genes in sea lice collected from Atlantic salmon farms.

The lice used in this study were cultured in our aquatic animal facilities and, therefore, unlike lice in the wild, were exposed to a stable "pristine" environment. Additional studies should now be directed toward determining the baseline levels of

expression of these two genes in both pre-adult and adult sea lice in the field. This baseline level should be conducted over the whole year to account for any possible influences due to temperature, light, etc. P-pg expression in oysters has been shown to be seasonal with elevations in summer and fall (Keppler and Ringwood, 2004). In this study P-gp seasonal expression was not correlated with exposure to any specific organic sediment pollutant but was coincident with elevated seawater temperature and algal blooms. Heat shock or elevated natural products in the water column from algal blooms could be responsible for seasonal P-gp expression observed.

Any future reports of EMB treatment failure could apply this tool to determine if over-expression of these genes, compared to this baseline expression level, is correlated to resistance development. Knowledge of the substrates transported by P-gp will allow alternative treatments to be considered that (i) are not exported by these transporters, and (ii) decrease the selection pressure favoring the over-expression of these drug transporters. By understanding and monitoring the potential resistance mechanisms and adapting the use of drugs accordingly will help in maintaining this highly efficacious drug use on Atlantic salmon farms a more feasible aspiration.

4.6. References

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CHAPTER 5. IDENTIFICATION OF THE GENES ENCODING FOR PUTATIVE GAMMA-AMINOBUTYRIC AND GLUTAMATE GATED-CHLORIDE CHANNEL ALPHA RECEPTOR SUBUNITS IN SEA LICE (*LEPEOPHTHEIRUS SALMONIS*)

Chapter accepted in part for publication:

Tribble ND, Burka JF, Kibenge F (2007) Identification of the genes encoding for putative gamma-aminobutyric acid (GABA) and glutamate gated-chloride channel (GluCl) alpha receptor subunits in sea lice (*Lepeophtheirus salmonis*). *Journal of Veterinary Pharmacology and Therapeutics*, **30**; 163-167.

5.1 Abstract

Emamectin benzoate, an avermectin, is currently one of the most effective drugs administered against sea lice (*Lepeophtheirus salmonis*) infestation on Atlantic salmon farms. The heavy use of this drug makes resistance development a major concern. Avermectins act by binding irreversibly to both ionotropic gamma-aminobutyric acid (GABA) receptors and glutamate gated chloride channels (GluCl's) resulting in paralysis and subsequent death to the parasite. The use of degenerate primers designed from conserved homologous regions of the same gene in different species generated PCR products which were subsequently extended using both 3' and 5` RACE. Both GABAα and GluClα receptors show sequence similarities to their respective receptors in other species. Topological analysis shows both sequences share features common to other ligand-gated chloride channels, having the expected four transmembrane regions as well as a characteristic long N-terminal extracellular domain and a very short C-terminal extracellular domain. Results of the BLAST analysis on both putative GABAα and GluClα amino acid sequences identified the highest similarity (approximately 85%) for their respective receptor subunits. Phylogenetic analysis, as was predicted, also grouped the *L. salmonis* GABAα and GluClα receptors with their respective channels in other invertebrates. The use of these sequences in gene expression and protein-protein interaction studies will expand the knowledge of emamectin drug-receptor interactions which will provide further understanding of mechanisms of drug resistance and how best to prevent and manage resistance development to avermectins.

5.2. Introduction

Ectoparasitic infection of Atlantic salmon from the sea lice *Lepeophtherius salmonis* continues to have a considerable effect on salmon farms in both Europe and North America (Pike and Wadsworth, 1999). Control of these parasites relies heavily on the use of chemotherapeutics, specifically the avermectin, emamectin benzoate (SLICE®) (Westcott *et al.,* 2004). Macrocyclic lactones, which include avermectins and milbemycins, act by irreversibly binding to, and opening, both gamma-aminobutyric acid (GABA) receptors and glutamate gated chloride channels (GluCl's) causing paralysis and subsequent death of the parasite (Arena *et al.,* 1995, Bloomquist, 2003).

$GABA_A$ and GluCl channels belong to a gene superfamily of ligand-gated ion channels which also includes glycine- and histidine- gated chloride channels to name a few. These receptors share certain structural and functional characteristics. These proteins consist of a number of subunits which, within the various members of the superfamily, share common sequence homology. Structurally all subunits are composed of a large N-terminal extracellular domain and four membrane spanning domains (M1-M4) with a large intracellular loop containing sites for regulation (Etter *et al.,* 1996, Smith and Olsen, 1995).

GluCl receptors are predicted to be the primary target for macrocyclic lactones in invertebrates. Ivermectin binds with high affinity to GluClα subunits in both nematodes and arthropods including *Haemonchus contortus*, *Caenorhabditis elegans* and *Drosophila melanogaster* (Cully *et al.,* 1994, Forrester *et al.,* 2003, Smith *et al.,* 2000). Forrester *et al.* (2003) demonstrated that ivermectin binds irreversibly to GluClα subunits in *H. contortus*. These studies expressed GluClα receptor subunits as homomeric

channels in *Xenopus laevis* oocytes and confirmed that avermectins bind irreversibly to these subunits. Thus chloride will continue to enter though this channel causing permanent hyperpolarization of nerve cells. Studies have also determined that the avermectin binding sites are located on the α-subunits of GABA receptors (Stephenson, 1995).

Knock-out studies of GluCl genes in *C. elegans* demonstrated a lack of sensitivity to ivermectin in these strains compared to wild type strains (Dent, 2000). Population studies have also found evidence for selection at GluCl and GABA receptor genes, with significant differences in allele frequencies detected between ivermectin selected and unselected genes in *H. contortus* and *C. oncophora* (Blackhall *et al.,* 1998, 2003; Njue and Prichard, 2004). Due to the heavy reliance on emamectin benzoate in Europe and North America for the control of *L. salmonis*, there appears to be a therapeutic pressure that could potentially lead to resistance development. Mutations in the α-subunits of the target receptors, particularly the GluCl channel, could therefore lead to a conformational change in the channel and decrease the affinity for avermectin binding, thus causing resistance. Evidence for this is, however, not supported by the limited literature available and it is possible that mutations may affect IVM activation of GluCl's or act to enhance allosteric glutamate activation.

The aim of this study was to identify the putative gene encoding for both GABAα and GluClα receptor subunits in *L. salmonis*. To specifically define where avermectins are acting in sea lice, these genes will be useful for both expression studies investigating emamectin benzoate binding affinity, as well as providing a foundation for future mutational studies if resistant populations arise in the wild. Further understanding of

avermectin-receptor binding may also provide insight into the optimal drug binding properties which could be applied directly to administration practices of emamectin benzoate for sea lice infestations to ensure optimum efficacy.

5.3. Materials and methods

5.3.1. Sea Lice Collection

L. salmonis were collected from the Atlantic salmon farms in the Bay of Fundy, NB, Canada. Lice were carefully removed using forceps and placed in a glass container with the seawater from the location of the farm being sampled. Once in the laboratory, live adult lice were placed in 300µl RNAlater® (Roche, Laval, QC) and stored at -20°C until processed.

5.3.2. Total RNA extraction from sea lice

Five adult female sea lice were removed from the RNAlater® and placed in a sterile DNase/RNase free 1.5 microcentrifuge tube containing 1ml Trizol. Sea lice were homogenized after which the sample was incubated for 5 min at room temperature. Two hundred microlitres of chloroform was then added and sample was shaken vigorously at room temperature for 3 min. After centrifugation at 12,000xg for 15 min, the upper colourless layer was transferred into a new 1.5ml tube ensuring absolutely none of the bottom pink layer containing the genomic DNA and proteins is removed. Isopropanol (500µl) was added to the supernatant and mixed vigourously. After 10 min incubation at room temperature the sample was centrifuged at 12,000xg for 10 min. The supernatant

was carefully removed and the RNA pellet was washed using 75% diethylpyrocarbonate (DEPC) treated water and centrifuged at 7,500xg for 5 min. The supernatant was removed and the pellet air dried before resuspending in 100µl DEPC treated water. RNA was stored at -80°C until required.

5.3.3. cDNA synthesis and PCR

First-strand cDNA for all samples was primed using oligo-dT primers (Superscript III first strand synthesis kit, Invitrogen, Burlington, ON). The cDNA served as a template for the initial amplification of both GABA and GluCl fragments using PCR and degenerate primers. The degenerate primers were designed based on an alignment of known GABA receptor sequences of two arthropods *Homarus americanus* and *D. melanogaster* (accession numbers AY098942 and AY017266, respectively), and an alignment of known GluCl sequences (Cully *et al.,* 1996). The GABA receptor forward and reverse primers used were 5`ATT TGG GTN CCT GAC CAN TTT3` and 5`GTR GTC ATK GTS TRC ACK GTW GT3` respectively, based on the conserved amino acids IWVPDTF and TTVLTMTT. The GluCl forward and reverse primers, based on the conserved amino acids WVCFW and IDVWIG, were 5`TGG GTN WSN TTY TGG TT3` and 5`GCN CCD ATC CAN ACR TCH AT3`, respectively. The PCR cycling conditions for the GABA receptor reaction comprised of an initial 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 55°C for 90 sec, extension at 72°C for 120 sec. A final extension step at 72°C for 10 min completed the cycle. The GluCl reaction was performed using touchdown PCR where cycling conditions comprised of 94°C for 10 min followed by 55°C for 90 sec, extension at 72°C

for 120 sec. There were 35 cycles during which the annealing temperature decreased 1°C every two cycles. The cycle was completed by a final extension at 72°C for 120 sec. From the resulting PCR product, 1μl was used as a template for re-amplification using the same primers and conditions as before. Subsequent PCR products were analyzed on a 1% agarose gel containing ethidium bromide and the expected bands were extracted directly from the gel, purified and cloned into a TOPO2.1 TA vector (Invitrogen) in preparation for plasmid DNA sequencing (all sequencing was commercially performed by ACGT Corp, Toronto, ON).

5.3.4. Rapid Amplification of cDNA Ends (5` and 3` RACE)

The sequence information obtained from these products was used to design gene specific primers for use in 3` and 5` RACE reactions. For the RACE reactions 5`/3` RACE kit, 2nd Generation (Roche) was used. Four sense and antisense primers were designed using Primer Detective (Clonetech, Version 1.0) for either the 3` or 5` reactions for both the GABA and GluCl genes. These primers were initially used in combination in a PCR reaction with a cDNA template to confirm primer specificity. After ensuring primer specificity, the primers were applied to total sea lice RNA using the 5`/3` RACE kit (Roche). There were slight modifications to the protocol to ensure the specificity of the amplified products. All RACE products were re-amplified using either 2 or 3 specific primers for the 3` and 5` RACE reactions respectfully after cDNA synthesis. During each round of re-amplification the specificity of the template was confirmed using gene specific internal primers. PCR products were analyzed on a 1% agarose gel containing SYBR Safe (Invitrogen). PCR products were cut out of the gel, purified, and cloned as

previously described. Clones containing the appropriate insert size were sent for DNA sequencing.

5.3.5. Phylogenetic analysis of GABA-, GluCl-, and GlyCl-α receptors

The gene database from National Center for Biotechnology Information (NCBI) was searched for nucleotide sequences encoding for GABA-, GluCl-, and GlyCl-α receptors. The sequences were examined using the NCBI ORF-Finder for the open reading frames and any non-coding sequences were removed. The subsequent sequences along with the *L. salmonis* genes were aligned using ClustalW on the BioEdit sequence alignment editor and a 119bp conserved nucleotide sequence encoding for the second transmembrane domain in GABA, GluCl, and glycine-gated chloride (GlyCl) receptors was used for phylogenetic analysis. The species used for this analysis were *Cooperia oncophora*, *C. elegans*, *H. contortus*, *Lucilia cuprina*, *D. melanogaster*, *Mus musculus*, *Morone americana*, *Danio rerio*, and *Drosophila simulans*. Phylogenetic analysis was performed using MEGA Version 3.1 (Kumar *et al.,* 1994).

5.3.6. Sequence Analysis

A Basic Local Alignment Search Tool (BLAST) (Altschul *et al.,* 1990) was used to search for sequence similarity to the sequences generated. The NCBI database was searched for amino acid sequences corresponding to either GABAα or GluClα receptors. Selected sequences were aligned to the corresponding *L. salmonis* receptor using ClustalW (Thompson *et al.,* 1994). The species used to align with the *L. salmonis*

GABAα subunit (accession number DQ916036) were *D. melanogaster* (accession number M69057), *D. simulans* (accession number AY017266), *C. elegans* (accession number AFAF498370), and *Carassius auratus* (accession number X94342). The *L. salmonis* GluClα subunit (accession number DQ916037) was aligned with *C. elegans* (accession number AAA50785), *Cyclicocyclus nassatus* (accession number AY727925), *Dirofilaria immitis* (accession number DIM581672), *Cooperia oncophora* (accession number AY372756), *H. contortus* (accession number AF119791), *Lucilia cuprina* (accession number AF081674), and *D. melanogaster* (accession number AF297500). The predicted amino acid sequences of each *L. salmonis* receptor was analyzed for transmembrane regions using TopPred (Vonheijne, 1992) and potential phosphorylation and glycosylation sites using NetPhos 2.0 Server (Blom *et al.*, 1999).

5.4. Results

Using degenerate primers based on conserved homologous regions of either GABAα or GluClα receptor amino acid sequences of other invertebrate species successfully amplified PCR products of 534bp and 153bp respectively. Touchdown PCR was used rather than conventional PCR because it amplifies less non-specific products which was necessary in order to generate sufficient GluCl PCR product to be visualized in an agarose gel. These putative GABAα and GluClα receptor cDNA sequences were successfully extended using both 5` and 3` RACE to yield 1434bp and 1332bp sequences (Fig 5.1 and 5.2). Protein products were predicted and analyzed for similarity to protein sequences in other species using BLAST analysis. Invertebrate GluClα and GluClβ subunits are known to share approximately 45% homology at the amino acid level (Etter

et al., 1996). Results of the BLAST analysis on both putative GABAα and GluClα amino acid sequences identified the highest similarity (approximately 85%) for their respective receptor subunits. More specifically the putative *L. salmonis* GABAα receptor showed the highest identity to *D. melanogaster* (87%) at the amino acid level. The putative GluClα *L. salmonis* receptor showed a high level of identity to the GluClα receptors of many insects and nematodes including *D. melanogaster*, *H. contortus*, and *C. elegans* (86%, 83%, and 82% respectively) which are targeted by avermectins and milbemycins. The putative GABAα subunit had on average only a 69% similarity to GABAβ subunits from other species and 57% similarity with glycine receptor α subunits. The putative GluClα subunit showed a 58% similarity to GluClβ and glycine receptor α subunits of other species. A conserved region located from the extracellular ligand-binding domain through the third transmembrane domain of the putative *L. salmonis* GABAα and GluClα receptors showed a 57% similarity with each other at the nucleotide level.

The amino acid sequence of the putative *L. salmonis* GABAα and GluClα were aligned to respective α subunits from other species (Fig. 5.3 and 5.4). The multiple sequence alignment identified a number of conserved regions particularly in transmembrane regions two and three. The most variable region appeared to be the region between transmembrane domains three and four, with only a moderate level of variation at the N-terminal end.

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        10         20         30         40         50         60         70         80         90        100
ttcagcggca attttaatga tgtaaacata tcggcaattt taaatagctt tcagatgggt tacgataaaa gagtacggcc aaactatggt ggtccacccg

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       110        120        130        140        150        160        170        180        190        200
tgacagtagg agttaccatg tacatcctat ccataagctc cgtgtccgaa gtcatgatgg actttaccct ggatatgtac tttcgtcaat tttggcaaga

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       210        220        230        240        250        260        270        280        290        300
tcctcgtcta tcctttactg gtcggcctgg acttgaaaaa cttgttgtag gagcggagta catacgactc atatgggtgc cagatacatt tttcgtgaat

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       310        320        330        340        350        360        370        380        390        400
gaaaaaactg catatttcca taaagccaca actgagaatc agtttcttcg tattcttcat actggagaaa tcctgaggag tataagatta acaatcacag

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       410        420        430        440        450        460        470        480        490        500
cttcgtgccc aatggatcta cagtacttcc ccatggatcg gcaactgtgt tatatagaaa tcgaaagttt cggttatact atgagtgata ttcggtacaa

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       510        520        530        540        550        560        570        580        590        600
gtggaacgac ggtctgaatt cggtgcaaat ttcttcggat gtatctttgc cacaattcaa agtactggga cataggcaga aaacaattga ggcgagccta

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       610        620        630        640        650        660        670        680        690        700
agtactggaa attactctcg tctagcttgc gagattcaat ttgttcgttc catgggttac tacttaattc aaatttatat accttcaagt ttaatcgtta

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       710        720        730        740        750        760        770        780        790        800
ttatttcttg ggtttcattt tggttaaata gaggagccgc tcctgctcgg gtcggtctcg gtgtgacaac agtgcacaca atgaccactc tcatggcctc

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       810        820        830        840        850        860        870        880        890        900
aactaatgct gctctaccca aaatttccta tgttaaatct atagacgttt atttaggagc ttgtttcttt atagtgtttg cttcacttct agaatacgct

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       910        920        930        940        950        960        970        980        990       1000
agtgtgggct acatggcaaa acgaattcaa atgcgtaaaa atcgtttttt ggccctacaa aaaatggcag aacaaaagaa gaatgaagcc atgaacgcta

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1010       1020       1030       1040       1050       1060       1070       1080       1090       1100
gtaatgcagc cttaaacgat ggcgatcacg gaatgcctaa gcaaactact gtccacttca aagttcatga tcccaagatg cattgtaaag gcagttctcg

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
agaaaatacg attagtcgct acactcatgg taaatacatg cctcgtcctc ctccagggcg ctatgacgat gaggccccac ctccgccgcc tcaaccccat

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
cacgtcgcac ctcaactacc cccttgccca ccacctcctg cacctcctcc accaaatcca gagtgcgata tgaaagatct gaataaaatg tttgggatga

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       1310       1320       1330       1340       1350       1360       1370       1380       1390       1400
cggcttcaga cattgataaa tactcgcgag tcatattccc tgttactttt acgtgtcttc aactcatgta ctggattatc tatcagcatc tgagtgatga

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|
       1410       1420       1430       1440       1450       1460
tgtagtagat gatcttgtct acttacaccc ggactaaaat acacaaacat aaaaaaaaaa aa
```

Figure 5.1. Sea Lice GABAα receptor subunit cDNA sequence (Genbank # DQ916036). UTR is in bold and underlined. Sequenced commercially by ACGT (Toronto, ON).

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        10        20        30        40        50        60        70        80        90       100
ctttttcttc tcgtgtattt taactcaaca gccctccaaa atagacgaga taagatagat tatcgtttga aggaaaaaca aatcctagac catgttttag

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       110       120       130       140       150       160       170       180       190       200
ggccactccg atatgacaaa aggattcgac cacctggatc cagcaatttg acaggcccat ctcctacagt tgtcacaata aacacgtatc tccgtgctat

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       210       220       230       240       250       260       270       280       290       300
agatcgcata gatgattata aaatggagta cagtgtacaa ttaacattca gagaaaattg gatggattcg cgtttaatgt ttaacgattt gaacggtaaa

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       310       320       330       340       350       360       370       380       390       400
ataaaatact taacattgac ggatgctgaa aaagtttgga tgcctgacac ttttttttcaa aatgagaaac ttggacattt ccataacatt attgtaccca

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       410       420       430       440       450       460       470       480       490       500
atgtttatgt ccgaatattt ccaacaggaa gtgttttata tagtataagg atttccttga cattagcttg tccaatggat ctgaagctat atcctttaga

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       510       520       530       540       550       560       570       580       590       600
tcgtcaagtc tgtgagatga gaatagctag ttatggatgg acaacggatg acttaatcta tagatggaaa agcaaggatc cagtgcaatt cgttcaggat

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       610       620       630       640       650       660       670       680       690       700
ttaaatcttc ctcgattcaa actcgagagc tttagtacat cgtattgtaa ttcaaaaact aacacagggg agtacagctg tttgaaaatc aatctagttt

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       710       720       730       740       750       760       770       780       790       800
tcaaaagaga attttcctac tatctactga ccatttatgt accttcctgt atgcttgtta tcatttcatg ggtgagtttc tggttagact cgaaatcggt

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       810       820       830       840       850       860       870       880       890       900
tcctgcacgg gtagctttag gtgtaacaac acttcttact atgtctactc aaacagctgg tgtaaataga tcactacctc cagtagctta cacaaaagca

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
       910       920       930       940       950       960       970       980       990      1000
atagacgtat ggatcggcgc atgtgttatt tttgtattta gtgccttatt agaatttgca tttgttaact atgcttcacg acatgatcga agaaagggaa

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
ggaaatctag atctgcaatg aattataaca tggatgacga tgaaatagat tatgatcaag gtcttgattg cacttcacga attcgttctc attatggaaa

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
acgaggatcc tttaactacg gagagaacgg taatatcctt ctgagctata ttgctggaga gggcgtgacg acgactaaaa aaaagtcctg gcttgctgaa

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
agatttccaa ggagatcaaa aagaattgat gtagtagctc gtattttatt tccgggcata tttgccatct ttaattttag ctattggttg tattatttgt

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
      1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
ccgcagaaca tgaatcaagg atttacaaat aa
```

tagttgat aaactggatg aattataatt gcaaactttt tttctcaaga tttgaaaagt accaacacaa

```
....|....| ....|....| ....|....| ....|....| ....|
      1410      1420      1430      1440
```

taaatatatgatggatggatccatccatcttgtgtaaaaaaaaaaaaaa

Figure 5.2. Sea Lice GluClα receptor subunit cDNA sequence (Genbank # DQ916037). UTR is in bold and underlined. Sequenced commercially by ACGT (Toronto, ON).

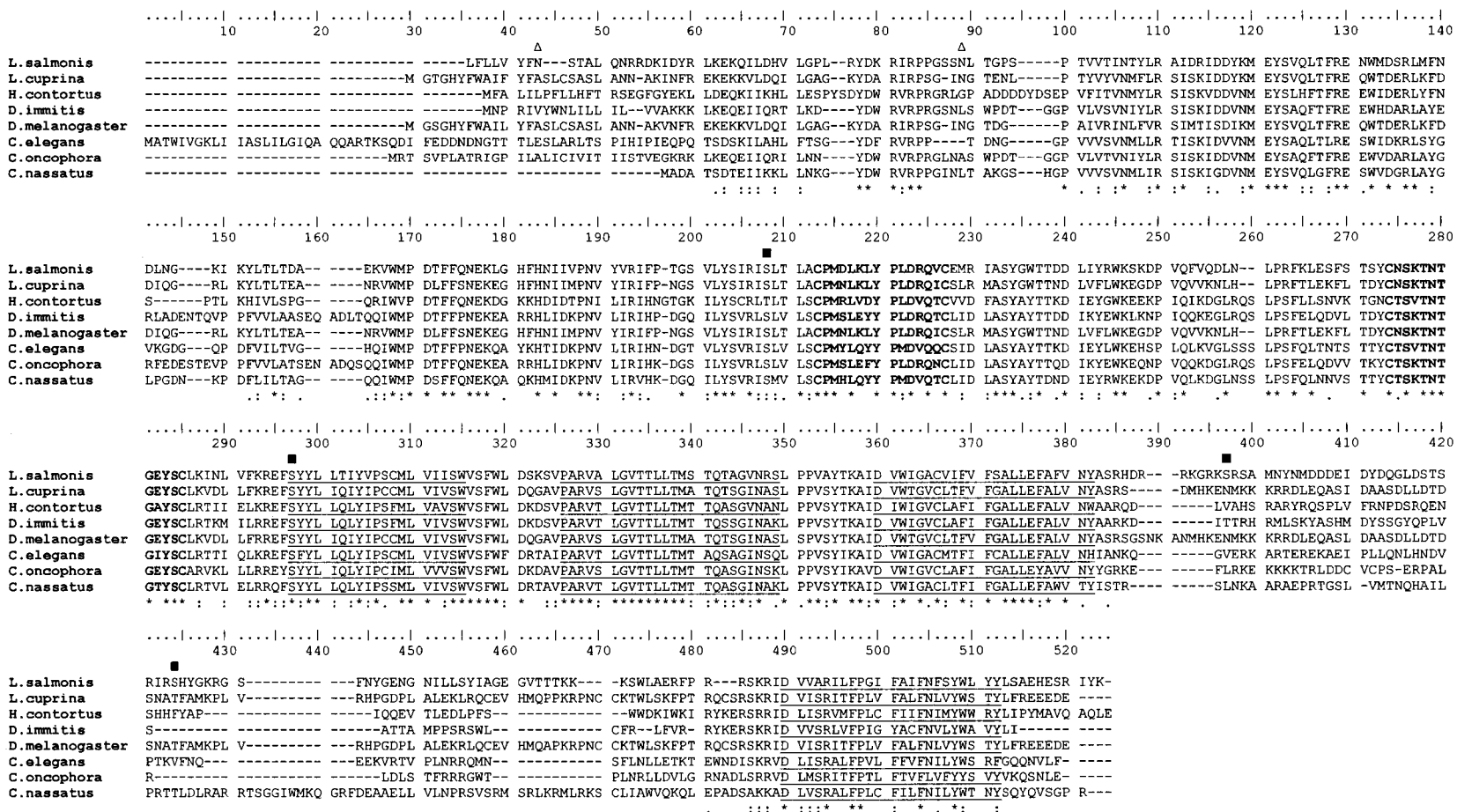

Figure 5.3. Amino acid alignment and topological arrangement of the *L. salmonis* GluClα receptor. Sequences aligned are: *L. salmonis* (DQ916037), *D. melanogaster* (accession number AF297500), *C. elegans* (accession number AAA50785), *C. nassatus* (accession number AY727925), *D. immitis* (accession number DIM581672), *C. oncophora* (accession number AY372756), *H. contortus* (accession number AF119791), *L. cuprina* (accession number AF081674), Conserved regions are indicated by (*), conserved substitutions by (:), and semi conserved substitutions by (.). Transmembrane regions are underlined and identified by TM1-TM4. Cys loop regions are identified in bold print. Predicted phosphorylation (protein kinase C (■)) and N-linked glycosylation sites (Δ) are shown.

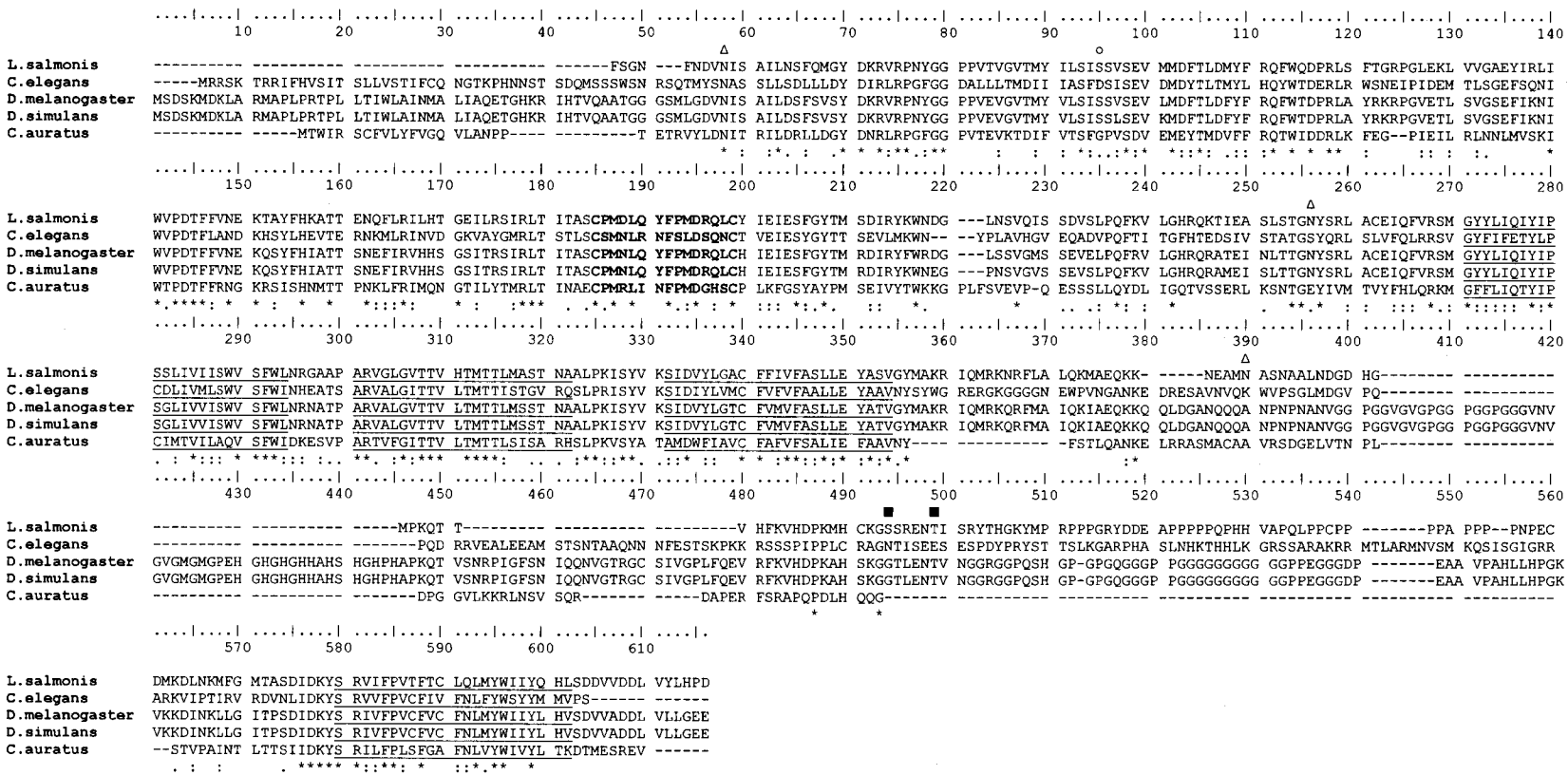

Figure 5.4. Amino acid alignment and topological arrangement of the *L. salmonis* GABAα receptor. Sequences aligned are: *L. salmonis* (accession number DQ916036), *D. melanogaster* (accession number M69057), *D. simulans* (accession number AY017266), *C. elegans* (accession number AFAF498370), *C. auratus* (accession number X94342). Conserved regions are indicated by (*), conserved substitutions by (:), and semi conserved substitutions by (.). Transmembrane regions are underlined and identified by TM1-TM4. Cys loop regions are identified in bold print. Predicted phosphorylation (protein kinase C (■) and casein kinase 2 (○)) and N-linked glycosylation sites (Δ) are shown.

Both putative *L. salmonis* receptor subunits share features typical of ligand gated chloride channels. Figure 5.5 shows a generalized topological representation of ligand gated ion channels. The GABAα and GluClα receptors contain four putative transmembrane regions which are the most conserved regions within the ligand gated chloride channels. The receptors also have a characteristic long N-terminal extracellular domain and a very short C-terminal extracellular domain. The GluClα and GABAα receptors also contain three N-linked glycosylation sites and three and four cysteine residues, respectively. The first two cysteine residues for both receptors are separated by 13 amino acids and are a common feature to all ligand gated chloride channels. The *L. salmonis* GABAα and GluClα subunits share a high level of similarity to the 13 amino acid cysteine residues seen in other respective channels of other species (Fig 5.6). The *L. salmonis* putative GluClα receptor also contains two cysteine residues present in the extracellular domain separated by 10 amino acids. The two *L. salmonis* receptors contain a number of putative phosphorylation sites, including two protein kinase C sites in the GABAα receptor and four in the GluClα receptor. The GABAα receptor also contains a putative casein kinase 2 site located close to the N-terminus (Fig 5.3 and 5.4).

A phylogenetic tree was constructed by the Neighbor-Joining method (Saitou and Nei 1987), with groupings supported by bootstrap analysis (Fig. 5.7). This analysis, as expected, grouped the *L. salmonis* GABAα and GluClα receptors with their respective channels in other invertebrates. High similarity between GluClα receptors of different species resulted in low scoring within this group making any further relationships within this family difficult. The *L. salmonis* GABAα receptor showed a close relationship with

that in *Drosophila* spp. This receptor also appears to have a distant relationship to the GABAα in *C. elegans*. The GluCl and GlyCl receptors appear to be sister groups more closely related to each other than to the GABA receptors.

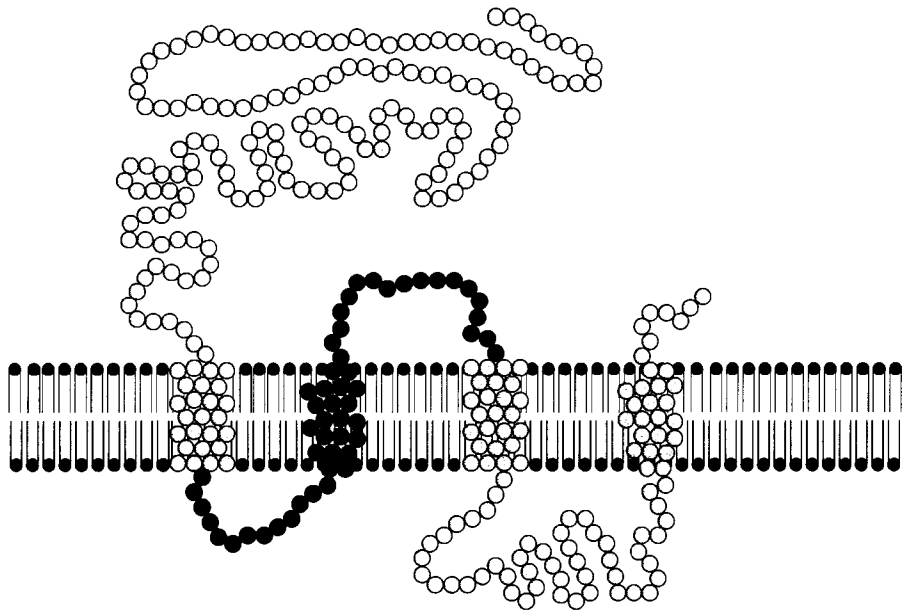

Figure 5.5. A cartoon representation of a single subunit of a ligand-gated chloride channel. These proteins are characterized by a large N-terminal domain and four transmembrane regions. The filled circles indicate residues of the M2 region that line the channel pore and the TM1-TM2 and TM2-TM3 loops that have been identified in channel activation.

```
L.salmonis GluCl          CPMDLKLYPL DRQVC
L.cuprina  GluCl          CPMNLKLYPL DRQIC
D.melanogaster GluCl      CPMNLKLYPL DRQIC
H.contortus GluCl         CPMRLVDYPL DVQTC
C.elegans GluCl           CPMYLQYYPM DVQQC
                          *** *  **: * * *

L.salmonis GABA           CPMDLQYFPM DRQLC
D.simulans GABA           CPMNLQYFPM DRQLC
H.americanus GABA         CPMNLQYFPM DRQLC
D.melanogaster GABA       CPMNLQYFPM DRQLC
C.auratus GABA            CPMRLINFPM DGHSC
                          *** *  *** * : *
```

Figure 5.6. Amino acid alignment of the Cys loop of both GABAα and GluClα subunits. The *asterisks* highlight residues conserved within the subunits.

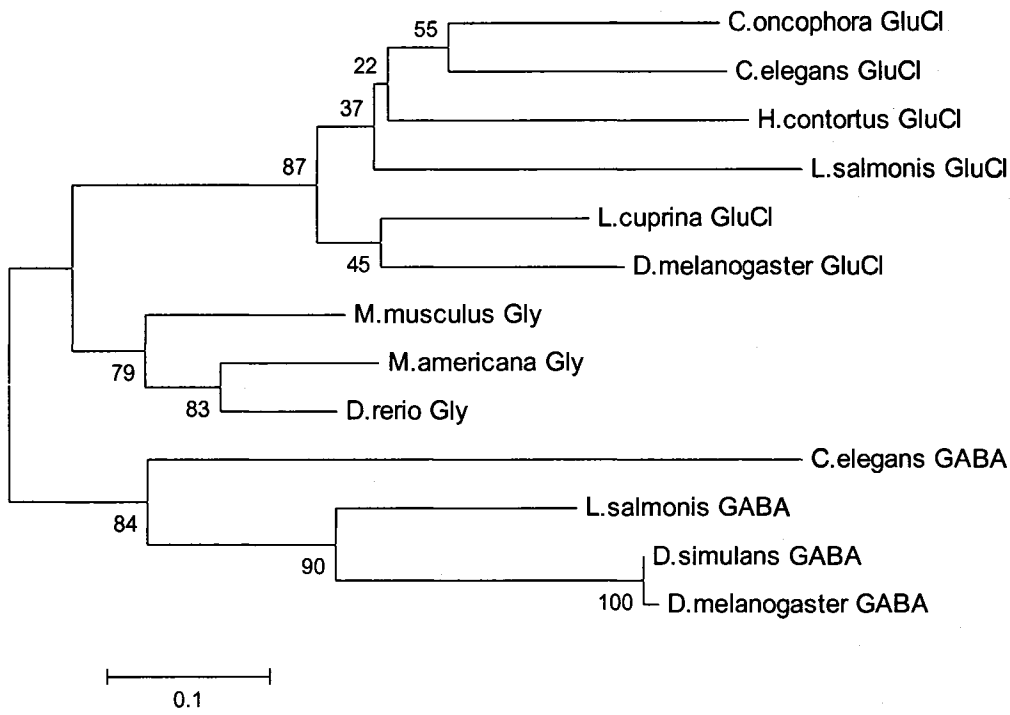

Figure 5.7. Unrooted phylogenetic tree showing the relationship of the two GABAα and Gluα receptor subunits to other species using a conserved 119bp sequence located in the second transmembrane domain in these receptors. These groupings were supported by bootstrap analysis.

5.5. Discussion

The invertebrate GluCl and GABA receptors share both structural as well as sequence similarities to $GABA_A$ receptors. Using degenerate primers based on conserved homologous regions of both the GABAα and GluClα receptor subunits of other species PCR products were generated from *L. salmonis* total RNA. The use of touchdown PCR, rather than conventional PCR generated sufficient GluCl PCR product to be visualized in an agarose gel due to the reaction amplifying less non-specific products. The GABAα and GluClα sequences were successfully extended using 5` and 3` RACE reactions to yield large open reading frames, which have the highest levels of identity to their respective receptor subunits. Phylogenetic analysis also supported this finding and offered an insight into the relationship between the two receptors. The GluClα receptor subunits appear to have a closer relationship to GlyClα receptors subunits than GABAα subunits. GlyCl receptors have only been identified in vertebrates and represent an additional inhibitory chloride channel in the vertebrate CNS (Langosch *et al.,* 1990). Both GluCl- and GABA- receptors are the closest homologes to GluCl and are likely sites of avermectin toxicity (Kane *et al.,* 2000). Previous evolutionary analysis has suggested that GABA is an ancesteral protein, and that GluCl and GlyCl represent evolutionarily newer receptors (Ortells and Lunt, 1995).

Member subunits of the ligand-gated chloride channel superfamily exhibit significant homology at both the DNA sequence and the protein levels. Invertebrate GluClα and GluClβ subunits are known to share approximately 45% homology at the amino acid level (Etter *et al.,* 1996). Results from this study show the *L. salmonis* GluClα

has a lower similarity to GluClβ receptors subunits of other species than is seen with GluClα subunits (58% compared to 85%). This pattern was also seen with *L. salmonis* GABAα receptor, sharing only a 69% similarity to GABAβ receptor subunits compared to an 85% similarity to GABAα in other species.

The *L. salmonis* putative GABAα and GluClα receptors subunits share characteristics displayed by all member subunits of the ligand-gated chloride channel superfamily; prominent features of these channels include a large N-terminal extracellular domain to which the agonist binds and four transmembrane segments (TM1–TM4), of which TM2 forms a putative pore lining α-helix (Vassilatis *et al.,* 1997). Among the notable extracellular domains present within the GABAα and GluClα *L. salmonis* subunits was a 15-residue signature loop (Cys loop) that is conserved among all ligand-gated chloride channels and is formed through a disulfide bond which links a pair of cysteine residues (Schofield *et al.,* 2003). The Cys loop contains a number of residues that are highly conserved, including a phenylalanine- proline motif in GABAα receptor subunit and a tyrosine- proline motif in GluClα subunits. These motifs are suggested to form a hairpin in the protein backbone and an adjacent, invariant aspartic acid residue (Cockcroft *et al.,* 1990). This same motif can be found in the GABAα subunit, but in the GluClα subunits the phenylalanine is replaced by another aromatic amino acid, tyrosine. Site-directed mutagenesis and functional expression studies have shown that residues within the Cys loop do not form the agonist binding site of glycine or $GABA_A$ receptors (Vandenberg *et al.,* 1993). To date, no study has delineated a specific functional role for the Cys loop; yet the highly conserved nature of this feature would imply that it is of critical importance.

Studies involving *C. elegans*, *C. oncophora*, and *D. melanogaster* all demonstrate that certain mutations in these subunits can be responsible for reduced avermectin binding affinity and possible development of avermectin resistance (Blackhall *et al.,* 2003; Kane *et al.,* 2000; Njue *et al.,* 2004; Njue and Prichard, 2004). It should be noted, however, that mutations in the target receptors is not the only proposed mechanism for avermectin resistance. Studies also support the increase in expression of the multiple drug transporter P-glycoprotein which reduces avermectin uptake and limits access to the target receptors (Xu *et al.,* 1998).

Future investigations should be directed toward a better understanding of the mechanism of action of emamectin benzoate in *L.* salmonis. This should involve expression of these genes to examine emamectin-binding potential. This would be extremely useful in determining to which receptor emamectin binds strongest and, thus, will give an indication of how significant potential mutations of these receptors will be on avermectin resistance development. Pharmacological studies focusing on the emamectin binding characteristics of these proteins will allow us to further understand the mechanism of action in *L. salmonis* and assist in possibly improving the therapeutic efficacy of these compounds. This research also provides the basis for future mutational analysis in these receptors as a potential for resistance development as has been done in other species (Blackhall *et al.,* 2003; Njue and Prichard, 2004). Thus we have set the basis for expanding the knowledge of emamectin drug-receptor interactions which will provide further understanding of how best to prevent and manage resistance development to avermectins. Thus we have set the basis for expanding the knowledge of emamectin

drug-receptor interactions which will provide further understanding of how best to prevent and manage resistance development to avermectins.

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CHAPTER 6. GENERAL DISCUSSION

6.1. Project summary

Sea lice (*Lepeophtheirus salmonis*) are persistent, aggressive ectoparasites of Atlantic salmon and thus continue to be a major concern to Atlantic salmon farms in many areas of the world. Presently the use of good husbandry practices, assisted by the use of chemicals and drugs to treat salmon helps to prevent sea lice outbreaks (Grant, 2002; Wall, 2005). Physical methods alone have not been sufficient to control *L. salmonis* infestation and therefore chemotherapeutic approaches are heavily relied on to maintain low lice burdens on salmon farms (Burka *et al.,* 1997; Pike and Wadsworth, 1999). Emamectin benzoate (EMB), an avermectin, is an orally administered drug that, when given at a dose of 50μg/kg/day for a seven day period, will kill all parasitic stages of sea lice with no adverse toxic effects on the salmon (Stone *et al.,* 2000; Ramstad *et al.,* 2002) and limited negative impact on the environment (Burridge, 2003). It is currently the major, and often the only, drug used in salmonid aquaculture to control sea lice (Wall, 2005).

Avermectins have been used to treat humans, pets, and livestock against various parasites (Vercruysse and Rew, 2002). Resistance to avermectin compounds has become increasingly documented in numerous organisms, most commonly in nematodes (Echevarria *et al.,* 1992; Vermunt *et al.,* 1995; Kaplan, 2002). The resistance mechanisms involved for different species, particularly gastrointestinal nematodes of ruminants and horses, has been extensively investigated. Understanding these resistance mechanisms as well as the factors influencing the spread of resistance development is desirable when implementing management programs. Avermectin resistance is suggested to be multi-factorial involving the following potential mechanisms: decreased penetration, increased

excretion, increased oxidative metabolism, altered target site, GST (glutathione S-transferase)-dependent conjugation and β-tubulin (Rohrer *et al.,* 1990; Clark *et al.,* 1995; Bloomquist, 1996; Jagannathan *et al.,* 1999; Grant, 2000; Eng *et al.,* 2006). Previous studies indicate target site mutations and/or over-expression of P-glycoproteins (P-gps) to be the predominant mechanisms involved in the development of an avermectin resistance phenotype (Xu *et al.,* 1998; Molento and Prichard, 2001). This research represents the first to investigate these potential EMB resistance mechanisms in *L. salmonis*. In this study we isolated the genes encoding for both putative *L. salmonis* EMB target GABA and GluCl alpha receptor subunits. GABAα and GluClα receptor subunits were identified in *L. salmonis* using degenerate primers applied in a "touch-down" PCR. These sequences were extended using both 3` and 5` rapid amplification of cDNA ends (RACE) to yield products of 1434bp and 1332bp, respectfully. Results of BLAST analysis on both GABAα and GluClα receptor amino acid sequences identified the highest similarity (approximately 85%) for their respective receptor subunits. Both GABAα and GluClα *L. salmonis* receptor subunits characterized share features typical of ligand gated chloride channels including four putative transmembrane regions, a long N-terminal extracellular domain and a very short C-terminal extracellular domain. Phylogenetic analysis, as expected, grouped the *L. salmonis* GABAα and GluClα receptors with their respective channel in other invertebrates.

Studies involving *Caenorhabditis elegans*, *Cooperia oncophora*, and *Drosophila melanogaster* all demonstrate that certain mutations in these GABA or GluCl receptors can be responsible for reduced avermectin binding affinity and possible development of avermectin resistance (Kane *et al.,* 2000; Blackhall *et al.,* 2003; Njue and Prichard,

2004). The isolation of the genes encoding putative GABAα and GluClα subunits provides the basis for future mutational analysis in these receptors as a potential for avermectin resistance development. No comparison could be made with EMB resistant sea lice due to its absence in the field and a lack of EMB driven resistance in the lab. Studies with increased numbers of sea lice especially in regions with reported need for increased SLICE® administration may demonstrate sea lice receptor polymorphisms or point mutations. A previous study used this approach to investigate *L. salmonis* from areas of pyrethroid treatment failure and identified a novel point mutation in the sodium channel target receptor which could be associated with resistance to pyrethroids in this species (Fallang *et al.,* 2005). Studies investigating avermectin resistance have identified possible mutations conferring resistance in *C. oncophora* (Njue and Prichard, 2004).

Future studies will focus on the ligand binding properties of the *L. salmonis* GABA and GluCl alpha subunits expressed in cell lines. This information will give further insight into the pharmacology of avermectins in sea lice and may offer itself as a means to possibly improve the therapeutic efficacy. As well as an improved knowledge of the pharmacology of emamectin drug binding characteristics, determining at which receptor emamectin has the highest affinity will give an indication of how influential potential mutations of these receptors will be on avermectin resistance development. These expressional studies can be expanded further if EMB resistant sea lice are obtained showing mutations in these target receptor subunits.

The major focus of this research project was directed toward ATP Binding Cassette (ABC) drug transporters. ABC proteins form one of the largest protein families with members being identified in every organism characterized to date, with P-gp falling

in ABC subfamily B. Active membrane transporters in this family have a minimal structural requirement of two transmembrane domains (six transmembrane helices in each) and two nucleotide binding domains involved in ATP binding. Although a large majority of these proteins are active pumps and subsequently transport substances against a concentration gradient, there are several examples that deviate from this function. An example is the GCN20 protein belonging to the ABC subfamily F. These proteins consist of two fused nucleotide binding domains and do not contain transmembrane regions. They are located intracellularly and do not function as a transporter but have nuclear signal properties which in humans have been suggested to play a role in aminoacyl-tRNA binding. As these proteins lack any transmembrane domains they are considerably smaller in size than the membrane transporter proteins (~70kDa smaller).

Various genetic studies indicate that the helminth *C. elegans* has at least fourteen P-gp homologs (Bard, 2000); it is therefore possible that there are numerous ABC proteins in *L. salmonis* that could actively serve to eliminate EMB. This study identified two *L. salmonis* ABC transporter genes, SL0525 and SL-Pgp1. The SL0525 gene was identified in a sea lice expressed sequence tag (EST) database whereas the SL-Pgp1 gene was generated by RT-PCR using degenerate primers previously designed by Xu *et al.*, (1998). A BLAST search showed SL-Pgp1 had a ~73% homology to other P-gps whereas the SL0525 had a relatively lower level of homology (~35%). Further analysis of both the SL-Pgp1 and SL0525 sequences identified distinctive conserved ABC transporter regions known as Walker A/B and signature motifs confirming their identity as ABC proteins. BLAST analysis suggested that the SL0525 protein may belong to the ABC-F subfamily based on its amino acid composition.

A GST expression system was used for the expression of the partial SL0525 gene. The resulting SL0525 GST-fusion protein was purified to immunize a rabbit for the production of SL0525 specific antiserum. After confirming the specificity of this antibody against SL0525 it was used to determine the size of the SL0525 full-length protein. In conjunction with this polyclonal antibody (pAb), we also used two commercially available P-gp specific monoclonal antibodies (mAb), C219 and JSB-1. C219 recognizes an internal, highly conserved amino acid sequence VQEALD and VQAALD, corresponding to the C-terminal and N-terminal regions, respectively, found in isoforms of P-gp (van den Elsen *et al.,* 1999). JSB-1 recognizes a highly conserved cytoplasmic epitope of the human P-gp (Scheper *et al.,* 1988). These antibodies have been used for the detection of P-gp in numerous organisms, including a variety of aquatic organisms (Hemmer *et al.,* 1995; Lyons-Alcantara *et al.,* 2002). These antibodies were applied to sea lice membrane protein fractions in Western blot analysis. The results show the detection of a 160kDa protein for each of these antibodies, which resemble P-gp with respect to the expected molecular mass. The size of this protein corresponds to a "full-transporter" which suggests that the SL0525 full-length protein does not belong to ABC subfamily F as it is too large for these types of proteins. Detecting a protein of the estimated size for P-gp is only the first step and it was far more important to determine where these proteins are expressed in the sea lice.

Methods were developed for the immunolocalization of P-gps in *L. salmonis* and its Atlantic salmon host (*Salmo salar*) using these mAbs and pAb. During this investigation American lobster organs were used as a crustacean control. Both sea lice and lobsters showed many similarities in the staining pattern and signal intensity

generated for each of the antibodies used. Importantly for this study, both expressed P-gp on the epithelial lining of the intestinal tract with the greatest intensity being seen with JSB-1 and SL0525. C219 only generated a weak signal suggesting that epitope presentation in the intestinal tract does not favor the binding of this antibody. The localization of P-gp in the intestinal tract of the sea lice was the primary interest as the intestine is the initial absorption site for EMB and thus a critical site for decreased drug absorption should P-gp be over-expressed. Smith and Prichard (2002) also localized P-gp primarily in the intestinal tract of the nematode worm *H. contortus* and suggested over-expression as a potential mechanism of ivermectin resistance. The localization of the SL0525 to the intestinal epithelial lining further suggests that this protein does not belong to the ABC subfamily F, but raises the question what substrate(s) does it transport and would EMB exposure induce upregulation of this protein?

In mammals, P-gps are present in renal tubular cells, hepatocyte canulicular cells, intestinal epithelium, and the blood brain barrier. This expression plays a significant role in reducing drug absorption and enhancing drug elimination (Fromm, 2000). Previous studies in teleosts have shown P-gp distribution in different tissues to resemble those seen in mammals (Hemmer *et al.,* 1995). The salmon tissues in this study showed expression of P-gp in all three tissues (liver, kidney, and intestine) investigated. The staining patterns generated by C219 compare well to those seen in other vertebrate species. C219 was the only antibody able to specifically detect P-gp in all three tissues with JSB-1 only generating a weak signal in the intestinal tissue. The significance of P-gp expression in salmon tissues has implications on the pharmacokinetics of EMB. Increased P-gp

expression could reduce the amount of drug present in the salmon mucus, skin and blood thus lowering the actual concentration of drug to which the sea lice are exposed.

The SL0525 and SL-Pgp1 genes were further investigated in Chapter 4 where quantitative RT-PCR (Q-RT-PCR) was used to measure their relative levels of expression in sea lice in relation to a previously validated reference gene, the translation eukaryotic elongation factor 1 alpha (eEF1α) (Frost and Nilsen, 2003). In the absence of sea lice which show an EMB resistant phenotype we wanted to investigate the potential role of both SL0525 and SL-Pgp1 proteins in EMB resistance. Lice were applied to an EMB bioassay previously developed by Westcott *et al* (2006). The surviving sea lice from 10ppb and 30ppb EMB exposure were used to determine if these genes had increased mRNA levels in response to EMB exposure. The results showed that gene expression of both SL0525 and SL-Pgp1 genes significantly increased in response to 10ppb EMB exposure. At the 30ppb EMB exposure no significant increase in gene expression could be seen, although a trend toward increased expression was evident. The marked difference in gene expression between 10ppb and 30ppb exposure groups could be explained by the sea lice being moribund at the higher EMB concentration.

To date Q-RT-PCR studies in aquatic organisms have primarily only focused on immune response to infectious agents (viral and bacterial) (Holland *et al.,* 2003; Morrison *et al.,* 2006; Bridle *et al.,* 2006; Hauton *et al.,* 2007). Previous studies examining changes in P-gp expression in invertebrates have used methods that are far less sensitive than Q-RT-PCR, such as Northern blot, semi-quantitative PCR and RNase protection analysis (Lincke *et al.,* 1993; Xu *et al.,* 1998; Huang and Prichard, 1999). This study is the first to document that putative P-gps in *L. salmonis* increase in transcription in response to a

xenobiotic, in this case EMB, and also the first to examine expression levels of P-gp in a parasitic organism in response to drug exposure using Q-RT-PCR.

The lice used for Q-RT-PCR analysis were cultured in our aquatic animal facilities and, therefore, unlike lice in the wild, were exposed to a stable "pristine" environment. Additional studies should now be directed toward determining the baseline levels of expression of these two genes in both pre-adult and adult sea lice in the field. Any future reports of EMB treatment failure could then apply this information to determine if constitutive over-expression of these genes, compared to this baseline expression level, is correlated to resistance development.

Although the Q-RT-PCR was used with great success in this study, the use of mRNA quantification to predict changes in actual protein levels is of course an assumption that is made where Western blot analysis is not performed in parallel. Future investigations could use the methods developed in this study to examine P-gp expression both at the mRNA and protein level in parallel. These studies would however require far greater numbers of pre-adult sea lice which could be a limitation, especially when the lice are being collected from the field rather than a lab culture population. Additional studies could also involve examining lice over a time series at 10ppb EMB to determine how rapidly these genes increase in expression. This may give an indication to which transporter is more active in terms of tolerance to EMB.

The results of this study represent a significant finding in terms of understanding the potential for EMB resistance development in *L. salmonis*. Interestingly, although the SL0525 gene showed a lower than expected similarity to P-gps found in other species, this transporter was shown not only to be of the same size as expected for P-gp in *L.*

salmonis, but is also localized on the epithelial lining of the sea lice intestinal tract and increases in its transcription in response to EMB exposure, possibly to limit absorption. This strongly suggests that the SL0525 gene encodes a *L. salmonis* P-gp like protein, although further work possibly involving expressional studies to determine its drug binding properties would help to confirm this.

Future research could be directed at expressional and immunological studies of the SL-Pgp1 protein to confirm whether protein size and localization fit with P-gp in sea lice. *In situ* hybridization using DIG labeled SL-Pgp1 probes and/or immunohistochemistry using specific antidodies may also allow for protein and mRNA localization.

6.2. Future Directions

RNA interference (RNAi) is a new technique for specific gene inhibition. RNAi is a post-transcriptional gene-silencing mechanism that can be initiated by double-stranded RNA homologous in sequence to the targeted gene (Milhavet *et al.,* 2003). This method has been used to manipulate the expression of both *MDR1* mRNA and its P-gp protein product in tumor cells (Lage, 2006). This gene suppression restored chemotherapeutic sensitivity in these cells caused by *MDR1* constitutive over-expression (Lage, 2006). It is possible future research could investigate the use of RNAi to suppress the expression of the SL0525 and/or SL-Pgp1 proteins to investigate if there is an increase in EMB sensitivity. This would also help further characterize the involvement of these two proteins in EMB tolerance and their potential role in resistance development.

P-gp blockers such as vinblastine, which act as non-competitive inhibitors, and verapamil, which is a competitive P-gp inhibitor (Garrigos *et al.,* 1997), have been shown to reduce resistance to benzimidazole or avermectin in resistant nematode populations (Kerboeuf *et al.,* 2003). However, the use of P-gp blockers to counter resistance in parasites has to date been unsuccessful due to systemic side effects in the host (von Samson-Himmelstjerna and Blackhall, 2005). On the other hand, the use of such chemicals could be applied to investigate possible increased sensitivity to EMB exposure using the same bioassay setup as was used in this study. This would also further our understanding of the binding specificities of P-gp in sea lice and their role in limiting EMB absorption.

This study focused on two of the predominant mechanisms of avermectin resistance, it is important to recognize that resistance development is a multi factorial process potentially involving mechanisms outside the scope of this research. Other investigations have suggested changes in enhanced metabolic enzyme activity and another recent study showed possible selection of specific β-tubulin alleles in ivermectin resistance nematodes (Eng *et al.,* 2006), although its actual function in providing resistance is not understood. These mechanisms should not be ignored and warrant further investigations to fully understand the big picture of how resistance development may occur.

Efficacious vaccines to protect animals against parasitic infections are few and difficult to produce, due to the antigenic variability created by the different stages of many parasites. The development of vaccines against sea lice is still an area being investigated. There is still no sign of a vaccine being available in the near future. It is

therefore important to better understand the mechanism of action and the potential for EMB resistance development. This knowledge can be transferred to the aquaculture industry to offer both diagnostic methods for the early detection of resistance and possibly offer advice for alternative treatments. Decreasing the selection pressure for such resistance, thus maintaining EMB use in an already limited available drug pool, is therefore important for the Atlantic salmon industry.

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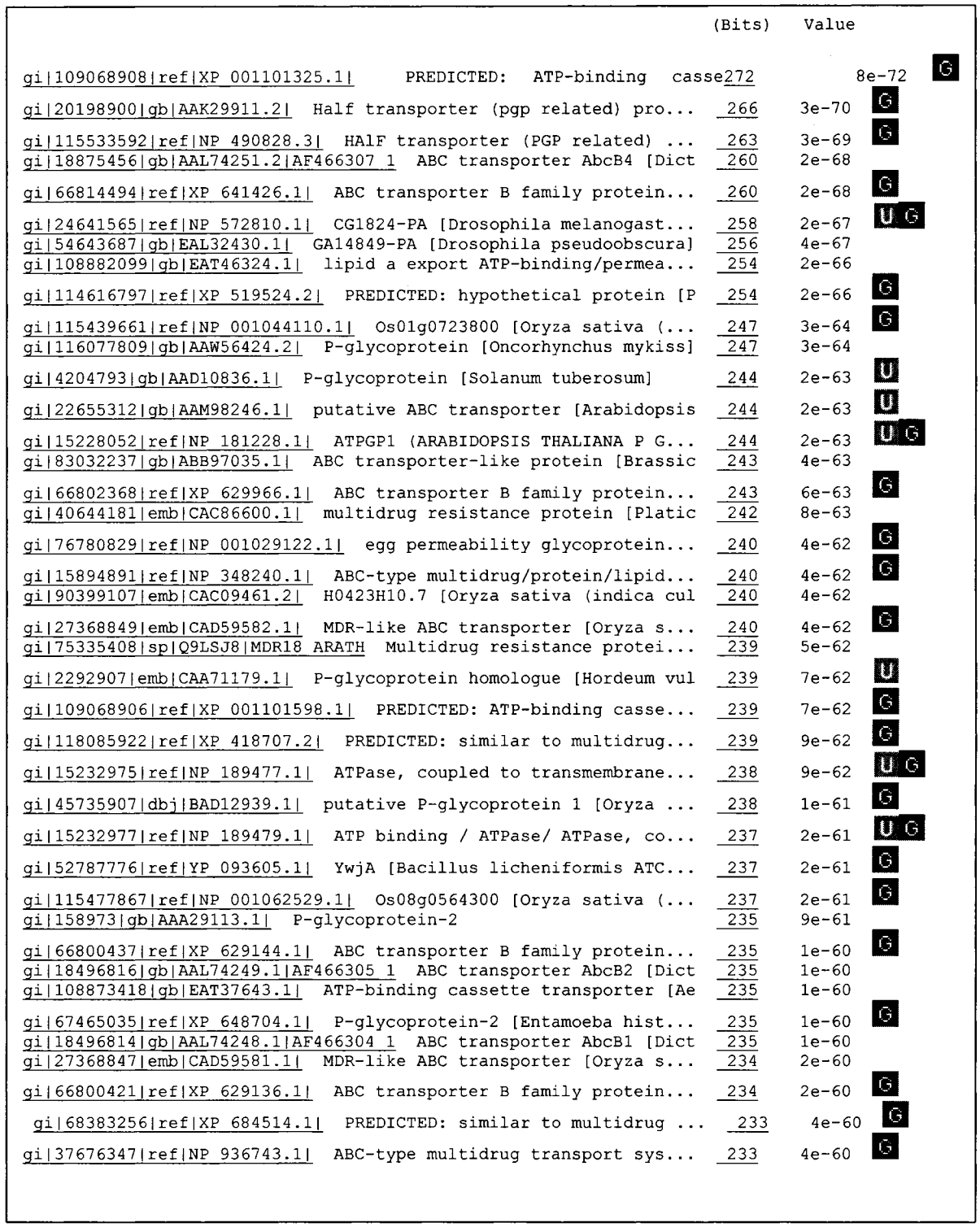
APPENDIX

APPENDIX A. BLAST RESULTS

BLAST analysis showing the top 40 sequences producing significant alignments to SL0525

	(Bits)	Value	
gi|21711697|gb|AAM75039.1| LD35151p [Drosophila melanogaster]	375	1e-102	U
gi|66513776|ref|XP_623255.1| PREDICTED: similar to CG1703-PA iso	373	4e-102	G
gi|24641342|ref|NP_572736.1| CG1703-PA [Drosophila melanogast...	367	2e-100	U G
gi|58393761|ref|XP_320293.2| ENSANGP00000016545 [Anopheles gambi	366	4e-100	G
gi|54643963|gb|EAL32706.1| GA14282-PA [Drosophila pseudoobscura]	365	6e-100	
gi|108883568|gb|EAT47793.1| ATP-dependent transporter [Aedes aeg	363	3e-99	
gi|116116875|gb|EAA00265.3| ENSANGP00000016545 [Anopheles gambia	363	4e-99	
gi|91076670|ref|XP_971562.1| PREDICTED: similar to CG1703-PA [Tr	356	5e-97	G
gi|57907380|ref|XP_551949.1| ENSANGP00000025805 [Anopheles gambi	344	2e-93	G
gi|39583900|emb|CAE63990.1| Hypothetical protein CBG08583 [Caeno	298	1e-79	
gi|17559834|ref|NP_506192.1| Temporarily Assigned Gene name f...	295	1e-78	U G
gi|115699067|ref|XP_781808.2| PREDICTED: hypothetical protein...	291	1e-77	G
gi|74139663|dbj|BAE31683.1| unnamed protein product [Mus musculu	286	7e-76	U G
gi|56404330|sp|Q767L0|ABCF1_PIG ATP-binding cassette sub-fami...	285	2e-75	
gi|73972119|ref|XP_532056.2| PREDICTED: similar to ATP-bindin...	284	3e-75	U G
gi|10863747|gb|AAG23960.1|AF293383_1 ABC50 [Rattus norvegicus]	283	4e-75	U G
gi|109509122|ref|XP_001062137.1| PREDICTED: similar to ATP-bi...	283	4e-75	G
gi|109509674|ref|XP_001056151.1| PREDICTED: similar to ATP-bi...	283	4e-75	G
gi|62088708|dbj|BAD92801.1| ATP-binding cassette, sub-family ...	282	8e-75	U G
gi|50949460|emb|CAH10648.1| hypothetical protein [Homo sapiens]	282	9e-75	U G
gi|109070245|ref|XP_001100719.1| PREDICTED: ATP-binding casse...	281	1e-74	G
gi|39930335|ref|NP_038882.1| ATP-binding cassette, sub-family...	281	2e-74	U G
gi|55700811|dbj|BAD69766.1| ATP-binding cassette, sub-family ...	281	2e-74	
gi|108860671|ref|NP_001035838.1| ATP-binding cassette, sub-fa...	280	3e-74	G
gi|55777326|gb|AAH46965.1| ATP-binding cassette, sub-family F...	280	5e-74	U G
gi|55961304|emb|CAI18157.1| ATP-binding cassette, sub-family ...	279	7e-74	G
gi|69354671|ref|NP_001020262.1| ATP-binding cassette, sub-fam...	279	7e-74	U G
gi|21759807|gb|AAH34488.1| ATP-binding cassette, sub-family F...	279	7e-74	U G
gi|10947135|ref|NP_001081.1| ATP-binding cassette, sub-family...	279	7e-74	U G
gi|47085973|ref|NP_998351.1| ATP-binding cassette sub-family ...	275	9e-73	U G
gi|51703659|gb|AAH81034.1| MGC81714 protein [Xenopus laevis]	269	1e-70	U G
gi|46189243|gb|AAH68282.1| Abcf1 protein [Mus musculus]	251	2e-65	U G
gi|18410084|ref|NP_567001.1| ATGCN4 [Arabidopsis thaliana] >g...	213	6e-54	U G
gi|21537128|gb|AAM61469.1| putative ABC transporter [Arabidopsis	210	5e-53	U
gi|52207936|emb|CAE47098.1| ABC transporter [Populus tremula x P	210	5e-53	U
gi|47204268|emb|CAG13733.1| unnamed protein product [Tetraodon n	210	6e-53	
gi|115453721|ref|NP_001050461.1| Os03g0441500 [Oryza sativa (...	206	7e-52	G
gi|17861928|gb|AAL39441.1| GM14873p [Drosophila melanogaster]	204	4e-51	U
gi|18859989|ref|NP_573057.1| CG9281-PB, isoform B [Drosophila...	202	8e-51	U G

BLAST analysis showing the top 40 sequences producing significant alignments to SL-Ppg1



	(Bits)	Value
gi|109068908|ref|XP_001101325.1| PREDICTED: ATP-binding casse	272	8e-72
gi|20198900|gb|AAK29911.2| Half transporter (pgp related) pro...	266	3e-70
gi|115533592|ref|NP_490828.3| HAlF transporter (PGP related) ...	263	3e-69
gi|18875456|gb|AAL74251.2|AF466307_1 ABC transporter AbcB4 [Dict	260	2e-68
gi|66814494|ref|XP_641426.1| ABC transporter B family protein...	260	2e-68
gi|24641565|ref|NP_572810.1| CG1824-PA [Drosophila melanogast...	258	2e-67
gi|54643687|gb|EAL32430.1| GA14849-PA [Drosophila pseudoobscura]	256	4e-67
gi|108882099|gb|EAT46324.1| lipid a export ATP-binding/permea...	254	2e-66
gi|114616797|ref|XP_519524.2| PREDICTED: hypothetical protein [P	254	2e-66
gi|115439661|ref|NP_001044110.1| Os01g0723800 [Oryza sativa (...	247	3e-64
gi|116077809|gb|AAW56424.2| P-glycoprotein [Oncorhynchus mykiss]	247	3e-64
gi|4204793|gb|AAD10836.1| P-glycoprotein [Solanum tuberosum]	244	2e-63
gi|22655312|gb|AAM98246.1| putative ABC transporter [Arabidopsis	244	2e-63
gi|15228052|ref|NP_181228.1| ATPGP1 (ARABIDOPSIS THALIANA P G...	244	2e-63
gi|83032237|gb|ABB97035.1| ABC transporter-like protein [Brassic	243	4e-63
gi|66802368|ref|XP_629966.1| ABC transporter B family protein...	243	6e-63
gi|40644181|emb|CAC86600.1| multidrug resistance protein [Platic	242	8e-63
gi|76780829|ref|NP_001029122.1| egg permeability glycoprotein...	240	4e-62
gi|15894891|ref|NP_348240.1| ABC-type multidrug/protein/lipid...	240	4e-62
gi|90399107|emb|CAC09461.2| H0423H10.7 [Oryza sativa (indica cul	240	4e-62
gi|27368849|emb|CAD59582.1| MDR-like ABC transporter [Oryza s...	240	4e-62
gi|75335408|sp|Q9LSJ8|MDR18_ARATH Multidrug resistance protei...	239	5e-62
gi|2292907|emb|CAA71179.1| P-glycoprotein homologue [Hordeum vul	239	7e-62
gi|109068906|ref|XP_001101598.1| PREDICTED: ATP-binding casse...	239	7e-62
gi|118085922|ref|XP_418707.2| PREDICTED: similar to multidrug...	239	9e-62
gi|15232975|ref|NP_189477.1| ATPase, coupled to transmembrane...	238	9e-62
gi|45735907|dbj|BAD12939.1| putative P-glycoprotein 1 [Oryza ...	238	1e-61
gi|15232977|ref|NP_189479.1| ATP binding / ATPase/ ATPase, co...	237	2e-61
gi|52787776|ref|YP_093605.1| YwjA [Bacillus licheniformis ATC...	237	2e-61
gi|115477867|ref|NP_001062529.1| Os08g0564300 [Oryza sativa (...	237	2e-61
gi|158973|gb|AAA29113.1| P-glycoprotein-2	235	9e-61
gi|66800437|ref|XP_629144.1| ABC transporter B family protein...	235	1e-60
gi|18496816|gb|AAL74249.1|AF466305_1 ABC transporter AbcB2 [Dict	235	1e-60
gi|108873418|gb|EAT37643.1| ATP-binding cassette transporter [Ae	235	1e-60
gi|67465035|ref|XP_648704.1| P-glycoprotein-2 [Entamoeba hist...	235	1e-60
gi|18496814|gb|AAL74248.1|AF466304_1 ABC transporter AbcB1 [Dict	235	1e-60
gi|27368847|emb|CAD59581.1| MDR-like ABC transporter [Oryza s...	234	2e-60
gi|66800421|ref|XP_629136.1| ABC transporter B family protein...	234	2e-60
gi|68383256|ref|XP_684514.1| PREDICTED: similar to multidrug ...	233	4e-60
gi|37676347|ref|NP_936743.1| ABC-type multidrug transport sys...	233	4e-60

BLAST analysis showing the top 40 sequences producing significant alignments to GABA alpha receptor

	(Bits)	Value	
gi|24661416|ref|NP_523991.2| Resistant to dieldrin CG10537-PA...	298	5e-80	U G
gi|405123|gb|AAA19249.1| GABA receptor	298	5e-80	U
gi|44887891|sp|Q9BLY8|GBRB_DROSI Gamma-aminobutyric-acid rece...	298	7e-80	
gi|110758209|ref|XP_001120292.1| PREDICTED: similar to Resist...	297	1e-79	G
gi|110555508|gb|ABG75734.1| GABA-gated chloride channel [Apis me	297	1e-79	G
gi|5762320|gb|AAD51101.1|AF172352_1 GABA receptor subunit [Cerat	296	2e-79	
gi|58388146|ref|XP_316071.2| ENSANGP00000022957 [Anopheles gambi	296	3e-79	G
gi|91093188|ref|XP_968857.1| PREDICTED: similar to CG10537-PA...	296	3e-79	U G
gi|2245656|gb|AAB62563.1| GABA-gated chloride channel isoform a2	296	3e-79	
gi|49205114|dbj|BAD16658.2| GABA-gated chloride channel subunit	295	4e-79	
gi|3719468|gb|AAC63381.1| GABA receptor Rdl subunit [Apis mellif	295	5e-79	U G
gi|2245681|gb|AAB62572.1| GABA-gated chloride channel isoform a3	295	6e-79	
gi|2565319|gb|AAB81966.1| gamma-aminobutyric acid receptor subun	295	8e-79	
gi|114650274|dbj|BAF31884.1| GABA-gated chloride channel subunit	294	1e-78	
gi|4691327|emb|CAB41615.1| Gaba-gated chloride channel [Heliothi	293	2e-78	
gi|24661412|ref|NP_729461.1| Resistant to dieldrin CG10537-PC...	290	2e-77	U G
gi|24661420|ref|NP_729462.1| Resistant to dieldrin CG10537-PB...	290	2e-77	U G
gi|157472|gb|AAA28556.1| GABA-alpha receptor	289	3e-77	U
gi|157473|gb|AAA28557.1| GABA-alpha receptor	289	4e-77	U
gi|157474|gb|AAA28558.1| GABA-alpha receptor	289	4e-77	U
gi|108875659|gb|EAT39884.1| gaba receptor invertebrate [Aedes ae	288	6e-77	
gi|110555506|gb|ABG75733.1| GABA-gated chloride channel [Apis me	288	9e-77	G
gi|58388144|ref|XP_316070.2| ENSANGP00000024940 [Anopheles gambi	287	1e-76	G
gi|58388148|ref|XP_316072.2| ENSANGP00000005863 [Anopheles gambi	287	1e-76	G
gi|881590|gb|AAA68961.1| GABA receptor subunit	287	2e-76	U
gi|15291147|gb|AAK92842.1| GH09619p [Drosophila melanogaster]	285	8e-76	
gi|2245658|gb|AAB62564.1| GABA-gated chloride channel isoform a1	269	3e-71	
gi|3228680|gb|AAC23602.1| GABA receptor subunit [Musca domestica	266	3e-70	
gi|103170|pir||A41145 gamma-aminobutyric acid receptor A, cyc...	266	3e-70	
gi|83779000|gb|ABC47324.1| GABA receptor [Tetranychus urticae]	253	2e-66	
gi|57165040|gb|AAW34359.1| GABA receptor [Plutella xylostella]	241	1e-62	
gi|57165036|gb|AAW34357.1| GABA receptor [Plutella xylostella...	241	1e-62	
gi|2555168|gb|AAB81523.1| GABA receptor subunit [Tribolium casta	212	4e-54	
gi|71984488|ref|NP_498532.3| F11H8.2 [Caenorhabditis elegans]...	201	1e-50	U G
gi|68359755|ref|XP_690068.1| PREDICTED: glycine receptor, alpha	200	2e-50	G
gi|47222620|emb|CAG02985.1| unnamed protein product [Tetraodon n	200	2e-50	
gi|76672871|ref|XP_610178.2| PREDICTED: similar to Glycine re...	200	2e-50	G
gi|39593954|emb|CAE70064.1| Hypothetical protein CBG16500 [Caeno	199	3e-50	
gi|1363142|pir||A49970 glycine receptor alpha-4 chain - mouse (f	199	3e-50	
gi|817957|emb|CAA53468.1| glycine receptor subunit alpha 4 [Mus	199	3e-50	G

BLAST analysis showing the top 40 sequences producing significant alignments to GluCl alpha receptor

	(Bits)	Value	
gi|110555514|gb|ABG75737.1| glutamate-gated chloride channel [Ap	164	2e-39	G
gi|118150476|ref|NP_001071277.1| glutamate-gated chloride cha...	164	2e-39	G
gi|91092750|ref|XP_973383.1| PREDICTED: similar to CG7535-PB,...	163	3e-39	U G
gi|3559846|emb|CAA05260.1| DrosGluCl [Drosophila melanogaster]	163	3e-39	
gi|66524280|ref|XP_391998.2| PREDICTED: similar to CG7535-PB, is	163	4e-39	G
gi|24648249|ref|NP_732447.1| GluClalpha CG7535-PB, isoform B ...	163	4e-39	U G
gi|62472736|ref|NP_001014641.1| GluClalpha CG7535-PC, isoform...	162	7e-39	U G
gi|110277453|gb|ABG57261.1| glutamate-gated chloride channel [Dr	158	1e-37	U
gi|62901525|sp|Q94900|GLUCL_DROME Glutamate-gated chloride chann	157	2e-37	
gi|24648251|ref|NP_650827.2| GluClalpha CG7535-PA, isoform A ...	157	3e-37	U G
gi|1507685|gb|AAC47266.1| glutamate-gated chloride channel [Dros	156	4e-37	U
gi|54639797|gb|EAL29199.1| GA20421-PA [Drosophila pseudoobscura]	156	4e-37	
gi|3420880|gb|AAC31949.1| glutamate gated chloride channel [Luci	156	4e-37	
gi|46409071|dbj|BAD16657.1| glutamate-gated chloride channel sub	155	1e-36	
gi|31242531|ref|XP_321696.1| ENSANGP00000009685 [Anopheles gambi	154	2e-36	G
gi|108881514|gb|EAT45739.1| glutamate-gated chloride channel [Ae	154	2e-36	
gi|31242533|ref|XP_321697.1| ENSANGP00000015782 [Anopheles gambi	154	2e-36	G
gi|116116437|gb|EAA01752.2| ENSANGP00000009685 [Anopheles gambia	154	2e-36	
gi|11875639|gb|AAG40735.1|AF297500_1 glutamate-gated chloride ch	153	4e-36	U
gi|34481588|emb|CAE46429.1| glutamate-gated chloride channel ...	145	8e-34	
gi|12002197|gb|AAG43232.1|AF119791_1 glutamate-gated chloride ch	142	5e-33	
gi|12002199|gb|AAG43233.1|AF119792_1 glutamate-gated chloride...	142	6e-33	
gi|4234776|gb|AAD13405.1| putative glutamate-gated chloride c...	142	6e-33	
gi|7159696|emb|CAA74622.2| GBR-2A protein [Haemonchus contortus]	140	4e-32	
gi|78771903|gb|AAR19751.2| glutamate-gated chloride channel A [H	139	5e-32	
gi|78771904|gb|ABB51214.1| glutamate-gated chloride channel B [H	139	7e-32	
gi|39595290|emb|CAE60327.1| Hypothetical protein CBG03919 [Caeno	138	1e-31	
gi|71980440|ref|NP_001020962.1| altered AVeRmectin sensitivit...	136	4e-31	U G
gi|1262893|gb|AAC25481.1| inhibitory amino acid receptor subu...	135	7e-31	U
gi|2285910|emb|CAA04171.1| GluClalpha2A protein [Caenorhabditis	132	9e-30	U
gi|72000175|ref|NP_001024076.1| altered AVeRmectin sensitivit...	132	9e-30	U G
gi|39593159|emb|CAE64628.1| Hypothetical protein CBG09387 [Caeno	131	1e-29	
gi|25395285|pir||C87791 protein B0207.12 [imported] - Caenorhabd	131	1e-29	
gi|72000177|ref|NP_001024077.1| altered AVeRmectin sensitivit...	130	2e-29	U G
gi|2285912|emb|CAA04170.1| GluClalpha2B protein [Caenorhabditis	130	2e-29	U
gi|39592961|emb|CAE62575.1| Hypothetical protein CBG06688 [Caeno	130	3e-29	
gi|1363140|pir||C49970 glycine receptor alpha-1 chain - mouse	129	5e-29	
gi|47226189|emb|CAG08336.1| unnamed protein product [Tetraodon n	129	5e-29	
gi|74006489|ref|XP_857033.1| PREDICTED: similar to Glycine re...	129	5e-29	U G
gi|17561822|ref|NP_504441.1| Glutamate-gated ChLoride channel...	129	6e-29	U G

APPENDIX B. PRE-IMMUNIZATION CONTROL REACTIONS

No reactivity was seen using the pre-immunization serum on the *Lepeophtheirus salmonis* intestine (A), *Homarus americanus* intestine (B) and hepatopancreas (C), *Salmo salar* intestine (D), kidney (E), and liver (F).