BINDING CHARACTERISTICS OF EMAMECTIN BENZOATE TO THE PUTATIVE GLUTAMATE-GATED CHLORIDE CHANNELS OF *LEPEOPHTHEIRUS SALMONIS*

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ABSTRACT

The objective of the present study was to analyze some of the binding characteristics of emamectin benzoate (EMB) to the glutamate-gated chloride channels (GluCl) of the salmon louse, *Lepeophtheirus salmonis*. This drug is used to control sea lice infestation in farmed Atlantic salmon.

Membranes from both sea lice and the CHSE-214 cell line were used in a binding assay to determine binding characteristics. The putative gene encoding the GluCl of *L. salmonis* was transfected into the cell line. The assay involved incubating the membranes at concentrations of 0, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 nM of [^{3}H]EMB for a minimum of two hours at 15°C and then terminating the reaction by filtration through glass fibre filter paper. The radioactivity of the protein retained on the filter paper was measured using a liquid scintillation counter. The calculated dissociation constant (K_D) of EMB on membranes extracted from the sea lice was 1.377 ± 0.205 nM. No binding was detected in the cell line membranes, and no GluClα protein was detected by Western blotting, indicating a potential inability of the CHSE cells to express GluCl. Binding assays were repeated on the membranes extracted from sea lice at 5°C and 20°C. The K_D calculated at these temperatures was 1.921 ± 0.715 nM and 1.578 ± 0.145 nM respectively. There was no significant change in the K_D, but there was an increase in non-specific binding at 5°C which resulted in high variability. The maximum number of receptors bound (B_{MAX}) was 9.049 ± 0.464 pmol/mg of protein at 15°C, 8.588 ± 0.268 pmol/mg of protein at 20°C, and 9.642 ± 0.822 pmol/mg at 5°C.

One of the potential mechanisms of EMB resistance development in sea lice involves a reduction or loss of drug affinity for the GluCl receptor due to a mutation

which changes the conformation of the avermectin binding site. This study confirmed the presence of one or more binding sites in the membrane of the sea lice and provided a measurement of the level of affinity. This site may correspond to a GluClα receptor. The results indicate that temperature has no significant effect on the affinity of the drug to the substrate. These data, coupled with a cell line expressing the GluCl receptor, can be used to develop a model to determine whether or not affinity is a cause for loss of sensitivity in resistant organisms.

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List of Abbreviations

°C	degrees Celsius
[^{3}H]	tritium
µM	micromolar
µg	microgram
µl	microliter
APS	ammonium persulfate
AVC	Atlantic Veterinary College
BLAST	Basic Logic Alignment Search Tool
B_{MAX}	maximum number of receptors bound
BSA	bovine serum albumin
bp	base pair
cDNA	complimentary DNA
CHSE	Chinook salmon embryo
cm	centimeter
CNS	central nervous system
CPM	counts per minute
Cv	coeffecient of variation
ddH_2O	deionized distilled water
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPM	dissociations per minute
ECL	electrochemiluminescence
EDTA	ethylenediaminetetraacetic acid
EMB	emamectin benzoate
EtOH	ethanol

FBS	fetal bovine serum
g	standard gravity
GABA	gamma amino-butyric acid
GABAα	GABA receptor α-subunit
GFP	green fluorescent protein
GluCl	glutamate-gated chloride channel
GluClα	GluCl receptor α-subunit
HCl	hydrochloric acid
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hour
IGR	insect growth regulators
K_D	dissociation constant
M	molar
m	meter
min	minute
mg	milligrams
$MgCl_2$	magnesium chloride
ml	milliliter
mM	millimolar
$NaCH_3COO$	sodium acetate
NaCl	sodium chloride
NaOH	sodium hydroxide
nM	nanomolar
nm	nanometers
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
pmol	picomole
ppt	parts per thousand
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase

sec	second
SOC	super optimal catabolite
TBE	Tris/borate/ethylenediaminetetraacetic acid
TBS	tris buffered saline
TEMED	tetramethylethylenediamine
U	units
UV	ultraviolet
V	volts

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INTRODUCTION

1.1. Sea Lice

1.1.1. Introduction to Sea Lice

Sea lice are ectoparasitic aquatic animals belonging to the phylum Arthropoda, class Crustacea, order Copepoda. Certain species of sea lice, with *Lepeophtheirus salmonis* being the major species of concern in salmon farms across the North Atlantic and British Columbia coast, are parasitic towards salmonid species and represent one of the primary ongoing concerns in the development of salmon aquaculture (as reviewed by Pike and Wadsworth, 1999). Sea lice infestation in salmon farms became a major concern in Norway throughout the 1960s, Scotland and Ireland in the late 1970s, and Canada in the latter part of the 1980s. By the 1990s, sea lice infestation was prevalent to the point of major yield and financial loss in the Atlantic Canadian aquaculture industry (MacKinnon, 1997). The development of improved therapeutic and management strategies have since brought sea lice infestation to levels of reduced impact, but the limited options and risk of environmental contamination cause sea lice to continue to be a major concern for the future development of aquaculture.

With the increasing economic importance of sea lice in the aquaculture industry, knowledge in the developmental biology of the parasite and host interactions has also increased.

1.1.2. Life Cycle

The life cycle of *L. salmonis* is composed of ten stages: two free-swimming

nauplius stages, a free-swimming infective copepodid stages, four attached chalimus stages, two attached pre-adult stages, and the final adult stage (Schram, 1994). Each stage is separated by a molt, which is controlled by both the endocrine and neurosecretory systems. Temperature and salinity have been shown to be the biggest factors in determining the survivability and rate of development, with high temperatures (greater than 10°C) and high salinity (30 ppt) leading to increased survival and faster development (Tully, 1989).

The first stage of the sea louse life cycle upon hatching, the nauplius I stage, is free swimming and non-feeding, depending instead on internal reserves for nutrients (Johannessen, 1978). The louse has noticeable appendages which carry over into the nauplius II stage, which is much less active. The louse spends ½ - 2 days in the nauplius I stage, and 1½ - 7 day in the nauplius II stage depending on the temperature of the water. The louse then molts to the copepodid stage, where it then possesses the capacity to infect the fish host. In this stage, the appendages are much less noticeable and the organism attaches itself to the host's skin using prehensile antennae and the maxillipeds. Once attached, the organism is able to feed due to the presence of a functional mouth (as reviewed by Pike and Wadsworth, 1999).

The next molt brings the louse into chalimus stages, which represent the first solely parasitic stages of the organism (Johnson and Albright, 1991). It is during this stage that the sea louse develops a frontal filament which allows the sea louse to anchor to the fish host (Gonzalez-Alanis *et al.*, 2001). During the chalimus IV stage, the male and female lice differentiate allowing them to be distinguished one from the other based upon size and shape. After spending 3-4 weeks in the four chalimus stages, members of the *L. salmonis* species develop through two pre-adult stages before fully maturing. The

pre-adults are not anchored to the host using the frontal filament, but are mobile and can move over the surface of the host. A temporary frontal filament is used only to attach the louse securely to the host during molting. After approximately a week, the organism develops into the adult stage. Both pre-adult and adult female lice are larger than the males, and both can transfer between hosts. The entire life cycle takes from 7-8 weeks at 10°C (Wootten *et al.,* 1982).

1.1.3. Feeding Habits

The feeding mechanism of sea lice causes injury to the host fish in the form of skin lesions. These lesions may range in severity from minor discolorations to large wounds, depending on the parasitic stage. The copepodids will cause small local responses that can be viewed as small black spots resulting from the accumulation of melanocytes (Wootten *et al.*, 1982). The chalimus stages cause similar damage with additional epidermal damage due to the attachment of the frontal filament. The pre-adult and adult stages feed far more aggressively and will consume mucus, epithelial tissue, subcutaneous tissue, and blood, which causes significantly greater damage (Wooten *et al.*, 1982). Severe cases will result in lesions that consist of removal of the skin from the fish. This can result in hemorrhages and secondary infections. These infections can be either fungal or viral in nature, and lead to the possibility that *L. salmonis* presents not only a parasitic problem, but also a vector for disease transmission (Rolland and Nylund, 1998).

1.1.4. Mating Habits

Adult *L. salmonis* males develop earlier than females, appearing approximately

one day earlier. The males generally prefer to mate with newly molted, virgin adult females rather than other more numerous females (Hull *et al.*, 1998). The reproductive organs of both sexes are situated in the cephalothorax behind the level of the eyes. The male will attach itself to the pre-adult II female's genital segment where it remains until the female molts to the adult stage, and copulation will then occur (Ritchie, 2006). Egg production begins approximately 9 days after copulation in the form of egg string pairs that can eventually contain from 100 – 500 eggs (average of about 350) per string (Johannessen, 1978; Wootten *et al.,* 1982). Adult females that have egg strings are called gravid females, and the egg strings may be more than twice the length of the female.

1.2. Sea Lice Management

There are several methods used to treat sea lice outbreaks. These methods can either be chemical, biological, or physical, and are most effective when a combination of various methods are used. Even if the infestation is completely eliminated from a farm, the nature of the marine environment allows sea lice to be reintroduced from a wild host.

1.2.1. Physical Methods

There are many physical management techniques that are helpful in preventing sea lice infestations. Site location, for instance may affect sea lice in terms of water flow, depth, and salinity. Adequate water flow can help prevent build up of lice larvae and disperse excess chemotherapeutants after a treatment has ended. It has been shown that sea lice infection is reduced when fish are kept in deeper waters as well, and as feeding technologies develop it may be possible to feed at depths below 4 m (Hevrøy *et al.*, 2003). Sea lice also develop optimally in salinity of 30 ppt, and lower salt

concentrations result in slower development and higher mortality in sea lice larvae. Farms should also be situated away from potential sources of infection such as areas where wild salmon would be present in high concentration, bringing in replacement parasites (Pike and Wadsworth, 1999) or to prevent infection of wild salmon should a sea lice infestation occur.

Another technique, fallowing, is performed by allowing a site to be free of stocked fish. Copepods need to attach to a host fish before its energy reserves run out; therefore, with no available hosts, the copepodids will starve and effectively end the life cycle. The fallow period should be at least 30 days in the winter to allow enough time for all newly hatched lice to die, and longer fallow periods increase the amount of time needed before lice numbers rise to the point where chemical intervention is required (Hogans, 1995).

Clean farming practice also may help prevent the build up of sea lice. Sites should be constantly monitoring the level of sea lice present in order to be able to take necessary measures to halt a rising infestation. Removing dead fish prevents the lice from having an easily accessible food source while introducing clean and healthy smolts will lower the overall ratio of lice to fish. Routinely cleaning nets to allow a better flow of water has been shown to lower the level of infestation.

1.2.2. Biological Methods

There are other ways of controlling sea lice infestation without the use of chemotherapeutants. One example is the use of wrasse, a cleaner fish. There are four species of wrasse that have been shown to be effective in clearing sea lice from salmon: goldsinny (*Ctenolabrus rupestris*), rock cook (*Centrolbrus exoletus*), corkwing

(*Crenilabrus melops*), and cuckoo (*Labrus mixtus*). There has been evidence to suggest that the rock cook wrasse is the most effective cleaner of the four species (Bjordal, 1991). Studies have shown that a salmon farm stocked with 1 – 4 % wrasse can reduce the amount of sea lice by over 80 % (Treasurer, 1994; Treasurer, 2002). However, these fish are able to escape from the net pens and need to be restocked in order to maintain the population (Pike and Wadsworth, 1999). Also, introduction of wrasse into areas where they are not indigenous, such as Atlantic Canada, is inadvisable due to a potential disruption of the ecosystem.

Another method of biological control being developed is vaccination. The ability to vaccinate Atlantic salmon against sea lice would offer significant advantages over a chemotherapeutic approach, since re-application would not be necessary and the effects of residual or excess drug would be eliminated. The vaccine would immunize the host fish against essential internal parasite antigens that the fish would normally never be exposed to, and therefore not develop any antibodies towards these antigens (Fast *et al.*, 2003). These vaccines could result in sea lice ingesting these antibodies and bind antigens in the intestinal tract, thereby disrupting the ability of the louse to perform many necessary functions and potentially killing the louse due to an inability to feed. There are many obstacles to overcome in the development of the vaccine, however. Unfortunately, blood is not a major component of the sea lice diet and only a minor fraction of lice will actually ingest blood. Also, sea lice are able to produce proteolytic enzymes to aid in digestion of proteins for nutrition (Fast *et al.*, 2003). The presence of these proteases would result in digestion of the antibodies and increase the amount of antibody required to have the desired effect. The pH and osmolarity of the sea louse

intestine are also known to be different than that of salmon, potentially reducing the efficacy of any ingested antibody (Grayson *et al.*, 1995; Raynard *et al.*, 2002).

Studies done using various antigens to immunize Atlantic salmon against sea lice have shown immunization can reduce the number of ovigerous females; however there was no observable significant effect against other stages (Grayson *et al.*, 1995). Other ongoing studies looking at the possibility of trypsin as the therapeutic target of these vaccines may prove to be more effective (Kvamme *et al.,* 2004). The successful development of a sea lice specific vaccine will eliminate the need to use chemotherapeutants to treat sea lice infestation (Fast *et al.*, 2003; Kvamme *et al.*, 2004).

A final method of biological control may be in the selective breeding of salmon naturally resistant to sea lice infestation. This solution will reduce the need for chemotherapeutants and provide an environmentally safe mechanism of sea lice control. However, difficulty arises in determining which fish are displaying resistance, as well as the 4 year generation cycle of salmon resulting in several years needed for this breeding plan to produce usable results (Glover *et al.* 2005).

1.2.3. Chemical Methods

While the avermectin, emamectin benzoate (EMB), is currently the drug of choice (Westcott *et al.* 2004), there are four other major types of antiparasitic compounds that have been used against sea lice infections. These drugs include pyrethroids, organophosphates, hydrogen peroxide, and insect growth regulators (IGRs).

Pyrethroids are synthetic derivatives of pyrethrin, which is a naturally occurring drug. These drugs have a high affinity for insect sodium channels to which they bind and maintain in a permanently active state (Narahashi, 1971). This causes neuronal

depolarization and results in paralysis due to the inability to repolarize. Though pyrethroids have a much higher affinity for insect sodium channels and are safe for mammalian consumption, toxicity has been shown in fish at high concentrations (as reviewed by Burka *et al.,* 1997; Grant, 2002). Newer derivatives, such as cypermethrin, have a higher therapeutic index making them safer for use in prevention of sea lice. These drugs are used in rotation with EMB in Norway in order to reduce the potential development of resistance to either drug (Sea Lice Conference 2008, Chile). Resistance has been shown to occur due to a point mutation in the sodium channel, resulting in an alteration in the conformation of the pyrethroid binding site (Fallang *et al.*, 2005). Pyrethroids have a wide range of activity in sea lice, and can affect all stages of the organism. They are given as a bath treatment, resulting in equal amounts of the drug being distributed to all target organisms.

Organophosphates are synthetic drugs that inhibit cholinesterases thereby interfering with neuromuscular transmission in the target organism. They are useful in treating all mobile stages of lice, but are ineffective against the chalimus stages, potentially due to differing pharmacokinetics of the larval stage. Organophosphates are given as a bath treatment, providing the same concentration of drug to all target organisms. Azamethiphos (Salmosan®) is a newer organophosphate that has a higher therapeutic index and is effective at much lower concentrations than organophosphates previously used in sea lice treatments. The drug is eliminated quickly from the fish resulting in a low withdrawal time, and has little to no effect on non-target organisms (Grant, 2002). Resistance to azamethiphos was reported in Norway and Scotland after treatment failures persisted (Fallang *et al.*, 2004). Possible mechanisms that could be involved in organophosphate resistance include decreased penetration, enzymatic

detoxification and alteration of the target enzyme. A study on organophosphate resistance demonstrated that modified acetylcholinesterase was a mechanism involved in resistance, potentially due to either point mutations or post-transcriptional modifications which altered the kinetic parameters of acetylcholinesterase hydrolysis (Fallang *et al.*, 2004). Resistance was not observed in Atlantic Canada where it was used extensively until 2000 (after which usage declined following the introduction of emamectin benzoate in 1999).

Hydrogen peroxide (H_2O_2) is a strong oxidizing agent that can be used to control chalimus and mobile stages of sea lice. Peroxide causes large amounts of oxygen to accumulate in the haemolymph and intestine, causing mobile stages of sea lice to float to the surface (Grant, 2002). Treatment results are inconsistent as even concentrations high enough to cause mortalities to Atlantic salmon may still result in surviving lice. This drug has a very low therapeutic index which is also temperature dependant, and is not recommended for use in farms with water temperatures above 14°C. Peroxide is very safe to use in the environment; however, as it breaks down rapidly into water and oxygen (Thomassen, 1993).

Insect growth regulators (IGRs), also known as benzoylureas, act by the inhibition of the incorporation of chitin into the exoskeleton (Blagburn and Lindsay, 1995).This prevents the molting process from being successful; however it also makes these compounds useless against adult lice as they have already completed all their molting stages. These drugs have no toxic effect on vertebrates due to the specificity for chitin inhibition, giving the drugs a high therapeutic index and making them very safe for mammals and fish. Teflubenzuron (Calicide®) has shown a high level of efficacy against all molting stages of sea lice. It is given as an in-feed treatment, making the

availability to the target organism dependant on feeding. 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 (Grontvedt, 1997). Exposure to teflubenzuron also appears to have a severe negative impact on the development of egg strings, preventing a new generation of sea lice from developing properly (Grøntvedt, 1997). Unfortunately, while the drug is easily eliminated from the fish, it appears to be fairly persistent in the environment, with a half-life anywhere from 35 days up to 6 months. Due to its mode of action, this compound is considered to be a potential risk to sediment dwelling crustaceans, thereby making the use of this drug less desirable in sensitive areas (Bloomquist, 2003).

1.2.4. Avermectins

Avermectins are a group of macrocyclic lactone drugs derived from avermectin, a naturally occuring substance produced by the bacterium *Streptomyces avermitilis* (Hotson, 1982). Avermectins are commercially important drugs in both human and veterinary medicine. One of the common uses of avermectins is as an anti-parasitic drug to control parasitic invertebrates, particularly nematodes and arthropods (Banks *et al.*, 2000; Bloomquist, 2003).

Avermectin compounds act as a parasiticide by interfering with the nervous system of insects and nematodes (Bloomquist, 2003). This causes the target organism to become paralyzed and eventually leads to the organism's death. Some avermectins, such as ivermectin, have extremely low toxicity in birds and mammals, but are toxic in fish due to an increased ability of ivermectin to cross the fish blood-brain-barrier and affect the GABA receptors in the brain (Høy *et al.*, 1990).

L. salmonis is one of the species that is controlled by the use of avermectins. Each year a significant amount of salmon yield is lost due to sea lice infections and the addition of chemical treatments to other management practices seem to be the best way of preventing even further loss (Westcott *et al.,* 2004). Ivermectin was the first avermectin used in control of sea lice, and has also been used as a parasiticide in humans and many animals, including cattle, sheep, horses, dogs, pigs, and salmon. Ivermectin proved to be highly effective at controlling sea lice, but eventually showed an increased mortality rate in salmon smolts and higher incidence of CNS depression in the fish (Roy *et al.,* 2000). Ivermectin is now rarely used due to its toxicity to the fish.

1.2.5. Emamectin Benzoate

Emamectin benzoate (SLICE®; Schering-Plough Animal Health, Pointe Claire, QC) is used in controlling sea lice in Canada (Westcott *et al.,* 2004). In 2003 in New Brunswick, emamectin benzoate (EMB) was reported to be the most extensively used chemical as a control for sea lice (Westcott *et al.,* 2004). Emamectin benzoate has not been registered for use in Canada, but is available through Emergency Drug Release (EDR), which is an authorization to provide limited amounts of unregistered drugs. Due to its high effectiveness and the low risk to the environment, EMB is now used as the primary control for sea lice in Canada, Europe, and Chile. However, continued reliance on EMB has raised concern about the potential for sea lice to develop resistance to the drug (Westcott *et al.,* 2004).

Emamectin benzoate was originally developed for pest control in edible plant crops due to its enhanced activity against the southern army worm, *Spodoptera eridania*, compared with abamectin, another avermectin that had been previously used. Compared

with the narrow safety margins of ivermectin, EMB caused no adverse effects in treated salmon up to 3.5 times the recommended dosage and no mortality in up to 7 times the recommended dosage in an in-feed treatment (Roy *et al.,* 2000). Another study reported that there were no differences in feeding responses, coordination, or histological appearance of smolts treated with the recommended dosage of EMB and smolts that received no treatment at all (Stone *et al.,* 1999, Stone *et al.,* 2002). Emamectin benzoate also had no adverse effects on exposed lobster when used at the recommended dosage, making it safe for use without posing a risk to the lobster fishing industry (Burridge *et al.,* 2004).

Emamectin benzoate was more effective at controlling sea lice than ivermectin or teflubenzuron (a chitin synthesis inhibitor) (Ramstad *et al.,* 2002). It is theorized that the benzoate salt confers increased stability to the compound which allows it to be effective for longer periods of time (Kim-Kang *et al.,* 2004). Use of EMB can potentially decrease the amount of sea lice in a population by 95% within 7 days from the initial treatment (Stone *et al.,* 2000), at which point it is most effective and at its highest concentration in the fish (Sevatdal *et al.,* 2005).

Both EMB and ivermectin are synthetic avermectins, and have the characteristic rigid 16-membered ring (Figure 1.1). Both compounds are highly lipophilic and dissolve readily in the fatty tissues of the fish. Because avermectins are highly insoluble in water, both EMB and ivermectin are given as in-feed treatments. Emamectin has been shown to have improved efficacy over ivermectin, while having fewer side effects on the host salmon as it does not readily cross the blood-brain barrier (Kim-Kang *et al.*, 2004, Sevatdal *et al.,* 2005). Though no studies have

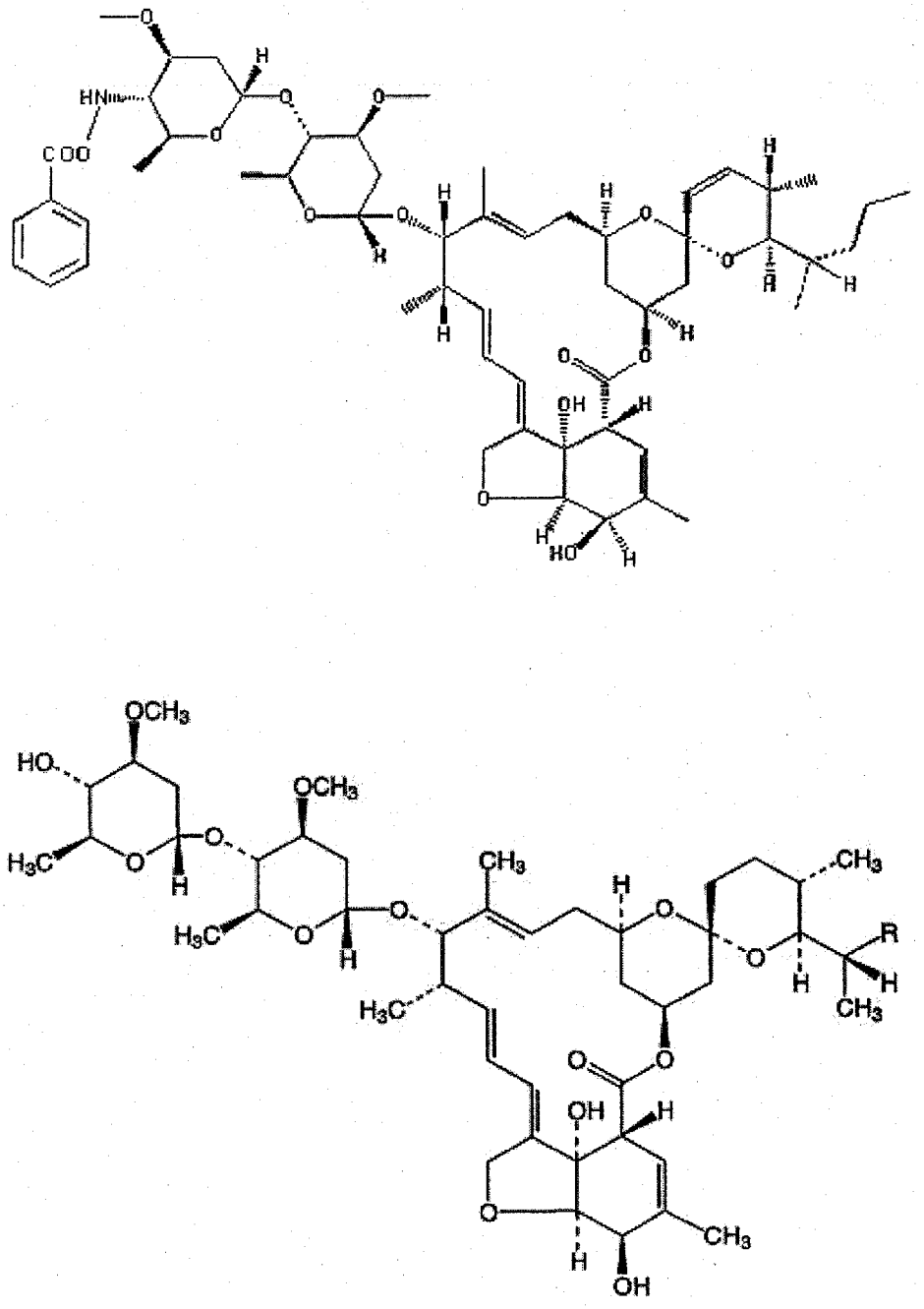

Figure 1.1. The chemical structures of EMB (top) and ivermectin (bottom) (Tribble, 2007).

been done on the mechanism of action of EMB, it is expected to be the same as other avermectins in other organisms.

Sea lice control strategies should include preventative as well as treatment measures, and each method has advantages and disadvantages associated with its use. In chemical control, the spectrum of action and the specificity (low toxicity for non-target organisms) are two of the major factors in determining the desirability of a drug. Both EMB and cypermethrin are the only compounds currently available that are both effective against all stages of the parasite as well as having low host toxicity (Figure 1.2). EMB also has a longer duration of action, making it potentially the best compound currently available as a control for sea lice. However, in an ideal situation a combination of drugs should be used in a rotation system in order to slow the potential development of resistance to either drug. Norway was the first country to use this approach, using both EMB and cypermethrin as chemotherapeutants, but Scotland and Chile have begun to adopt a multi-drug approach recently as well. Other countries, notably Canada, rely solely on EMB, thereby increasing the risk for resistance development.

1.3. The GluClα Receptor

1.3.1. GluCl and GABA

Avermectins are known to act on at least two different receptors found in invertebrates. The first is the glutamate-gated chloride (GluCl) channel and the second is the γ-aminobutyric acid (GABA) receptor. The avermectins are considered to act by binding irreversibly to the GluCl channels, increasing neuronal permeability to chloride ions at invertebrate inhibitory synapses, resulting in paralysis and death.

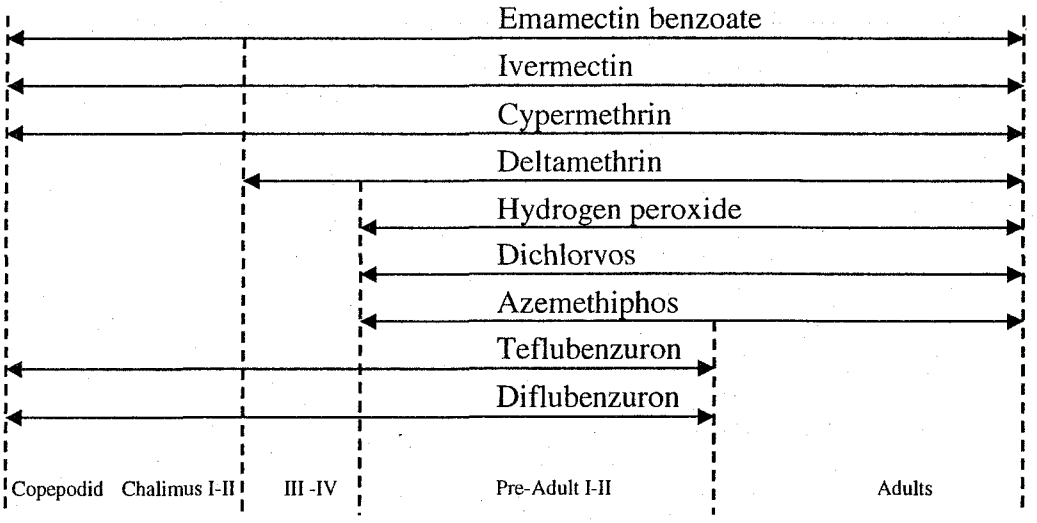

Figure 1.2. The spectrum of action of chemicals used in treatment of sea lice versus the different stages of the sea lice life cycle (Tribble, 2007).

The GABA receptor was originally thought to be the primary receptor involved in the avermectin mechanism of action. When the GluCl channels were discovered, it left a question unanswered about whether it was GABA or GluCl which mediated the paralysis caused by avermectins (Blackhall *et al.,* 1998). GABA receptors are found in the central nervous systems of vertebrates, and in the neuromuscular junctions of nematodes and arthropods, and were known to be the target of many drugs that caused paralysis in invertebrates and CNS depression in fish and mammals. There are three types of GABA receptors, but the one thought to be involved in avermectin mode of action was $GABA_A$. $GABA_A$ receptors belong to the superfamily of ligand-gated ion channel receptors that also includes the nicotinic and glycine receptors (Stephenson, 1995). It consists of three separate subunits with an α, β, γ arrangement similar to that found for neuronal nicotinic receptors (Egebjerg, 2003). The ligand binding site is located at the interface between the α and β-subunits (Stephenson, 1995; Egebjerg, 2003). Resistance to many cyclodiene insecticides is caused by a mutation in the $GABA_A$ receptor, leading to the conclusion that avermectins may work through GABA as well.

Avermectins were shown to irreversibly bind to GABA receptors which caused neuronal cells to hyperpolarize through the influx of chloride ions, leading to paralysis. It was proposed that the avermectin bound to the α-subunit which forced a channel to open between the α and the β subunits (Boileau *et al.,* 1999). The inability of the channel to then close causes hyperpolarization and inhibits the ability of the neuron to generate action potentials. This work supported the theory of avermectins working on GABA receptors; however this work was done primarily on mammals and did not consider invertebrates.

Further research showed significant differences in the structure of $GABA_A$ receptors of mammals and of invertebrates (Ozoe *et al.,* 1998). The receptor in invertebrates was shown to more closely resemble the structure of vertebrate $GABA_C$ receptors. Subsequently, a new ligand-gated ion channel was discovered that was also a chloride channel but was mediated by glutamate (Cully *et al.,* 1994; Vassilatis *et al.,* 1997b). The GluCl was found to be unique to invertebrate systems and allowed for a potentially new mechanism for the action of avermectins on invertebrate systems (Cully *et al.,* 1994; Cully *et al.,* 1996). The subunits of this channel, GluClα (including three subtypes GLC-3, avr-14, and avr-15) and GluClβ were isolated from the nematode *Caenorhabditis elegans* and expressed in *Xenopus* oocytes. The avermectins bound to the GluClα subunits (encoded by the glc-3, avr-14, and avr-15 genes) with an extremely high affinity (Cully *et al.,* 1994; Laughton *et al.,* 1997; Dent *et al.,* 2000). The GluClβ subunit of the receptor responded to glutamate and glycine, but was insensitive to avermectin (Laughton *et al.,* 1995). Cully *et al.* (1994) showed that the presence of ivermectin potentiated glutamate binding affinity in the GluClβ subunit, and glutamate was also able to potentiate the affinity of ivermectin for the GluClα subunit. It was also shown that the subunits were more sensitive to ligand binding when expressed alone than were the receptors formed by coexpression of the α and β subunits. Further research showed that native glutamate-gated chloride channels expressed in the pharynx of *C. elegans* were in fact more sensitive to ivermectin than when they were cloned in *Xenopus* oocytes (Cully *et al.,* 1994; Laughton *et al.,* 1995). Recombinant receptors, therefore, do not react the same in an expressed environment as they do in their natural cell types.

Even though avermectins were shown to still act on GABA receptors, binding with GluCl receptors are now considered to be the primary mode of action for avermectins in invertebrates (Cully *et al.,* 1994). Much like the GABA receptors, avermectins irreversibly bind to the receptor and force an inward flow of chloride ions. It is generally accepted that this causes a hyperpolarization of the membrane (Dent *et al.,* 2000), but certain experiments have indicated that the membrane equilibrium may shift into a permanently depolarized state instead (Pemberton *et al.,* 2001). However, there is a clear advantage to having identified these receptors. GluCl receptors are theorized to be a divergent trait from the vertebrate glycine receptors, but are found only in nematodes and arthropods (Vassilatis *et al.,* 1997a). These receptors provide a clear target for pesticides since any drug targeting these receptors would be selective for invertebrates (Vassilatis *et al.,* 1997a; Bloomquist, 2003), providing selective toxicity.

1.3.2. Current Research

The experiments with GluCl receptors generated new research into the study of avermectins. Novel chloride channels were identified in *Drosophila melanogaster* that expressed similarities to GABA (Henderson *et al.,* 1994). The gene for the glutamate-gated chloride channel was isolated from *Drosophila* and expressed in *Xenopus* oocytes to demonstrate functionality (Cully *et al.,* 1996). The final protein of the GluClα subunit in *Drosophila* showed a 48 % similarity to the GluClα expressed in *C. elegans*. Studies showed that only the GluClα subunit was required for avermectin binding in all species examined. However, while only the GluClα was needed for glutamate binding in *Drosophila* (Cully *et al.,* 1996; Pomes *et al.,* 1997), both GluClα and GluClβ were needed for glutamate binding in *C. elegans* (Cully *et al.,* 1994; Cully *et al.,* 1996). Cully

also observed an inhibitory effect of ivermectin in the glutamate response in *Drosophila*, possibly due to allosteric interference with the glutamate binding site, even though there was a potentiated effect with glutamate and ivermectin in *C. elegans.*

Blackhall *et al.* (1998) published a study on the glutamate-gated chloride channels of *Haemonchus contortus*, a parasitic nematode. Three subunits were identified, including two GluClα and one GluClβ. The GluClβ subunit was shown to have no interaction with ivermectin, whereas the GluClα subunits did, as in *C. elegans* (Cheeseman *et al.*, 2001). Further study showed that the avr-14 subunit, one of the subunits sensitive to avermectin, had an 80% homology to the avr-14 subunit of *C. elegans* (Jagannathan *et al.,* 1999; Cheeseman *et al.,* 2001). Ivermectin was shown to inhibit pharyngeal pumping in *H. contortus*, giving support to the idea that the glutamate-gated chloride channel was involved in neuromuscular function in a similar fashion to the GABA receptor (Paiement *et al.,* 1999). Much like the *Drosophila* experiment, however, ivermectin was shown to inhibit the glutamate response in the GluClβ subunit which contrasts with the potentiated response in *C. elegans* (Paiement *et al.,* 1999).

Further research into *H. contortus* involved a ligand binding study of ivermectin to the GluClα subunit (Forrester *et al.,* 2002). Since it had already been shown that only the GluClα subunit was required, this study focused entirely on ivermectin and moxidectin (a milbemycin parasiticide) interaction with the GluClα subunit. It was revealed that while interacting with the GluClα subunit, low to moderate concentrations of glutamate potentiated the binding of ivermectin to the receptor (Forrester *et al.*, 2002). This gave further insight into the possible conformational changes caused by the interaction of avermectins and glutamate with glutamate-gated chloride channels.

Forrester *et al.* (2002) proposed that the conformational changes induced by glutamate allowed for greater high-affinity binding of ivermectin. It was then shown that the EC_{50} (effective concentration which produces half of the maximum effect) of ivermectin and glutamate was much lower in *H. contortus* than in *C. elegans*, and more similar to the values found in *Drosophila* (Forrester *et al.,* 2003). Forrester then proposed that there were two mechanisms by which ivermectin could bind to the GluClα subunit. The first was a low-affinity, reversible binding. The second was a high-affinity, virtually irreversible binding due to the slow dissociation. The conformational change induced by the first binding mechanism, and that of any other ligand, increases the affinity of ivermectin for the receptor and allows the second irreversible binding to occur (Forrester *et al.,* 2004). The GluCl receptor of *H. contortus* is localized to the pharynx (Liu *et al.,* 2004), so the irreversible binding of ivermectin would prevent pharyngeal pumping due to the hyperpolarization of the neuronal membrane and eventually starve the nematode.

Another possibility that exists is that GABA and GluCl receptors may interact to increase binding affinity of avermectins. Homo-oligomeric GABA receptors (receptors made up all of the same subtype, generally the α subunit) have been shown consistently not to have any affinity for GABA or avermectins (Boileau *et al.*, 1999, Feng *et al.*, 2002) but hetero-oligomeric receptors (receptors made up of two or more subtypes) are fully functional and display affinity for avermectins. Co-expression of an α subunit from *H. contortus* and β subunit from *C. elegans* were able to form a fully functional receptor capable of expressing varying degrees of sensitivity (Feng *et al.,* 2002). In *Drosophila*, it was shown that an α subunit of the GABA receptor was capable of interacting with the GluClα subunit to increase the binding affinity for avermectins (Ludemerer *et al.,* 2002). Since GABAα is incapable of binding to avermectin in a homo-oligomeric state, the

results suggested that GABAα may have in fact formed a co-association with GluClα (Ludemerer *et al.*, 2002). This creates the possibility that GABA receptors may still play an important role in both avermectin binding and the potential development of avermectin resistance, despite not being the primary target of avermectins.

To date, only the efficacy of avermectins against sea lice has been studied. Tribble *et al.* (2007a) identified two genes from *L. salmonis* that show high homology to the genes encoding the GluClα and GABAα subunits studied in *H. contortus*, *C. elegans*, and *D. melanogaster*, and closely related in a phylogenetic tree (Figure 1.3). Analyses of the sequences reveal the likely structures to have all the characteristics of a transmembrane ionotropic receptor, and it was suggested that further studies on these genes may assist in increasing the current knowledge of EMB's mechanism of action in *L. salmonis* (Figure 1.4).

1.4. Resistance Development

One of the major concerns in the usage of EMB to control sea lice has been the potential development of resistance to the drug. History has shown that the overuse of a single chemotherapeutant can accelerate the development of resistance if not properly managed or mutations develop, and the lack of a suitable alternative for EMB highlights the importance of preventing this from occuring. Currently, while there have been no reported treatment failures in Canada, signs of resistance have been reported in other salmon farming countries (Sea Lice Conference 2008, Chile).

The potential development of avermectin resistance is one of the major concerns which have driven the extensive research regarding the mechanisms of avermectin reaction and avermectin sensitivity in parasites. A study done on parasiticide use to

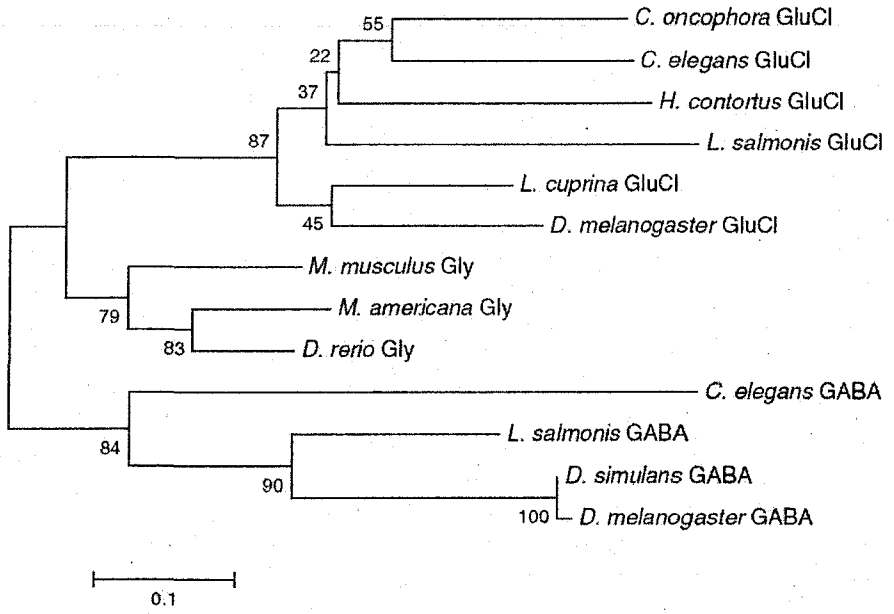

Figure 1.3. Unrooted phylogenetic tree showing the relationship of the GluClα (top) and GABAα (bottom) receptor subunits of sea lice to those other species as well as the vertebrate Glycine receptor (middle) using a 119-bp conserved sequence located in the second transmembrane domain in these receptors. The numbers were generated using the neighbor joining method, and are representative of divergence between the genes. (Tribble *et al.*, 2007a).

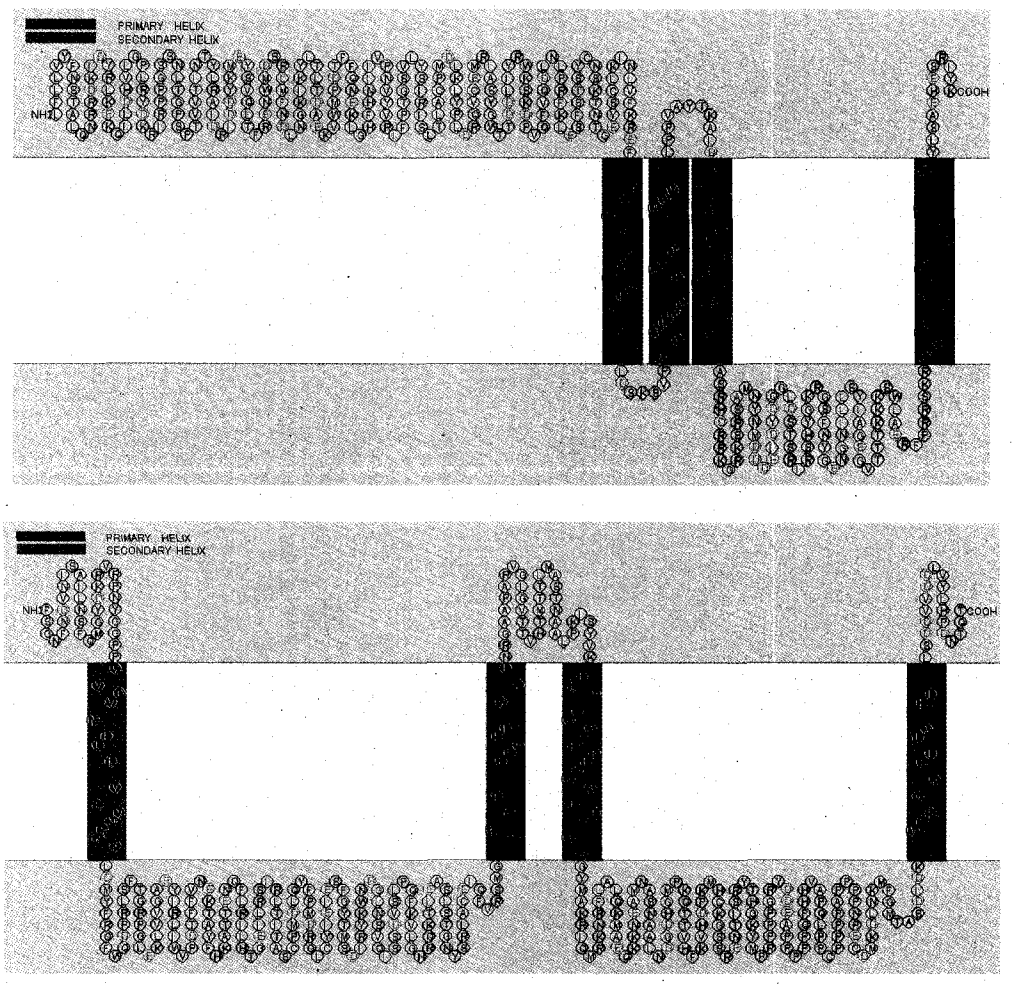

Figure 1.4. The likely structures of GluClα (top) and GABAα (bottom) based upon the amino acid sequences reported in Tribble *et al.* (2007a) using PredictProtein analysis software to identify the likely transmembrane domains.

control sea lice in the Bay of Fundy reported that nearly 80 % of the fish farms included in the survey used an avermectin as the primary treatment (71.1 % EMB, 8.4 % ivermectin) (Westcott *et al.* 2004). This has since increased to nearly 100 % usage of emamectin benzoate and elimination of ivermectin usage. Since EMB is now virtually the only treatment used for control of sea lice in Canada, extensive and repeated use may eventually select for avermectin resistant strains of sea lice.

1.4.1. Mechanisms of Resistance

There are several ways in which avermectin resistance could potentially develop. Mutations could induce a conformational change in the GluCl channel thereby decreasing or preventing avermectin-binding. There could also be a mutation which involves metabolizing the antiparasitic agent and rendering it ineffective. Finally, there could be a third interaction introduced which prevents the drug from interacting with the receptors.

Resistance to avermectins in invertebrates may arise from a variety of mutations in either the GABA receptors or the GluCl channels. For *H. contortus*, it was shown that ivermectin had equal affinity and bound equally well to GluClα receptors taken from both avermectin-sensitive and avermectin-resistant strains (Blackhall *et al.,* 1998). It is possible that a mutation in the GluClα receptor did not affect the binding affinity of ivermectin but instead changed the conformation of the receptor to react differently to ivermectin thereby preventing the depolarization of the cellular membrane. Dent *et al.* (2000) showed that mutations in any two of the three GluClα subtypes conferred little or no avermectin resistance in *C. elegans*, but a point mutation in all three subtypes conferred high resistance to avermectin. The degree of resistance was further influenced

by mutations in the genes *unc*-7 and *unc*-9 (Barnes and Hekimi, 1997; Dent *et al.,* 2000). It is possible that these two genes influence the degree of the excitability of the cellular membranes and reduces the effect of hyperpolarization and depolarization caused by avermectins (Barnes and Hekimi, 1997).

Further study was done in another parasitic nematode, *Cooperia oncophora*, on the genetics of avermectin resistance (Njue *et al.,* 2004, Njue and Prichard, 2004). Genetic variability was identified between the GluClα and GluClβ subunits of both avermectin-sensitive and avermectin-resistant strains of *C. oncophora.* Both subunits were very closely related to the same subunits that had previously been isolated from *H. contortus*. There appeared to be no differences in the GluClβ subunits isolated from both avermectin-resistant and avermectin-sensitive strains of *C. oncophora,* further supporting that avermectins act only on the GluClα subunits. Following this, there did appear to be some differences in the GluClα from the avermectin-resistant and avermectin-sensitive strains (Njue and Prichard, 2004). Mutations were noted in three different amino acid positions in the final protein (Njue *et al.,* 2004).

Studies were also done on the genetic mechanisms of resistance in *Drosophila.* Binding studies done with GluClα from avermectin-resistant strains of *Drosophila* revealed that ivermectin displayed a much lower affinity for the resistant allele than the wild-type (Kane *et al.,* 2000). Sequencing of the gene for this subunit revealed a proline to serine mutation from the wild-type. The effect of this mutation on ivermectin sensitivity was demonstrated by introducing the mutation into a recombinant GluClα gene and performing a second binding study with ivermectin and the receptor. The binding ability of ivermectin to the receptor was reduced by nearly 10-fold (Kane *et al.,* 2000). Studies were also done on the genes influencing cellular polarization, known as

the *ort* genes. Instead of finding ivermectin resistance to mirror the study with the *unc* genes, mutant *ort* alleles displayed hypersensitivity to treatment with ivermectin (Georgiev *et al.,* 2002).

Avermectins have been shown to degrade via numerous metabolic pathways, including the cytochrome P450 pathway in mammals (Zeng *et al.,* 1996). Cytochrome P450 is a common metabolic pathway to many domains of life, and is likely to be found in sea lice as well (though no genes encoding for the enzymes of this pathway have yet been identified). Mutations or upregulation in this or other currently unknown enzymatic pathways responsible for drug metabolism is a potential path to resistance development. The mechanisms of drug metabolism in sea lice are as of yet poorly understood, but provide an avenue of research into another mechanism that could potentially cause a loss of sensitivity to avermectin compounds.

A third potential mechanism of resistance to avermectins was reported in study based on multi-drug resistance (Blackhall *et al.,* 1998; Blackhall *et al.,* 2003). This other mechanism of resistance may alter drug concentrations at either or both the GABA or GluCl receptors. P-glycoprotein is an efflux pump that can remove hydrophobic compounds from cytoplasm by pumping them across cell membranes; avermectins are known substrates (Blackhall *et al.,* 2003). P-glycoprotein is expressed in vertebrate systems, but generally not expressed as extensively in invertebrate systems (Wolstenholme *et al.,* 2004). This may explain the selective toxicity of avermectins to invertebrates since P-glycoprotein may in fact remove the threat before avermectins cross the blood-brain barrier to affect GABA receptors in the vertebrate brain. Potential mutations may increase P-glycoprotein expression, thereby allowing for resistance to develop in invertebrate systems. In this situation, resistant invertebrates may express

avermectin-sensitive GABA and GluCl receptors and still not be affected since the resistance mechanism would take place before the drug reached the receptor. Tribble *et al.* (2007b) showed that sea lice could upregulate transcriptional levels of putative P-glycoprotein genes in response to EMB exposure, highlighting this route as a possible means of resistance development even without a mutation.

The mechanism by which resistance develops plays an important role in determining future development of chemotherapeutic control. Mutations in the GluCl receptor that reduces the ability of EMB to bind and effectively eliminates a therapeutic target for future drug development unless a new binding site is found. If the means of resistance is not in the receptor itself, the usefulness of GluCl as a therapeutic target may not be irreversibly affected. In the case of a mechanism that prevents the drug from reaching the target receptor, newer formulations may potentially be studied and developed that can bypass the organism's resistance mechanism. P-glycoprotein upregulation, however, is often a cause of multiple-drug resistance, potentially leaving few, if any, possible alternatives for chemotherapeutic control. This underscores the need to understand the mechanism for resistance, and the development of models which can be used to determine which mechanism is being employed. The results of these studies on various invertebrates display a great deal of variability in the mechanisms of avermectin resistance and emphasize a need for greater understanding on how avermectins interact with the organisms it is used to control.

1.4.2. Current State of Resistance Development

There have currently been no reported treatment failures due to potential loss of

sensitivity in Canada. However, reports of treatment failures in farms using EMB as a sole chemical control for sea lice have been prominent in Scotland and Chile (Sea Lice Conference 2008, Chile). Lees *et al.* (2008) reported that although infestations were reduced after EMB treatment in salmon farms across Scotland, not all treatments were effective. Although this does not prove the development of resistance, due to potential unknown factors, it does indicate the possibility and emphasizes the risk in using a single drug as the sole treatment in controlling a parasite. Since this does demonstrate a loss of efficacy, uncovering the mechanism by which this loss occurred should be a priority in understanding and taking steps in preventing further treatment failures.

1.5. Project Rationale and Hypotheses

This study is intended to provide a better understanding of the mechanism of action of EMB in sea lice, which will expand the current knowledge on the interactions between avermectins and their receptors, provide the potential for further development of prevention and management of resistance, and allow for further research into the genetics and molecular biology behind the development of avermectin resistance. This will allow further understanding of the mechanisms by which EMB acts on GluCl receptors, and provide further knowledge into measures to prevent the development of avermectin resistance in sea lice as well as other parasites (Vassilatis *et al.,* 1997b; Wolstenholme and Rogers, 2005). Controlling sea lice is important in order to maintain the health and welfare of farmed salmon and to decrease the impacts of farmed - wild fish interactions. Understanding the mechanism behind the primary means of control is vital in this protection.

The hypothesis of the current study is that EMB will display high affinity binding to membranes extracted from both sea lice and the cell line used to express the GluClα subunit. Based upon previous studies indicating a possible temperature effect, it is also hypothesized that binding affinity will vary depending on the temperature. There are three aims of this study that will be used to test the hypothesis:

1. First, to create a stable cell line expressing the GluClα in order to provide a model of EMB-GluClα binding.
2. The second aim is to determine the binding characteristics of avermectin receptors expressed in *L. salmonis*. These characteristics primarily consist of the number of receptors that bind to EMB (B_{max}) and the binding affinity of EMB to these receptors (K_m). It will also consider factors which may influence the ability of EMB to bind to these receptors, thereby leading to possible mechanisms of resistance. Based upon previous research, this study will operate on the hypothesis that EMB will demonstrate high affinity, irreversible binding to the α-subunits of GluCl receptors.
3. Finally, the effect of temperature on the binding characteristics of EMB will also be tested to determine whether or not there may be a potential seasonal effect in the efficacy of EMB to bind to the receptors.

   The results of this study will increase our current knowledge of the ability of EMB to control sea lice.

MATERIALS AND METHODS

2.1. Sea Lice Collection

Adult *L. salmonis* were collected from Atlantic salmon farms in the Bay of Fundy, NB, Canada. Lice were removed from the salmon using forceps and placed in a glass container with the seawater from the location of the farm being sampled. After transportation to the laboratory, the lice were placed in 300 µl of RNAlater and stored at -20°C for further use. A second sample of lice was also taken from the salmon farms, and after transportation to the laboratory, the abdomens were removed from each louse using a sterile surgical scalpel, and the cephalothoraxes were placed in a sterile 25 ml plastic screw-cap tube, 1.0 g of cephalothoraxes per tube. The tubes were then flash frozen and stored at -80°C until needed.

2.2. Molecular cloning of the putative GluClα

In order to establish a cell line expressing the putative gene for the GluClα of *L. salmonis*, the gene was cloned into a eukaryotic vector, pIRES2-AcGFP1 (Clontech) and transfected into the salmon embryo fibroblast cell line, CHSE-214.

2.2.1. RNA Isolation

Five adult lice were removed from RNAlater and placed in a sterile RNase/DNase free 1.5 ml microcentrifuge tube. In a fume hood, 1 ml of TRIzol reagent (Invitrogen) was added and the sample was homogenized using a tissue tearer. The sample was incubated for 5 min at ambient temperature to permit complete dissociation of nucleotide complexes. Two milliliters of chloroform was added, the sample shaken by hand for 15 sec, and then incubated for 3 min at ambient temperature. The sample was

centrifuged at 12000 x g for 15 min at 4°C and after separation the colorless upper aqueous phase was carefully transferred to a new sterile RNase/DNase-free microcentrifuge tube. The RNA was precipitated by adding 0.5 ml of isopropyl alcohol and incubated for 10 min at ambient temperature. The sample was then centrifuged at 12000 x g for 20 min at 4°C to form an RNA pellet at the bottom of the microcentrifuge tube. The supernate was discarded and 1 ml of 75 % ethanol was added to the tube to wash the pellet. The sample was mixed by vortexing and centrifuged at 7500 x g for 5 min at 4°C. After centrifugation, the supernate was discarded and the pellet was air-dried for 10 min. The pellet was then dissolved in 500 µl of sterile RNase-free water and incubated at 55°C for 10 min. An aliquot was taken to be analyzed via UV spectrophotometry in order to determine purity and concentration and the sample was stored at -80°C until needed.

2.2.2. cDNA Synthesis and PCR

cDNA synthesis was performed using a Superscript III cDNA Synthesis Kit (Invitrogen). Two and one-half microliters of RNA (0.65 µg/µl) was added to a sterile RNase/DNase-free 0.5 ml microcentrifuge tube containing 1 µl of oligo(dT) primer (50 µM), 1.0 µl of dNTP mix (10 mM), and 5.5 µl of 0.1 % DEPC-H_2O. The solution was incubated at 65°C for 5 min and then chilled at 4°C for 1 min. The cDNA synthesis mix was prepared by adding, in order, 2.0 µl of 10X RT Buffer, 4.0 µl of $MgCl_2$ solution (25 mM), 2.0 µl of DTT (0.1 M), 1.0 µl of RNaseOUT (40 U/µl), and 1.0 µl of Superscript III RT. The cDNA synthesis mix was added to the RNA/primer mixture and was gently mixed and centrifuged. The sample was then incubated at 50°C for 50 min and the reaction was terminated at 85°C for 5 min. The sample was cooled to 4°C and

1 µl of RNase H was added before incubating at 37°C for 20 min. The cDNA sample was then stored at -20°C until it was used in PCR.

Primers were designed based upon the putative sequences for GluClα of *L. salmonis* (accession number DQ916037) and GABAα of *L. salmonis* (accession number DQ916036) identified in previous work in our lab (Tribble *et al.*, 2007a). The sense primer for GluCl was 5' ATG CTT TTT CTT CTC GTG TAT TTT 3', and the antisense primer was 5' TTA TTT GTA AAT CCT TGA TTC ATG 3'. The sense primer for GABA was 5' ATG TTC AGC GGC AAT TTT AAT GAT 3', and the antisense primer was 5' TTA GTC CGG GTG TAA GTA GAC AAG 3'. The PCR cycling conditions for the GABA receptor reaction were an initial temperature of 94°C for 10 min followed by 34 cycles of denaturation at 94°C for 60 sec, annealing at 54°C for 90 sec, extension at 72°C for 120 sec, and a final extension step at 72°C for 10 min. The PCR cycling conditions for the GluCl receptor reaction were an initial temperature of 94°C for 10 min followed by 34 cycles of denaturation at 94°C for 60 sec, annealing at 52°C for 90 sec, extension at 72°C for 120 sec, and a final extension step at 72°C for 10 min. The PCR was performed using a combination of AccuTAQ, and IDPol TAQ in order for accurate replication with A overhangs.

The PCR products were analysed on a 1 % agarose gel (0.5 g agarose/50 ml, 0.5 % TBE) mixed with 4 µl of SYBR Safe (Invitrogen). The mixture was boiled to dissolve the agarose and allowed to set for 20 min at ambient temperature. The gel was then placed into an electrophoresis apparatus and covered with 0.5 % TBE. Ten microliters of DNA ladder was loaded into the first well and the samples were loaded into the remaining wells along with loading dye (17 µl of sample and 3 µl of loading dye

per well). The samples were run at 100V for 1 hr to allow for complete resolution of the bands.

2.2.3. DNA Purification

The PCR products on the gel were visualized using a UV box. Bands from the 1.4 kbp section were cut from the gel using a sterile scalpel at a size of approximately 100 mg and placed into a sterile RNase/DNase-free 1.5 ml microcentrifuge tube. The High Pure PCR Product Purification Kit (Roche) was used to purify the DNA from the gel. Three hundred microliters of the binding buffer was added to each microcentrifuge tube. The tubes were vortexed for approximately 30 sec, placed in a water bath at 56°C for 10 min, and vortexed briefly at 2 min intervals. After ensuring the gel had been completely dissolved, 150 µL of isopropanol was added to each tube and was again vortexed for 30 sec. High Pure filter columns were placed into 2.0 ml collection tubes and the contents of each microcentrifuge tube were pipetted into the upper reservoir of the tube. The collection tubes were centrifuged for 30 sec at 14000 x g at ambient temperature and the flow through was discarded. After the filters and the collection tubes had been reassembled, 500 µL of wash buffer was added to the upper reservoir of each tube and then centrifuged for 1 min at 14000 x g at ambient temperature. The flow through was discarded, another 200 µL of wash buffer was added to the upper reservoir, and the sample was centrifuged again for 1 min at 14000 x g at ambient temperature. The flow through and collection tubes were both discarded and the filter columns were each inserted into a sterile RNase/DNase-free 1.5 ml microcentrifuge tube. One hundred microliters of sterile water was added to the upper reservoir of each tube and centrifuged for 1 min at 14000 x g at ambient temperature. The filters were then discarded and 10 µL

of sodium acetate (pH 5.2) was added to each tube. After mixing briefly, 275 μL of ethanol was added to each tube and the solutions were vortexed thoroughly before storing at -80°C.

2.2.4. Addition of Restriction Sites and FLAG sequence

A second PCR was performed on each sample using extended primers that contained a SacI restriction site (bold) and a Kozak motif (underlined) on the sense primer, which were added to facilitate expression in a eukaryotic system, and a SalI restriction (bold) site as well as a FLAG (Poly-His for the GABA gene) sequence (italics) on the antisense primer. The added FLAG sequence allows the final protein to be detectable via Western blotting by using an anti-FLAG antibody. The start codon (ATG) was added after the Kozak motif, and the stop codon (TTA) was added before the FLAG/Poly-His sequence. The sense primer for the GluClα was 5' CT **GAG CTC** GCC ACC ATG CTT TTT CTT CTC GT 3' and the antisense primer was 5' CC **GTC GAC** TTA *CTT GTC ATC GTC GTC TTG TAG TCC* TTT GTA AAT CCT TGA TTC 3'. The PCR cycling conditions for the GluClα reaction were an initial temperature of 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 52°C for 90 sec, extension at 72°C for 120 sec, and a final extension step at 72°C for 10 min. The sense primer for the GABAα was 5' CT **GAG CTC** GCC ACC ATG TTC AGC GGC AAT TT 3' and the antisense primer was 5' CC **GTC GAC** TTA *CAC CAC CAC CAT CAT CAT* GTC CGG GTG TAA GTA GAC 3'. The PCR cycling conditions for the GABAα reaction were an initial temperature of 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 54°C for 90 sec, extension at 72°C for

120 sec, and a final extension step at 72°C for 10 min. The DNA was then purified by repeating the methods described in Section 2.2.3.

2.2.5. TA Cloning of PCR Products

Growth medium was made in a 500 ml glass screw top bottle. Into 300 ml of ddH_2O, 2.4 g of trypticase peptone, 1.5 g of yeast extract, 1.5 g of NaCl, and 4.5 g of agarose were added and then autoclaved for 1 hr. The agar was allowed to cool in a 55°C water bath for 30 min and then 6 ml of ampicillin (5.0 mg/ml) and 0.75 ml of X-gal (20 mg/ml) were added to the bottle which was then swirled gently in order to mix without creating bubbles. The solution was poured gently into sterile Petri dishes (approximately 15 ml in each dish) and allowed to set in a fume hood for 30 min. After setting, the dishes containing the medium were stored upside down at 4°C.

The PCR products obtained in Section 2.1.5 were removed from -80°C and centrifuged for 30 min at 14000 x g at 4°C after thawing. Once the DNA pellets had been formed at the bottom of the microcentrifuge tube, the supernatant was discarded and the pellets were washed by suspension in 1.0 ml of 70 % ethanol followed by centrifugation for another 5 min at 14000 x g at 4°C. The supernate was discarded and the pellets were allowed to air dry for 15 min to ensure the ethanol had all been removed.

After the pellets were dry, a solution was created using the chemicals found in the TOPO TA Cloning Kit (Invitrogen) by adding 1.0 µl of the salt solution, 4.0 µl of sterile H_2O, and 1.0 µl of the TOPO vector to each of the tubes containing the DNA pellets. These solutions were pipetted up and down to mix and then allowed to incubate at ambient temperature for 30 min. A vial of TOP10 competent *E. coli* for each sample

was removed from the -80°C freezer and thawed on ice. After the solution had completed incubation, 2.0 µl of each solution was added to a tube of the *E. coli*. The mixes were allowed to incubate on ice for 30 min, after which they were heat shocked in pre-heated water bath at 42°C for 30 sec. After being taken out of the bath, 250 µl of SOC medium was added to each tube and the tubes were placed in a shaker at medium at 180 rpm for 1 hr in a 37°C incubator.

Two of the agar plates for each sample were taken out of the refrigerator and allowed to warm up to room temperature. After the tubes had been incubated, 100 µl of sample was added to a plate (2 plates for each sample) and were spread with a glass rod, sterilizing the rod between each sample. The plates were the labeled and left to incubate overnight at 37°C to allow the TOPO-transformed bacteria to grow.

2.2.6. Plasmid DNA Isolation

Broth was prepared by adding 2.4 g of trypticase peptone, 1.5 g of yeast extract, 1.5 g of NaCl, and 300 ml of ddH_2O into a 500 ml glass screw top bottle and autoclaving for 1 hr. After cooling, the broth was distributed into sterile capped glass tubes by pipetting 5.0 ml of broth and 100 µl of ampicillin (5 mg/ml) to each tube. The plates containing the TOPO-transformed bacteria were retrieved from the incubator and 5 white bacterial colonies were marked for isolation on each plate. Fresh sterile plates (one for each sample) containing media made in section 2.1.6 were labeled with 5 separate areas. Each white colony of interest was taken from the sample plate using a sterile inoculation loop and inoculated to a corresponding labeled area on the fresh plate, and then swirled in a sterile tube containing sterile broth. After all marked colonies had been

inoculated onto a fresh plate and into a broth; both the plates and the broths were incubated overnight at 37°C. The broths were placed on a shaker running at 180 rpm.

The cultures were removed from the incubator, and the plate cultures were stored at 4°C. The plasmid was isolated from the broth cultures using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). The broth cultures were briefly vortexed, and 2 ml of each culture was transferred to a sterile 2 ml microcentrifuge tube. The tubes were centrifuged at 12000 x g for 1 min to form a bacterial pellet at the bottom of the tube. The supernate was discarded, and 200 µl of resuspension solution was added to each tube. The tubes were then vortexed to resuspend the cells to homogeneity. The cells were lysed by adding 200 µl of lysis solution to each tube and mixing by inverting gently 8 times. The solution was allowed to incubate at ambient temperature for 5 min and the cell debris was precipitated by adding 350 µl of neutralization/binding solution to each tube. The solution was mixed by inverting 5 times and the tubes were centrifuged at 12000 x g for 10 min to form a pellet of cell debris at the bottom of the tube. A sterile binding column was inserted into a sterile 2.0 ml microcentrifuge tube for each sample and 500 µl of column preparation solution was added to the upper reservoir of each column. The miniprep columns were centrifuged at 12000 x g for 1 min, and the column eluate was discarded. The clear supernate from the sample tubes were transferred to the upper reservoirs of each column and the cell debris pellets were discarded. The miniprep columns containing the supernate were centrifuged at 12000 x g for 1 min and the eluate was again discarded. Seven hundred and fifty microliters of a wash/EtOH solution (20 % wash solution, 80 % EtOH) was added to the upper reservoir of each miniprep column, and was centrifuged at 12000 x g for 1 min to remove the excess salt. The eluate was discarded and the miniprep columns were centrifuged at

12000 x g for 1 min to remove any remaining wash solution. The binding columns were then transferred to a clean, sterile 2 ml microcentrifuge tube and 100 µl of molecular grade H_2O was added to the upper reservoir. The columns were centrifuged at 12000 x g for 1 min, and the binding column was discarded. The microcentrifuge tubes containing the eluted plasmid were stored at -20°C.

2.2.7. Subcloning of GluClα cDNA into the Eukaryotic Expression Vector

A restriction digest was performed using SalI and SacI (Fermentas). For each of the solutions obtained in section 2.1.7, 5.0 µl of sample was added to a sterile 0.5 ml microcentrifuge tube. After thawing, 4.0 µl of 10x Tango Buffer, 1.0 µl of SalI (10 U/µl), 1.0 µl of SacI (10 U/µl), and 9.0 µl of molecular grade H_2O was added to each tube. After vortexing briefly, the digest solutions were incubated at 37°C for 16 hr and the digest terminated by incubation at 65°C for 20 min. The vector used to clone the gene was the pIRES2-AcGFP1 (Clontech, Figure 2.1), which was linearized by restriction digest. Two microliters of the plasmid solution was added to 4.0 µl of Tango Buffer, 1.0 µl of SacI, 1.0 µl of SalI, and 11.0 µl of molecular grade H_2O in a sterile 0.5 ml microcentrifuge tube. The solution was vortexed briefly and incubated at 37°C for 16 hr. After incubation, 1.0 µl of calf intestinal alkaline phosphatase (Invitrogen) was added to the solution in order to prevent the vector from self-annealing. After a brief vortex, the solution was incubated at 37°C for 30 min, and the reaction terminated by incubation at 65°C for 20 min. The samples and the plasmid were resolved by gel electrophoresis (100 V for 1 hr) following the protocol described in section 2.1.4. Bands shown under the UV box at 1.4 kbps for the lanes containing the samples and 5.2 kbps for the lane containing the plasmid were excised from the gel using a sterile scalpel and purified

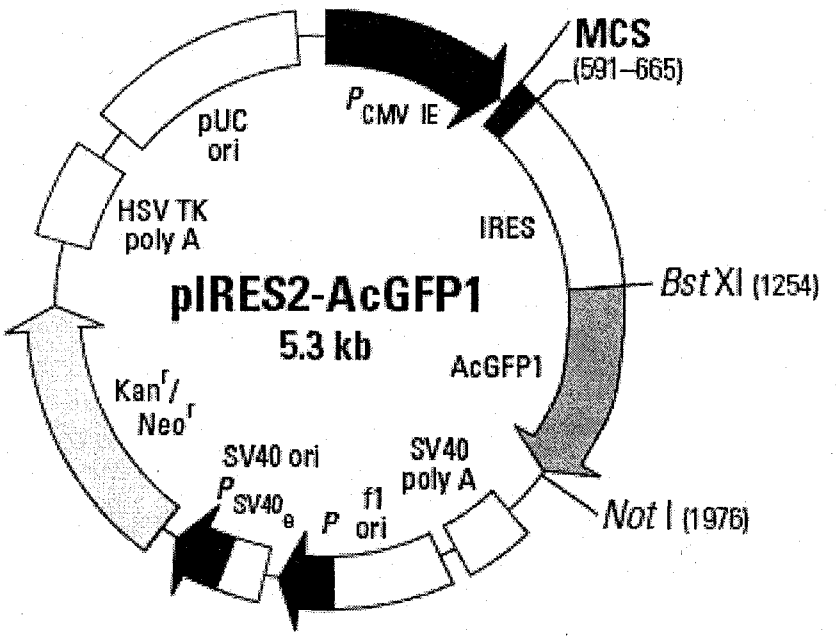

Figure 2.1. The pIRES2-AcGFP1 vector (Clontech) includes a gene encoding AcGFP1 for early detection of successful transfection and a Kanamycin/Neomycin resistance gene to allow for growth on Kanamycin or G418 treated media.

following the protocol of the High Pure PCR Purification Kit (Roche) as described in section 2.1.4. The EtOH-DNA solutions obtained from this procedure were centrifuged at 14000 x g for 30 min at 4°C to obtain DNA pellets in the bottom of the microcentrifuge tube. The supernate was discarded. The pellets were washed in 70 % ethanol and centrifuged at 14000 x g for 15 min at 4°C. The supernate was discarded and the pellets allowed to air dry for 15 min to remove all remaining EtOH. The pellets were dissolved in 20 µl of molecular grade H_2O. In a sterile 0.5 ml microcentrifuge tube, 10 µl of GluClα DNA, 5.0 µl of plasmid DNA, 4.0 µl of 5X Ligase Reaction Buffer, and 1.0 µl of T4 DNA Ligase was added and mixed by inverting. In a second tube, 10 µl of GABAα DNA, 5.0 µl of plasmid DNA, 4 µl of 5X Ligase Reaction Buffer, and 1.0 µl of T4 DNA Ligase (Invitrogen) was added and mixed. The solutions were incubated at ambient temperature for 30 min to allow ligation to form pIRES2-GluClα and pIRES2-GABAα constructs and then stored at 4°C until the next step could be performed.

2.2.8. Vector DNA Isolation

Growth medium was made in a 500 ml glass screw top bottle. Into 300 ml of ddH_2O, 2.4 g of trypticase peptone, 1.5 g of yeast extract, 1.5 g of NaCl, and 4.5 g of agarose were added and then autoclaved for 1 hr. The agar was allowed to cool in a 55°C water bath for 30 min and then 3 ml of kanamycin (5 mg/ml) was added to the bottle contents which were then swirled gently to mix without creating bubbles. The solution was poured gently into sterile Petri dishes (approximately 15 ml in each dish) and allowed to set for 30 min. After setting, the dishes containing the medium were stored inverted at 4°C.

One vial of MAX Efficiency DH5α competent *E.coli* (Invitrogen) was thawed on ice and resuspended by gently shaking. The cells were separated into two 100 µl aliquots and dispensed into separate sterile 1.5 ml microcentrifuge tubes. 2.0 µl of each construct was added to one of the tubes and chilled on ice for 30 min. The cells were then heat-shocked for 45 sec in a water bath pre-heated to 42°C and then chilled on ice for 2 min. A 900 µl volume of sterile SOC medium was added to each tube and the cells incubated at 37°C for 1 hr while being shaken at 225 rpm. Two of the kanamycin-agar plates for each sample were taken out of the refrigerator and allowed to warm up to room temperature. After the tubes had been incubated, 200µl of culture was added to a plate (2 plates for each sample) and spread with a sterile glass rod, re-sterilizing the rod between each sample. The plates were labeled and left to incubate overnight at 37°C to allow the pIRES2-transformed bacteria to grow.

Broth was prepared by adding 2.4 g of trypticase peptone, 1.5 g of yeast extract, 1.5 g of NaCl, and 300 ml of ddH_2O into a 500 ml glass screw top bottle and then autoclaving for 1 hr. After cooling, the broth was dispensed into sterile capped glass tubes by pipetting 5.0 ml of broth and 100 µl of kanamycin (5.0 mg/ml) into each tube. The plates containing the pIRES2-transformed bacteria were retrieved from the incubator and 5 bacterial colonies were marked for isolation on each plate. Fresh sterile plates (one for each sample) containing kanamycin-agar were labeled with 5 separate areas. Each colony of interest was taken from the sample plate using a sterile inoculation loop which was then used to inoculate a corresponding labeled area on the fresh plate and then swirled in a tube containing sterile broth. After all marked colonies had been inoculated onto a fresh plate and into broth cultures, both the plates and the broths were incubated overnight at 37°C with the broth cultures on a shaker at 180 rpm. The

constructs were purified from the culture using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich) following the procedure described in section 2.2.6. The final solutions were stored at -20°C with 10 µl aliquots of each solution sent to ACGT Inc. for sequencing to confirm proper sequence and orientation.

2.3. Transfection and Cell Line Development

2.3.1. Cell Line Maintenance

The cell line used for the transfection was the Chinook salmon embryo fibroblast (CHSE-214). The cell line was grown at temperatures equivalent to those the receptors would be exposed to in nature in order to maintain proper membrane fluidity and receptor conformation. The cell cultures were split 1:4 once every 7-10 days when at 95-100 % confluence. The media (Hanks Minimum Essential media, 10% FBS, 1x antibiotic/antimycotic) was aspirated via vacuum using a sterile Pasteur pipette. To wash the cells, 10 ml of 0.1 % sterile PBS was added and rotated to spread over the cells. The PBS was then aspirated via vacuum using a sterile Pasteur pipette. The cells were treated with 1.0 ml of trypsin (0.5% trypsin, 0.2% EDTA) and the flask was rotated gently to spread the trypsin over the cells. The flask was allowed to incubate at ambient temperate for 30 min and then given a brief shake to detach the cells from the flask surface. In the culture hood, 3.0 ml of medium was added and pipetted up and down to evenly disperse the cells. One ml of culture was then transferred to each of four sterile T-75 cm^2 flasks to result in a 1:4 dilution. Twenty-four ml of medium was then added to each flask and the flasks were then incubated at 16°C for 7-10 more days before another split was required.

2.3.2. Transfection

A transfected cell line was established based upon a study done by Forrester *et al.* (2002). The protocol used was taken and modified from the Fugene HD Transfection Reagent (Roche) product insert. During a cell splitting session, following the protocol described in section 2.3.1, 2 ml of the cell suspension was transferred to a sterile tissue-culture treated 6-well plate (350 µl/well) rather than two T-75 cm^2 flasks. The plate was incubated at 16°C until the cells reached 80-85 % confluence (approximately 3 days). To make the transfection solution, 100 µl of sterile molecular grade H_2O was added to each of six sterile 0.5 ml microcentrifuge tubes. The pIRES2-GluClα vector previously prepared in section 2.2.8 was thawed and 2.0 µl was added to each of the microcentrifuge tubes. Fugene HD Transfection Reagent was removed from the refrigerator and allowed to warm to ambient temperature. The reagent was pipetted directly into the vector solution without allowing the reagent to touch the sides of the microcentrifuge tubes to prevent nonspecific binding. The amounts of reagent added to the six microcentrifuge tubes were 3.0 µl, 4.0 µl, 5.0 µl, 6.0 µl, 7.0 µl, and 8.0 µl. The tubes were then briefly vortexed and allowed to incubate at ambient temperature for 30 min. The 6-well plate containing the cells was removed from the incubator and the entire contents of one of the microcentrifuge tubes was pipetted in a drop-wise manner into each of the wells. The plate was swirled gently in order to disperse the transfection complex throughout the media. The 6-well culture plate was incubated at 16°C for 48 hr. After incubation, the cells were transferred to T-25 cm^2 flasks. The media was aspirated from each well via vacuum using a sterile Pasteur pipette and washed with 2.0 ml of sterile 0.1 % PBS. The PBS was removed using another sterile Pasteur pipette and 150

µl of trypsin was added to each well. The plate was allowed to incubate at ambient temperature for 30 min, and then 2.0 ml of media was added to each well. The media was pipetted up and down to suspend and disperse the cells and then 150 µl of each well was transferred to a well on a sterile tissue-culture glass slide which was sealed. The remaining 2.0 ml from each well was transferred to a sterile T-25 cm^2 flask and 6 ml of media was added to bring the total volume to 8.0 ml. Both the flasks and the glass slide were incubated at 16°C for 24 hr after which the culture media was removed from glass slide and the cells were viewed under a fluorescence microscope to determine the expression of GFP. The flasks were treated with G418 (1 mg/ml) by adding 160 µl of 50 mg/ml Geneticin to each flask in order to select for transfected cells. The cultures were then incubated at 16°C for a period of 3 months. Once every 7-10 days, the media was replaced with fresh media and G418. After the cells became 95-100 % confluent, the cells in the flask that had originally been treated with the 8:2 ratio of Fugene to vector were transferred to a T-75 cm^2 flask. The media was removed via vacuum using a sterile Pasteur pipette and the cells washed using 4 ml of sterile 0.1 % PBS. The PBS was also removed using vacuum aspiration and 300 µl of trypsin was added. After incubation at ambient temperature for 30 min, 3 ml of media was added and pipetted up and down to disperse the cells. The cells were then transferred to a sterile T-75 cm^2 flask, and media was added to bring the total volume to 25 ml. The cells were again treated with G418 by adding 500 µl of 50 mg/ml Geneticin to make the concentration 1.0 mg/ml.

2.3.3. Membrane Isolation from Cell Line

A T-75 cm^2 flask of confluent cells were taken from the CHSE-pIRES2, the CHSE-GluClα, and the CHSE-214 cell lines. The media was aspirated from the flasks

via vacuum using a sterile Pasteur pipette. The cells of each flask were washed with 10 ml of sterile 0.1 % PBS which was then aspirated via vacuum as well. Each flask had 1 ml of trypsin added and then rotated gently to spread the trypsin over all the cells. After incubation at ambient temperature for 30 min, 9.0 ml of culture media was added to each flask and the contents then transferred to 25 ml plastic screw-capped tubes. The tubes were centrifuged at 2000 x g for 10 min at 4°C and the supernate discarded. The pellets formed in the bottom of the tube were resuspended in 10 ml of 50 mM of ice cold HEPES, and maintained on ice. The cells were lysed and dispersed using a sonicator for two 10 sec bursts on high settings and centrifuged at 2000 x g for 10 min at 4°C. The supernate was transferred into two fresh tubes (5.0 ml/tube) compatible with a 70.1ti ultracentrifuge rotor and the pellet was discarded. The samples were centrifuged at 40000 x g for 1 hr at 4°C, and the supernate was transferred to a fresh tube and stored at -80°C. The pellets were washed in 5.0 ml of 50 mM HEPES and centrifuged at 40000 x g for 1 hr at 4°C. The supernatant was discarded and the pellets were suspended in 1.0 ml of 50 mM HEPES and transferred to a sterile 1.5 ml microcentrifuge tube and stored at -80°C.

2.3.4. Protein Assay

A protein assay was conducted to determine the concentration of protein obtained from the isolation. Protein concentration was determined using a BioRad protein assay. A standard curve was created using six concentrations of BSA. BSA (1.0 mg/ml) was pipetted into sterile 150 µl microcentrifuge tubes in volumes of 0, 5.0 µl, 10 µl, 25 µl, 40 µl, and 50 µl. Each aliquot was adjusted to a final volume of 50 µl using 0.1 M PBS. Five microliters of each sample was added to three microcentrifuge tubes and

diluted by a factor of 10 (45 µl), 50 (245 µl), and 100 (495 µl) in 0.1 M PBS. The samples and standards were all vortexed and 5 µl of each was added to a flat-bottom 96-well plate in triplicate. A solution of BioRad dye reagent was prepared by diluting 2.0 ml of the stock solution in 8 ml of deionized water. Two hundred and fifty microliters of the diluted dye reagent was added to each well of the plate, and was tapped gently to mix. The plate was incubated at ambient temperature for 5 min, and then read in a chemoluminescent plate-reader at a wavelength of 595 nm. The concentration obtained was used to dilute each sample to 1.0 mg/ml by adding the necessary amount of 50 mM HEPES buffer, and both the membrane solution and the retained supernate were aliquoted out into 1.5 ml microcentrifuge tubes.

2.3.5. Western Blotting

Protein expression was confirmed using Western blotting. Ten microliters of the membrane solution was added into a 0.5 ml microcentrifuge tube along with 10 µl of 2x loading buffer and boiled for 5 min to denature the proteins. In a plastic tube, the separation gel was prepared using 3.3 ml of dH_2O, 2.5 ml of separation buffer (1.5 M Tris, pH 8.8) 100 µl of 10 % SDS and 4.0 ml of 30 % polyacrylamide which were added and swirled gently to mix. After mixing, 100 µl of 10 % APS and 5 µl of TEMED were added and quickly mixed. The gel mixture was then pipetted into the electrophoresis apparatus, overlayed with dH_2O, and left to set for 20 min. The stacking gel was then prepared using 3.0 ml of dH_2O, 1.25 ml of stacking buffer (0.5 M Tris, pH 6.8), 50 µl of 10 % SDS and 0.67 ml of 30 % polyacrylamide which were added and swirled gently to mix. The water overlay was removed from the separation gel and 50 µl of 10 % APS and 5 µl of TEMED were added to the stacking gel solution which was then mixed and

pipetted on top of the separation gel. The well comb was inserted, and the gel was allowed to set for 20 min. After the comb was removed, the gel was rinsed with cathode buffer (0.1 M Tris, 0.1 M Tricine, 0.1 % SDS) and the cathode buffer was used to fill the inner chamber of the apparatus. Two hundred milliliters of anode buffer (0.2 M Tris, pH 8.9) was added to the outer chamber of the apparatus. After the samples were loaded into the gel in triplicate, they were separated at 100V for 15 min through the stacking gel and then 150V for 60 min through the separation gel.

Once the separation was complete, the gel was removed from the apparatus and equilibrated in transfer buffer (0.2 M glycine, 25 mM Tris, 10 % EtOH) for 15 min before being transferred to nitrocellulose that had been pre-soaked in transfer buffer for 15 min using a transfer assembly. The assembly was placed into a mini-tank which was filled with transfer buffer and transferred using 100V for 1 hr. The nitrocellulose was removed cut into three separate sample pieces and placed face-up in a plastic container containing an excess of blocking buffer (1x TBS, 0.1 % Tween-20, 5 % skim milk powder) for 1 hr at room temperature with agitation. The nitrocellulose strips were then washed in washing buffer (1x TBS, 0.1 % Tween-20) for 1 hr three times. One of the strips was then incubated with the anti-FLAG M2 monoclonal antibody (7.0 μg/ml) overnight at 4°C while being agitated and the others were frozen at -20°C. This antibody was specific for the DYKDDDDK sequence encoded for on the reverse primer used in section 2.2.4. The incubated strip was washed with the washing buffer for 1 hr three times and was then incubated with the anti-mouse IgG secondary antibody (1:4000 dilution) for 1 hr at room temperature while being agitated. After three more 1 hr washes, the strip was incubated for 1 min while being covered in 3.0 ml of a mixture of 1.5 ml of ECL solution A and 1.5 ml of ECL solution B (Amersham). The ECL mixture

was then removed and the strip was wrapped in clear plastic wrap and then positive bands were detected by exposure to autoradiographic film for 20 min.

2.3.6. Confirmation of Gene Insertion

A T-75 cm^2 flask of cells was taken from the incubator, the media aspirated via vacuum using a sterile Pasteur pipette, and then washed in 10 ml of 0.1 % PBS. After removal of the PBS, 7.5 ml of TRIzol reagent was added and pipetted up and down several times to detach the cells from the flask surface. The sample was incubated for 5 min at ambient temperature to permit complete dissociation of nucleotide complexes and transferred to a plastic screw-cap tube. After incubation 1.5 ml of chloroform was added. The sample was shaken by hand for 15 sec and then incubated for 3 min at ambient temperature. The sample was centrifuged at 12000 x g for 15 min at 4°C and after separation the colorless upper aqueous phase was carefully transferred to a new sterile RNase/DNase-free screw-cap tube. The RNA was precipitated by adding 3.75 ml of isopropyl alcohol and incubating for 10 min at ambient temperature. The sample was then centrifuged at 12000 x g for 20 min at 4°C to form an RNA pellet at the bottom of the plastic tube. The supernate was discarded and 7.5 ml of 75 % ethanol was added to the tube to wash the pellet. The sample was mixed by vortexing and centrifuged at 7500 x g for 5 min at 4°C. After centrifugation, the supernate was discarded and the pellet was air-dried for 10 min. The pellet was then dissolved in 500 µl of sterile RNase-free water and incubated at 55°C for 10 min. After the removal of the colorless upper aqueous phase, the interphase and phenol phase were used to isolate DNA. Two and one quarter milliliters of 100% ethanol was added to the tube and mixed by inversion. The sample was incubated at room temperature for 3 min and then centrifuged at 2000 x g for 5 min

at 4°C. The supernate was discarded and the DNA pellet that formed in the bottom of the tube was washed twice using 7.5 ml in a solution of 0.1 M sodium citrate in 10 % EtOH. For each wash, the pellet was incubated in the washing solution for 30 min at ambient temperature and then centrifuged at 2000 x g for 5 min at 4°C. Following the two washes, the DNA pellet was suspended in 15 ml of 75 % ethanol and incubated for 15 min at ambient temperature, with a brief vortex every 2 – 3 min. The solution was then centrifuged at 2000 x g for 5 min at 4°C and the supernate was discarded. The pellet was air dried in an open tube in a fume hood for 15 min and then dissolved in 500 µl of 8 mM NaOH. The solution was centrifuged at 12000 x g for 10 min at 4°C and the supernate was transferred to a sterile RNase/DNase-free 1.5 ml microcentrifuge tube. The RNA sample was then used to create cDNA following the protocol described in section 2.2.2 and both the DNA and cDNA samples were used in a PCR using the conditions and the GluClα primers from section 2.1.3. A housekeeping gene, the 18S rRNA subunit gene, was used to determine the quality of the sample. The sense primer for the 18S subunit was 5' TGTGCCGCTAGAGGTGAAATT 3', and the antisense primer was 5' GCAAATGCTTTCGCTTTCG 3'. The PCR cycling conditions for the reaction was an initial temperature of 94°C for 10 min followed by 34 cycles consisting of denaturation at 94°C for 60 sec, annealing at 52°C for 90 sec, extension at 72°C for 120 sec, and a final extension step at 72°C for 10 min. The PCR product was analyzed via gel electrophoresis and the presence of the transcript was confirmed by the size of the band and comparison with a PCR product taken from a cell line transfected only with pIRES2-AcGFP1.

2.4. Binding Assays

2.4.1. Membrane Isolation from Sea Lice

When needed, a tube of sea lice that had been previously flash frozen was thawed on ice in 10 ml of 50 mM HEPES solution. The cephalothoraxes were homogenized using a tissue tearer, kept on ice until completely dissolved into the buffer. The tube was centrifuged at 2000 x g for 10 min at 4°C, and the supernate was transferred into two fresh tubes (5 ml per tube) compatible with a 70.1Ti ultracentrifuge rotor. The pellet was discarded. The samples were centrifuged at 40000 x g for 1 hr at 4°C and the supernate was transferred to a fresh tube and stored at -80°C. The pellets were washed in 5.0 ml of 50 mM HEPES and centrifuged at 4000 x g for 1 hr at 4°C. The supernatant was discarded and the pellets dissolved in 1.0 ml of 50mM HEPES and transferred to a sterile 1.5 ml microcentrifuge tube and stored at -80°C.

The protein assay used in section 2.3.4 was conducted to determine the concentration of protein obtained from the isolation. The concentration was diluted to 1.0 mg/ml by adding 50 mM HEPES and both the membrane solution and the retained supernate was aliquoted into 1.5 ml microcentrifuge tubes.

2.4.2. Saturation Binding Assay

Binding assays were conducted based upon methods used in Forrester *et al.* (2002) and Ludmerer *et al.* (2002). The incubation buffer solution consisted of using 50 mM HEPES, 0.1 mg/ml of bacitracin, and 2 % DMSO in deionized H_2O with the pH adjusted to 7.5 using 10 M HCl. A 5.0 ml aliquot of incubation buffer was pipetted into a glass test tube and 7.0 µl of [^{3}H]EMB (Amersham, 14000mCi/mol, 1mCi/ ml) was added, giving a final [^{3}H]EMB concentration of 0.1 µM, and mixed by briefly vortexing.

Using 10 ml glass test tubes, 1 ml of buffer was added to each of 24 different test tubes. An aliquot of the membrane protein solution was removed from the freezer and thawed on ice and 20 μg of membrane protein was added to each test tube. [^{3}H]EMB was then added to give eight final concentrations of 0, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 nM, with three replicates for each concentration. The solutions were covered to prevent evaporation and incubated at 15°C overnight.

A vacuum filtration system was set up using ceramic Buckner funnels. The binding reaction was terminated by vacuum filtering the solutions through glass-fiber filter papers (grade C) which had been pre-washed with 1.0 ml of a buffer to help facilitate protein binding to the filter paper (50 mM HEPES, 0.5 % Triton X-100, 0.1 % polyethyleneimine). The filter paper was washed using 3.0 ml of a 0.5 % Triton X-100 solution. The filter paper was removed and placed into a clean 1.5 ml microcentrifuge tube and 1 ml of scintillation fluid was added to it. This process was repeated for each solution. The filter papers were analyzed for ^{3}H in a liquid scintillation counter.

Non-specific binding was measured by repeating this protocol with the solutions incubated in the presence of a minimum of 1000-fold molar excess of unlabeled EMB for 2 hr before adding the [^{3}H]EMB. Five milligrams of powdered EMB was dissolved in 5.0 ml of the binding buffer and 4.0 μl of solution were added to each tube giving a final concentration of 4.0 μM of EMB in each tube before the [^{3}H]EMB was added. Specific binding was determined as the difference between the total binding and the non-specific binding using the results from the liquid scintillation counter. The results were analyzed by non-linear regression using Graphpad Prism 5.0 software in order to determine the B_{MAX} and K_D.

The assay was repeated twice, with the incubation temperature changed to 5°C and 20°C for the respective assays to determine if there was any change in the K_D as a function of temperature. The assay was also carried out using the membrane protein taken from the cell line. Counting efficiency was determined by plotting the CPM values taken from samples with known DPMs that had been set up in the same fashion as the experimental samples. Initial experiments in this section were performed in quadruplicate without using DMSO in the incubation buffer. All subsequent trials were performed in triplicate, and DMSO was a component of the incubation buffer.

2.5. Data Analysis

Data analysis was done using saturation curve non-linear regression in Graphpad Prism 5.0. Graphs were generated using Graphpad Prism 5.0 and Minitab Statistical Software 15.1.

RESULTS

3.1. Molecular Cloning of GluClα Gene

3.1.1. RNA Isolation and PCR

The RNA samples were analyzed by spectrophotometry to determine the concentration and purity of the samples (Table 3.1). The RNA was also analyzed via gel electrophoresis to determine its integrity, the results of which are shown in Figure 3.1. After the cDNA was created, the samples were tested by running a PCR using internal primers for the GABAα gene and ran through electrophoresis (Figure 3.2). The cDNA synthesized from the RNA of adult sea lice, shown in lane 3 of Figure 3.3, showed the best results and was selected as the basis for subsequent reactions. After the cDNA was purified, it was re-amplified using modified primers. Figure 3.4 shows the results of the restriction digest of the purified TOPO plasmids taken from the five selected bacterial colonies. The band from the first colony was used in the insertion of the gene into the pIRES2-AcGFP1 vector. After the extraction of the bands, and insertion into the pIRES2-AcGFP1 plasmid, a second restriction digest was used to confirm the presence of the putative GluClα gene (Figure 3.5). Plasmid DNA of sample 3 was sequenced and used in the transfection process.

3.1.2. Sequence Alignment

The pIRES2-GluClα plasmid that was to be used in the transfection process was sent to ACGT, Inc. for sequencing in order to confirm proper reading frame, orientation, and sequence. After the sequence was obtained, the results were used in BLAST to compare to the sequence identified by Tribble *et al.* (2007a). (Appendix A).

Table 3.1. The purity (A260/280) and concentration of RNA in 1 ml aliquots of samples extracted from sea lice and read by a UV spectrophotometer. A260/280 of highly pure RNA is 2.0.

RNA Sample	A260/280	Concentration (µg/µl)
Pre-Adult 1	1.33	0.16
Pre-Adult 2	1.64	0.63
Adult 1	1.75	2.14
Adult 2	1.69	2.31

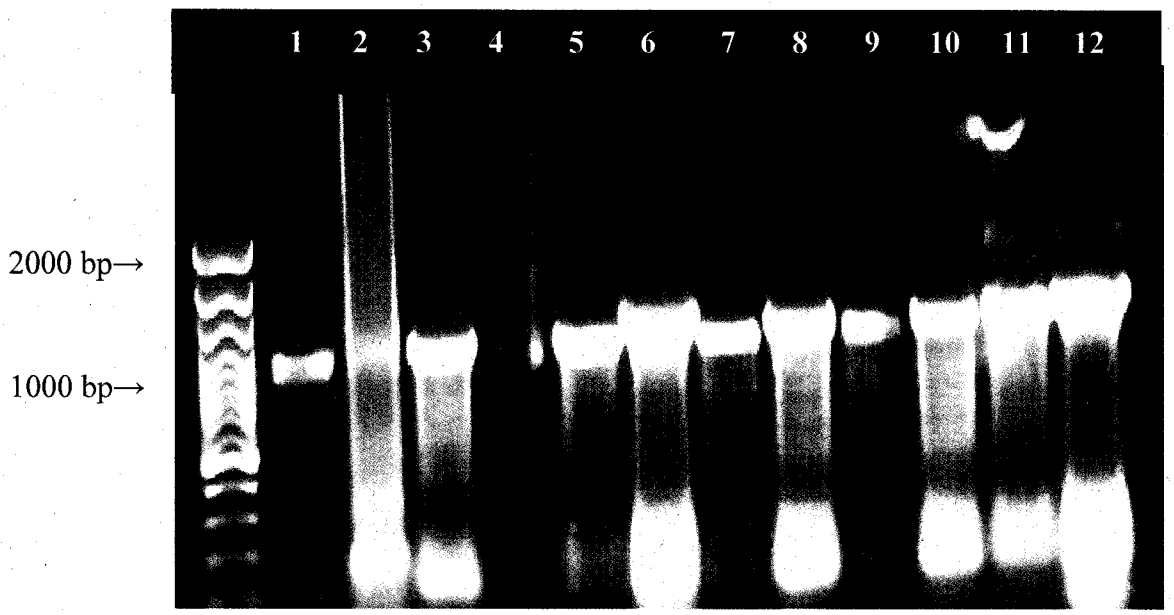

Figure 3.1. Gel electrophoresis of RNA samples taken from pre-adult (lanes 1-6) and adult sea lice (lanes 7-12), in 1.0 µl (lanes 1,4,5,7,9, and 11) and 8.0 µl (lanes 2, 3,6,8,10, and 12) amounts used in determining RNA sample quality.

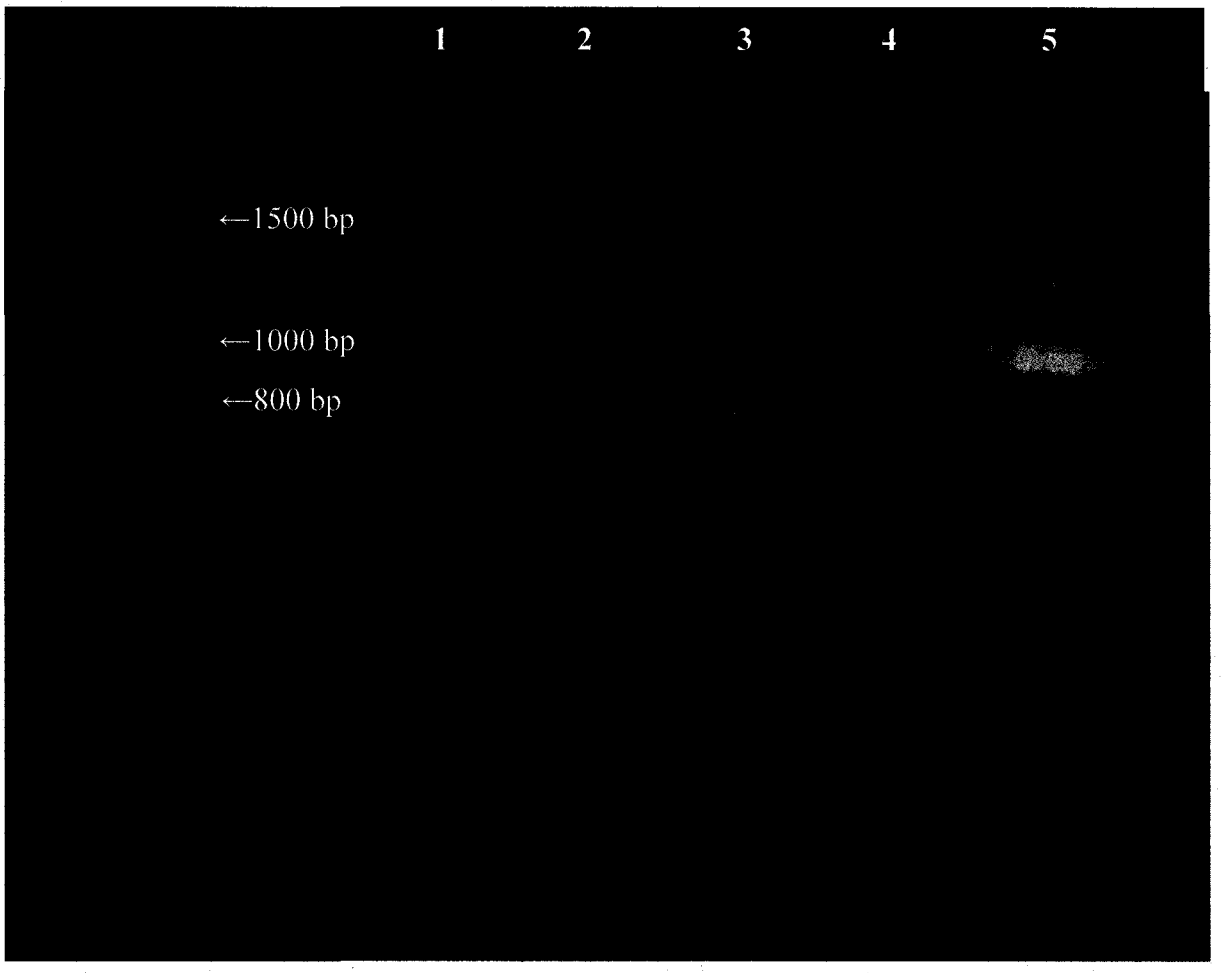

Figure 3.2. Gel electrophoresis of PCR products using internal GABA primers using cDNA created from RNA samples. Lanes 3 and 5 are PCR product of cDNA synthesized from adult louse RNA, lanes 2 and 4 are PCR product of cDNA synthesized from pre-adult louse RNA.

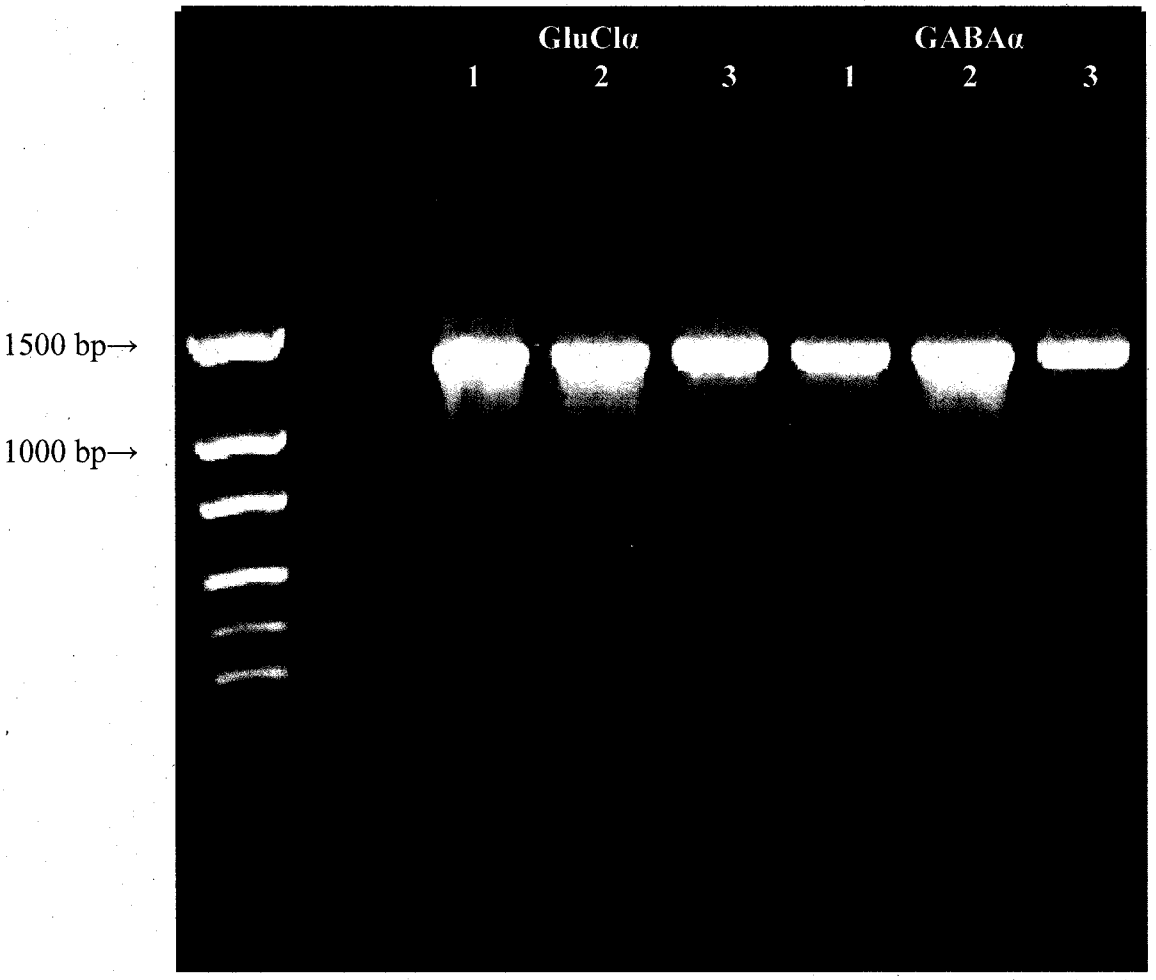

Figure 3.3. Gel electrophoresis of triplicate PCR product using internal GluCl (Lanes 1-3) and GABA primers (Lanes 4-6) using cDNA created from RNA samples.

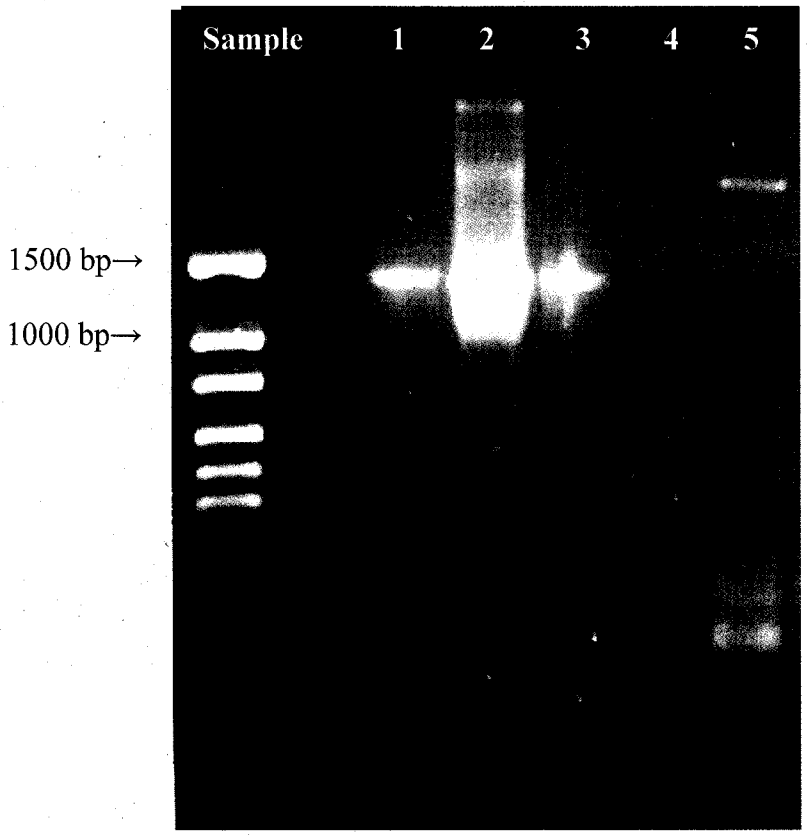

Figure 3.4. Gel electrophoresis showing restriction digest of plasmids taken from the five TOPO transformed E. coli colonies, with GluClα bands apparent at 1.4 kbp in lanes 1, 2 and 3.

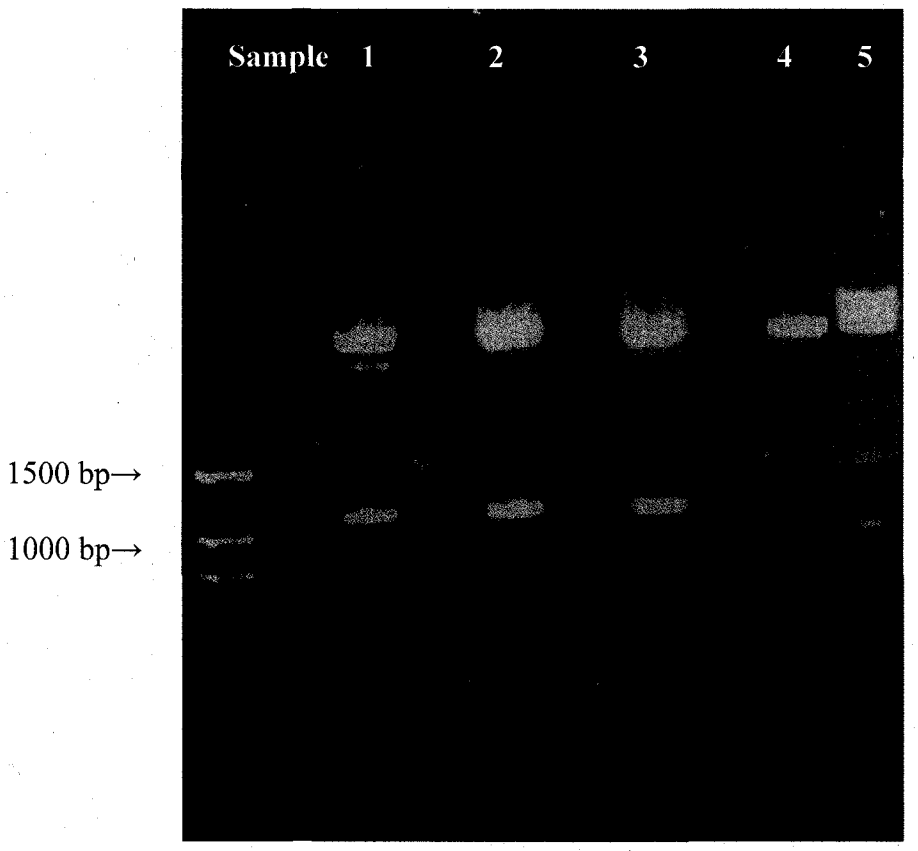

Figure 3.5. Restriction digest of pIRES2-GluClα using SacI and SalI, showing the putative presence of GluClα in lanes 1, 2, and 3.

3.2. Transfected Cell Line

3.2.1. Immunofluorescence

While being transferred to the T-25 cm^2 tissue culture flask, a small sample of cells was placed on a glass slide to examine for green fluorescence, which would be indicative of successful transfection. Fluorescence was observed in all transfection concentrations, with the highest observable amount of fluorescence in the 8:2 ratio of Fugene to vector. A comparison of the fluorescence of the 8:2 transfection well to the negative control well is shown in Figure 3.6. G418 selection was used to eliminate non-transfected cells (95 % of total cells) from the flask and an extended period of growth was needed for the remaining G418-resistant cells to become confluent. In the non-transfected cells, 100 % of the cells died and no further growth was observed. When the cells were transferred to the T-75 cm^2 flask, another small sample of cells was placed on a glass slide to confirm the green fluorescence. Figure 3.7 shows a comparison of fluorescence of the transfected cells after G418 selection to a sample of non-transfected cells.

3.2.2. Protein Assays

Protein concentration was determined using a chemiluminescent spectrophotometer. The concentrations obtained are provided in Table 3.2, and the standard curve had an R^2 value of 97.4 % ($p < 0.01$). All concentrations were modified to approximately 1.0 µg/µl in 50 mM HEPES for use in binding assays.

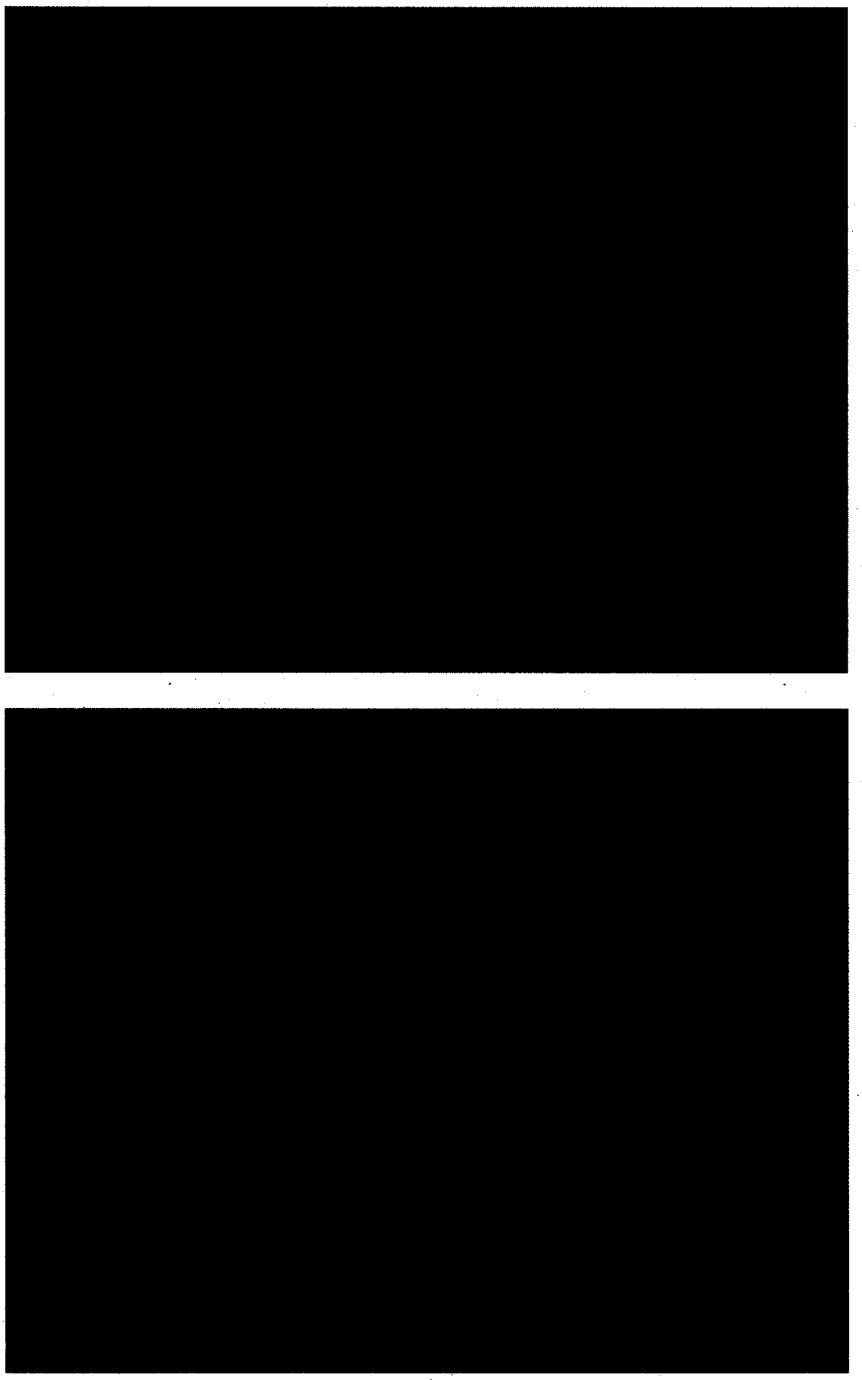

Figure 3.6. Non-transfected (top) and transfected (bottom) CHSE-214 cells observed under a fluorescence microscope after initial transfection (400x magnification).

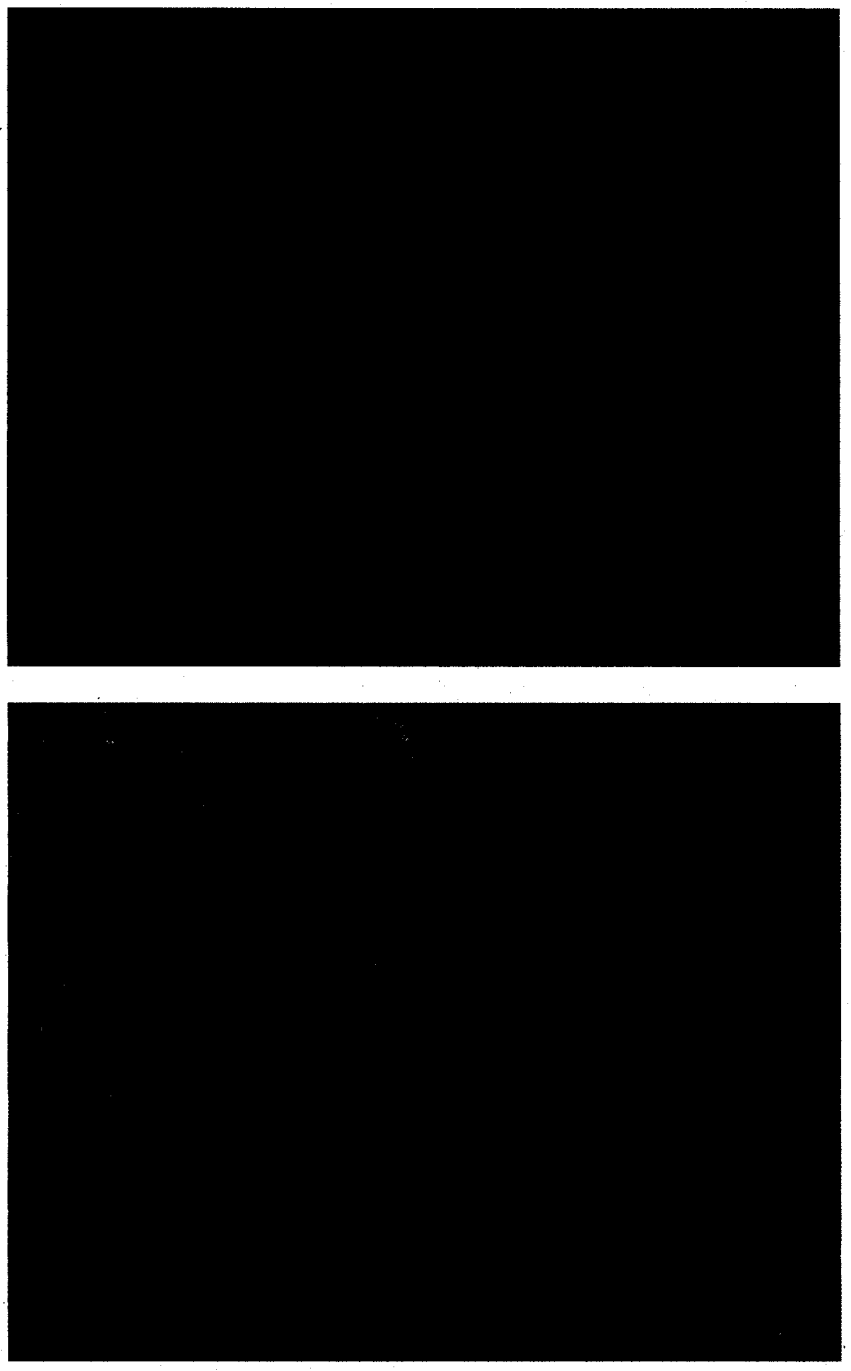

Figure 3.7. Non-transfected (top) and transfected (bottom) CHSE-214 cells observed under a fluorescence microscope after treatment with G418 (400x magnification).

Table 3.2. Concentration of protein samples extracted from all sources of membrane used before diluting to 1.0 μg/μl, and K_D of EMB versus the protein at 15°C.

SAMPLE	CONCENTRATION (μg/μl)	KD (nM)
Sea Lice Membrane	1.22	1.377 ± 0.205
Sea Lice Cytosol	1.51	$>1 \times 10^5$
CHSE-GluCl Membrane	2.32	$>1 \times 10^5$
CHSE-pIRES Membrane	1.47	$>1 \times 10^5$
CHSE-214 Membrane	2.67	$>1 \times 10^5$

3.2.3. Western Blotting

In order to determine if the GluClα receptor was being expressed in the cell line, the membrane purified from the transfected cells was analyzed via a Western blot using an anti-FLAG monoclonal antibody specific to the FLAG epitope added during PCR. Two different concentrations of anti-FLAG were used. The antibody bound nonspecifically to other proteins of the cell line; the control cells had higher levels of binding than the transfected cells (Figure 3.8). At the higher concentration of antibody, similar binding was observed in the negative control and the pIRES2-AcGFP1 only control cells, with again fewer bound proteins in the transfected cells. (Figure 3.9)

3.2.4. Genetic Confirmation

In order to determine whether or not the gene for GluClα was present in the cell line, a PCR was performed with both GluCl primers and housekeeping primers for the 18S ribosomal subunit (approximately 100bp). The resulting PCR fragments were analyzed by gel electrophoresis (Figure 3.10). Analysis of the gel revealed three bands in cDNA of the transfected cell line that were not present in the non-transfected cells. The largest band was approximately 1450 bp, which corresponds to the size of the predicted fragment. The two other bands were 1350 and 350 bp, respectively.

3.3. Binding Assays

3.3.1. Saturation Assays

The first assay done was to assess the efficiency of counting by analyzing the

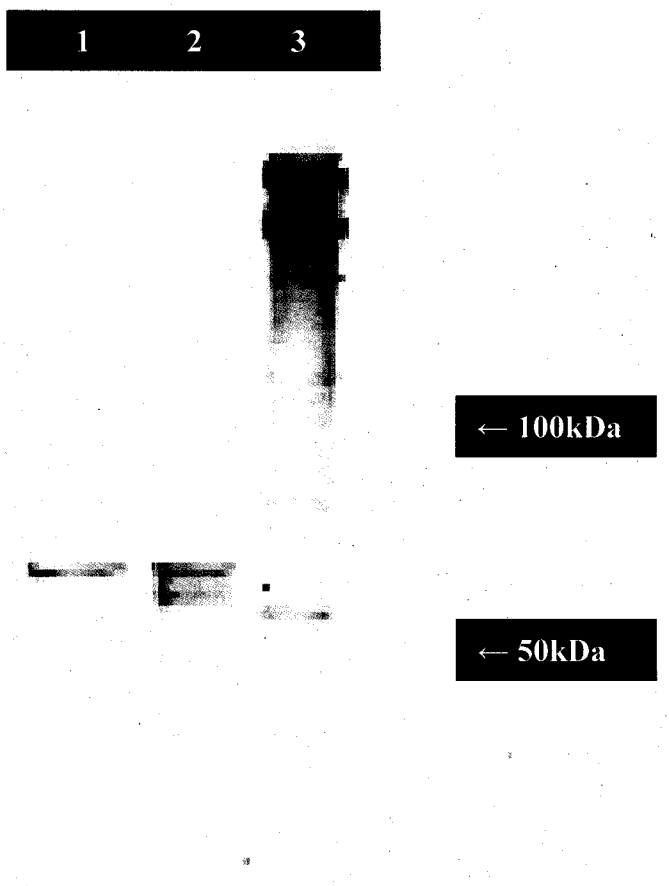

Figure 3.8. Western blot of membranes taken from transfected cells (Lane 1), non-transfected cells (Lane 2), and sea lice (Lane 3).

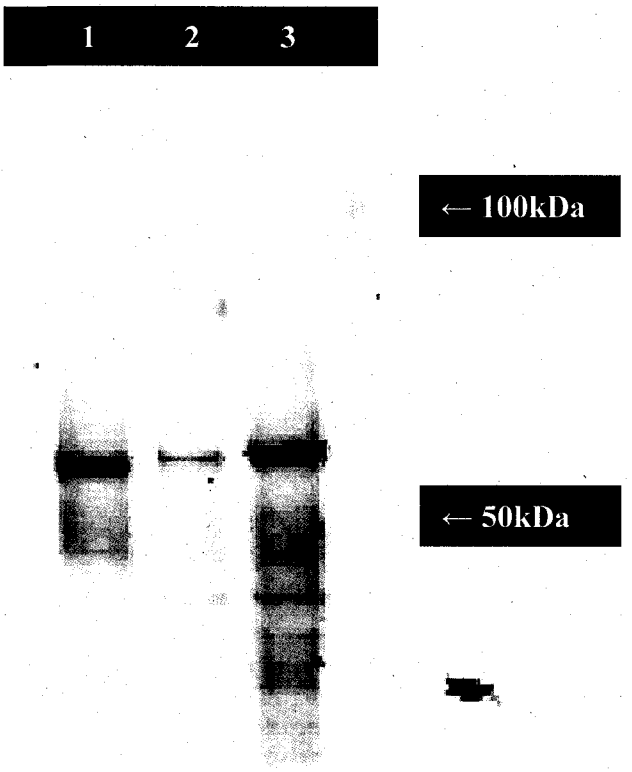

Figure 3.9. Western blot of membranes taken from transfected cells (Lane 1), pIRES2-transfected cells (Lane 2), and non-transfected cells (Lane 3).

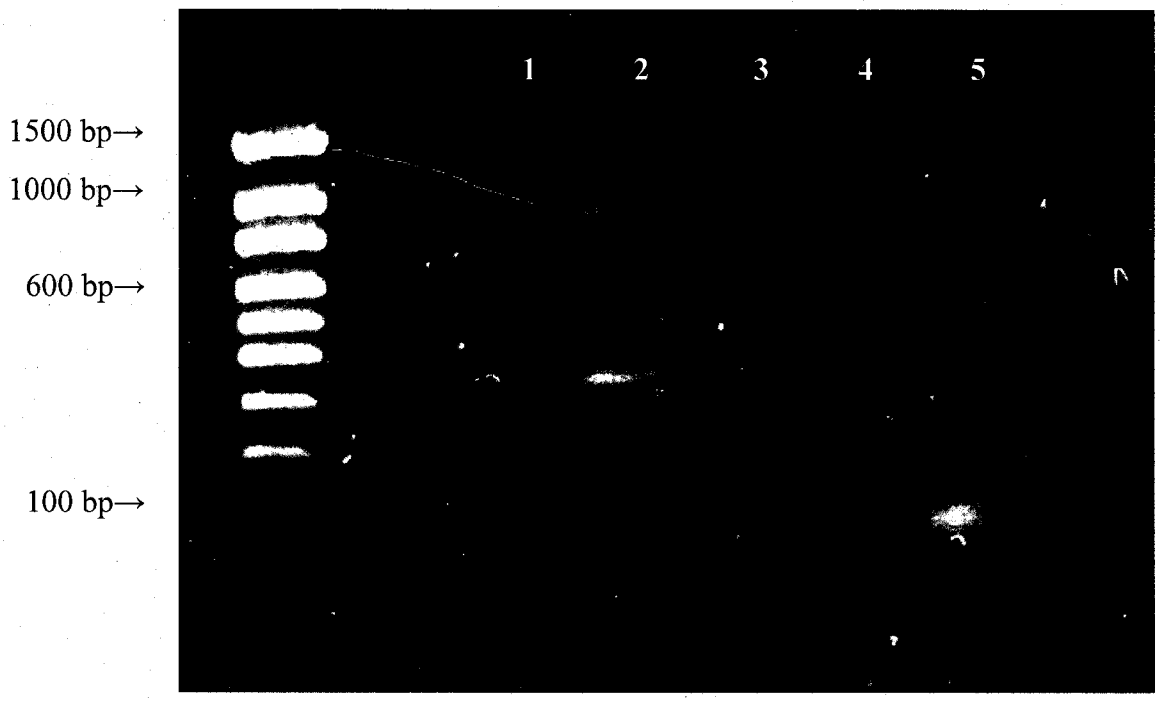

Figure 3.10. Gel electrophoresis of PCR product using GluClα primers of transfected cell line DNA (lane 1), cDNA (lane 2), pIRES2-transfected cell line DNA (lane 3), cDNA (lane 4), and of PCR product using 18S primers of pIRES2-transfected cell line cDNA (lane 5).

CPM vs DPM of known concentrations of [^{3}H]EMB using the same conditions as the experimental procedure. As the relationship was shown to be linear, the counting efficiency for this experiment was found to be 13.8 %, with an R^2 of 99.3 % ($p < 0.01$). This was used as a standard curve by which to convert experimental analyses into DPM in order to calculate the concentration of bound [^{3}H]EMB (Figure 3.11). Originally, no DMSO was used in the assay binding buffer, and lead to high non-specific binding and variability within the results (Figure 3.12). The protocol was modified to include DMSO, which resulted in less variability (Figure 3.13). Specific binding was calculated as total binding – non-specific binding. The supernate containing the cytosolic contents extracted from the sea lice cephalothoraxes had no significant specific binding (Figure 3.14), indicating no target receptor was present. Membranes taken from the transfected (Figure 3.15) and non-transfected (Figure 3.16) cell lines each displayed no significant specific binding, and had no significant difference to each other, confirming the Western Blot analysis that the GluClα subunit was not being expressed in sufficient amounts. Figure 3.13 already confirmed binding in the membranes extracted from the sea lice cephalothoraxes and these were then used in the temperature analysis.

3.3.2. Temperature Binding Assays

The cephalothorax membranes were analyzed at three different temperatures using the protocol modified to include DMSO in the binding buffer. As shown in Figure 3.17, at 15°C, the K_D was found to be 1.377 ± 0.205 nM and the B_{MAX} was 9.049 ±

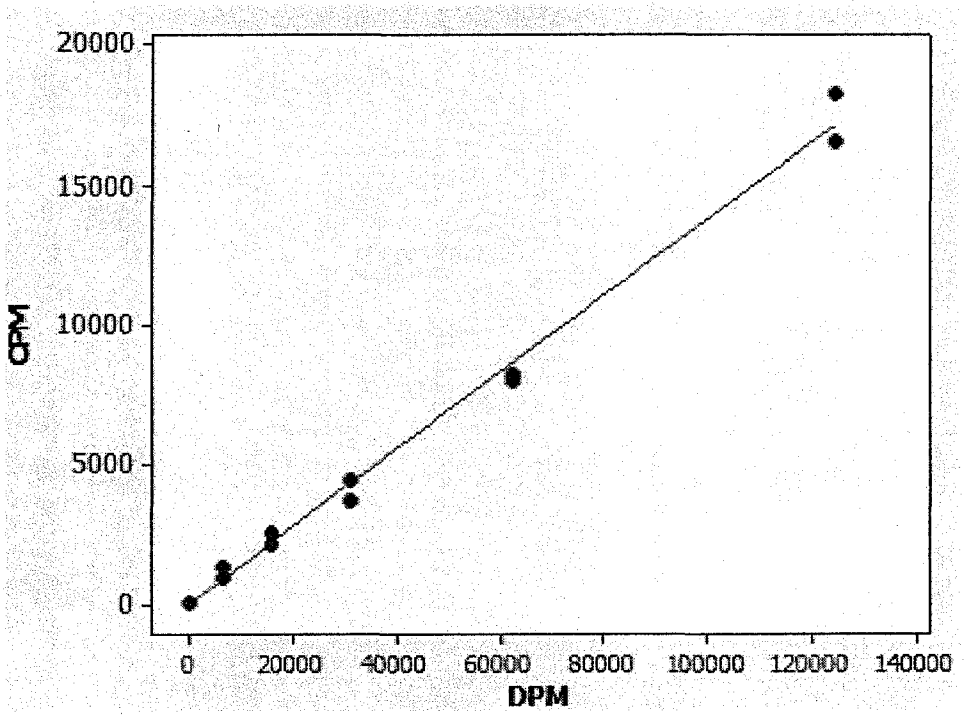

Figure 3.11. CPM vs DPM using known concentrations of [^{3}H]EMB to determine counting efficiency by comparing the output of the liquid scintillation counter to the concentration to demonstrate a linear relationship (n=2 per concentration, slope=0.138).

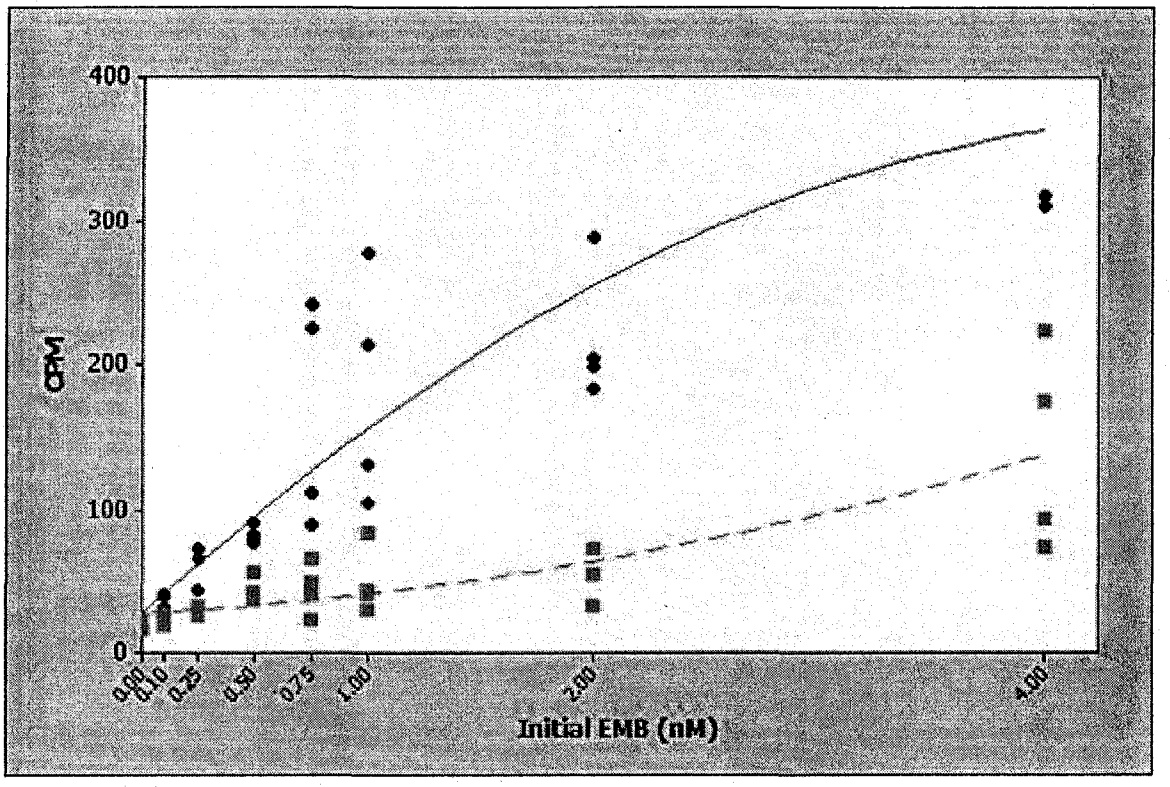

Figure 3.12. Total (●) and non-specific (■) binding of [^{3}H]EMB to membranes extracted from sea lice cephalothoraxes at a range of concentrations of EMB at 15°C in incubation buffer without DMSO (10 µg of membrane/sample, n=4). Average Cv of total binding is 25.8%, and of non-specific binding is 14.4%.

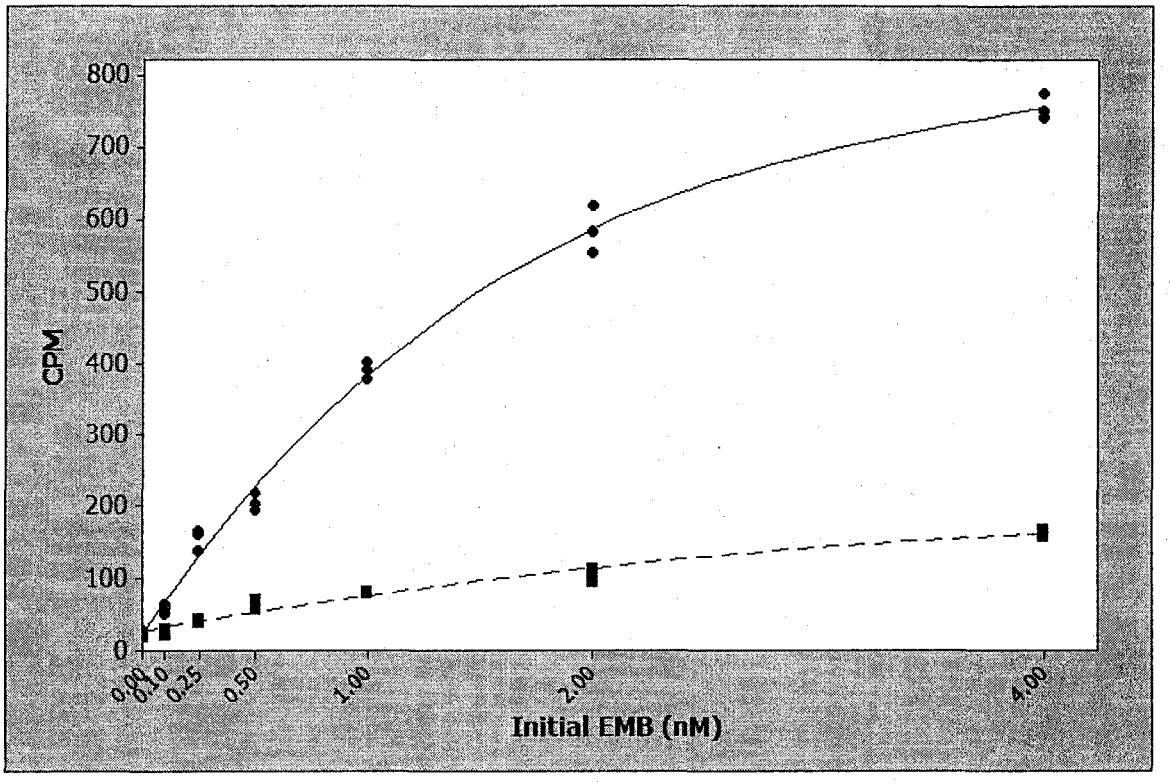

Figure 3.13. Total (●) and non-specific (■) binding of [^{3}H]EMB to membranes extracted from sea lice cephalothoraxes at a range of concentrations of EMB at 15°C in incubation buffer using DMSO (20 µg of membrane/sample, n=3). Average Cv of total binding is 7.42%, and of non-specific binding is 6.89%.

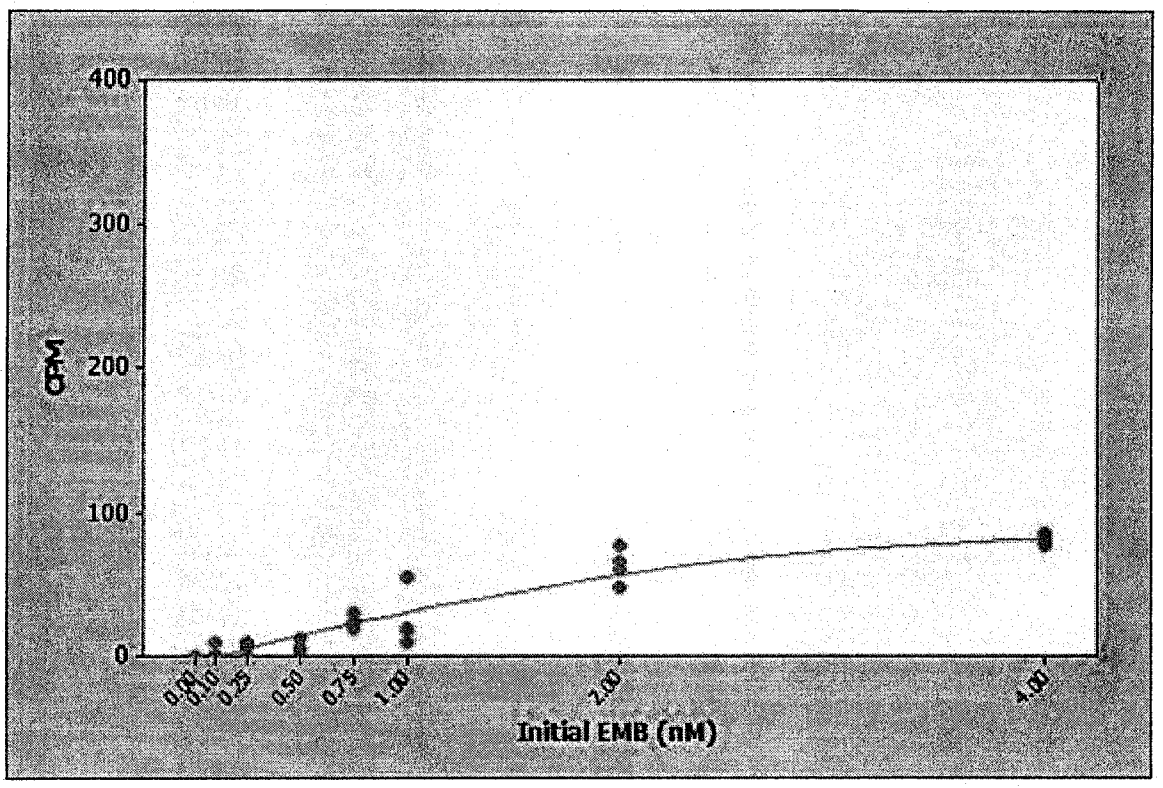

Figure 3.14. Specific binding of [^{3}H]EMB to cytosolic proteins of sea lice at various concentrations at 15°C read by a liquid scintillation counter (20 µg of membrane/sample, n=4).

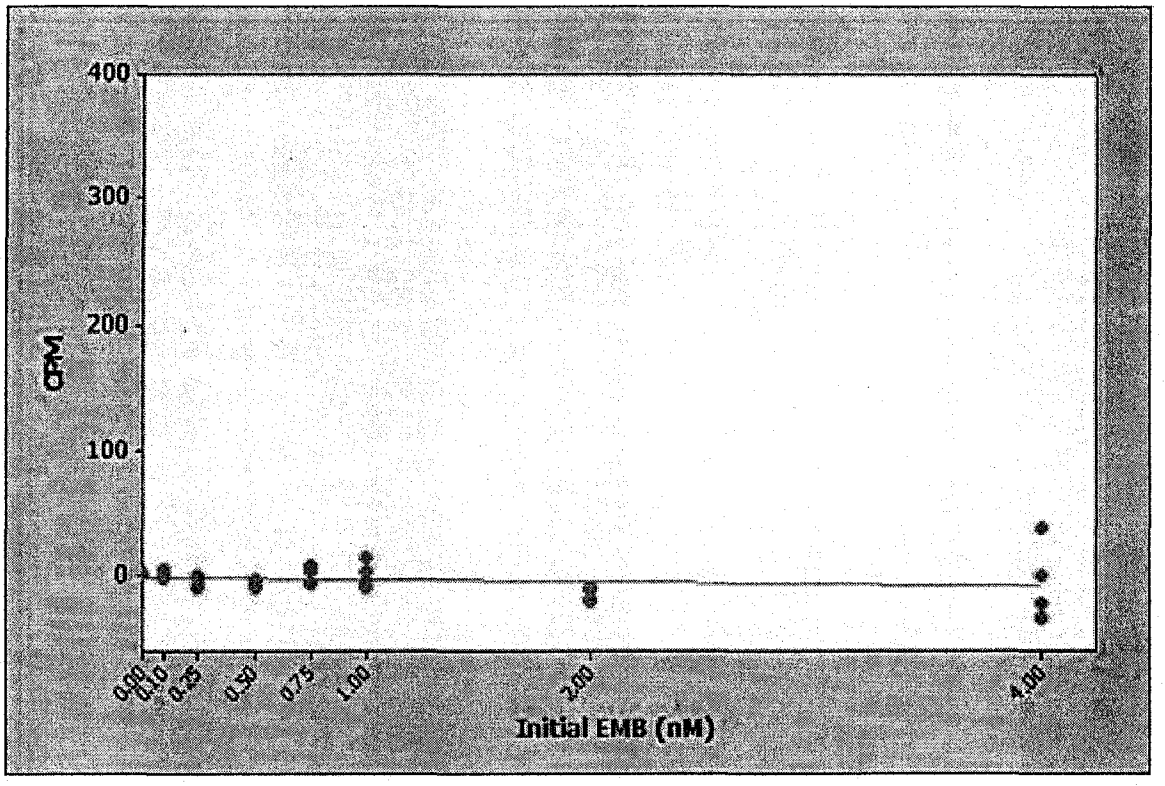

Figure 3.15. Specific binding of [^{3}H]EMB to membranes taken from transfected cells at various concentrations at 15°C read by a liquid scintillation counter (20 μg of membrane/sample, n=4).

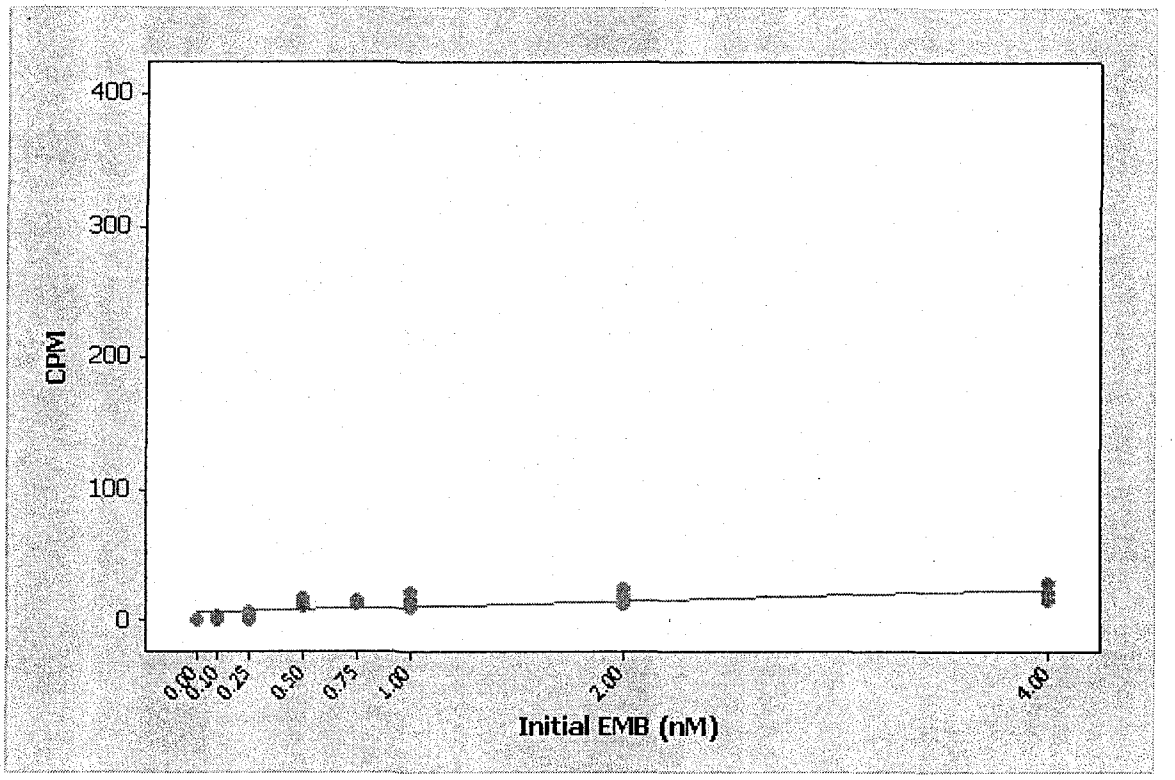

Figure 3.16. Specific binding of [^{3}H]EMB to membranes taken from non-transfected cells at various concentrations at 15°C read by a liquid scintillation counter (20 µg of membrane/sample, n=4).

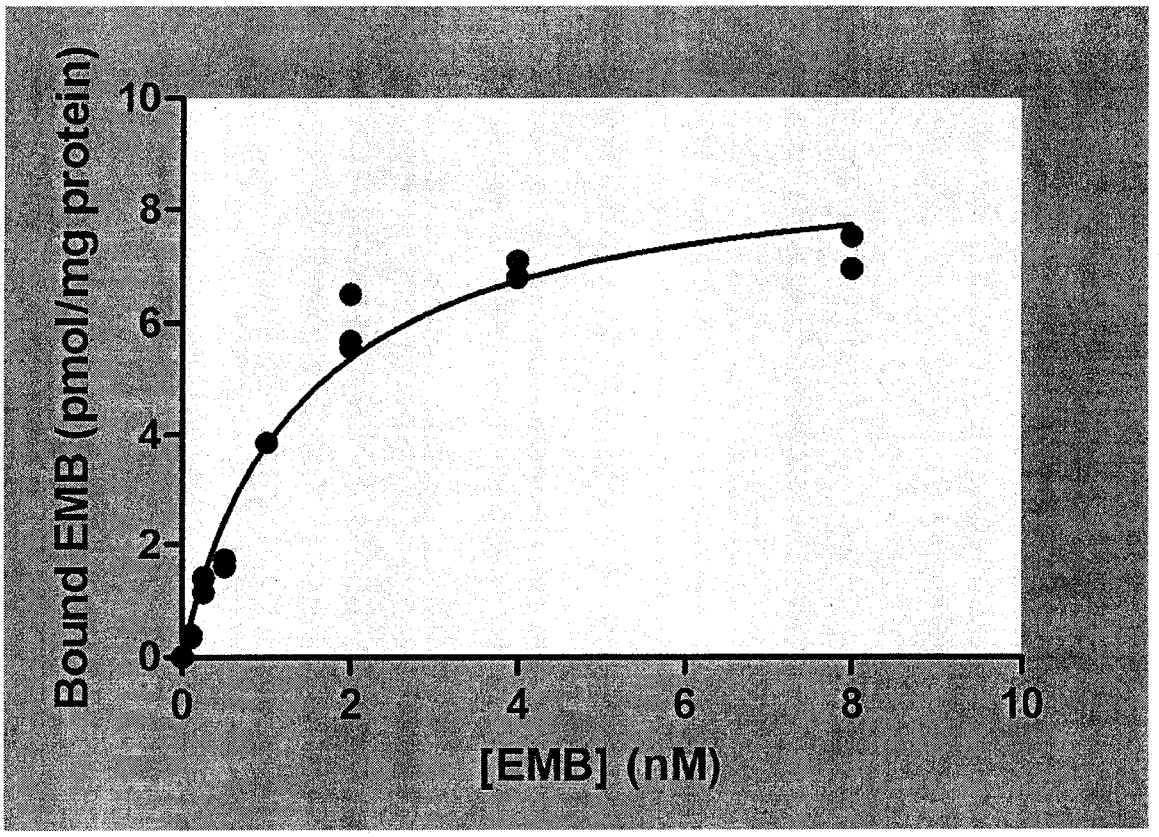

Figure 3.17. Binding of [^{3}H]EMB to membranes taken from sea lice at 15°C at various concentrations read by a liquid scintillation counter (20 µg of membrane/sample, n=4).

0.465 pmol/ mg of protein (R^2 = 97.7%, $p < 0.01$). At 20°C, the K_D was found to be 1.578 ± 0.145nM and the B_{MAX} was 8.588 ± 0.268 pmol/ mg of protein (Figure 3.18). At 5°C, the K_D was found to be 1.921 ± 0.715 nM and the B_{MAX} was found to be 9.642 ± 0.820 pmol/ mg of protein (Figure 3.19). All three temperatures expressed binding characteristics that were within standard error of each other, indicating that temperature change of this magnitude did not play a role in the affinity of the drug for the target.

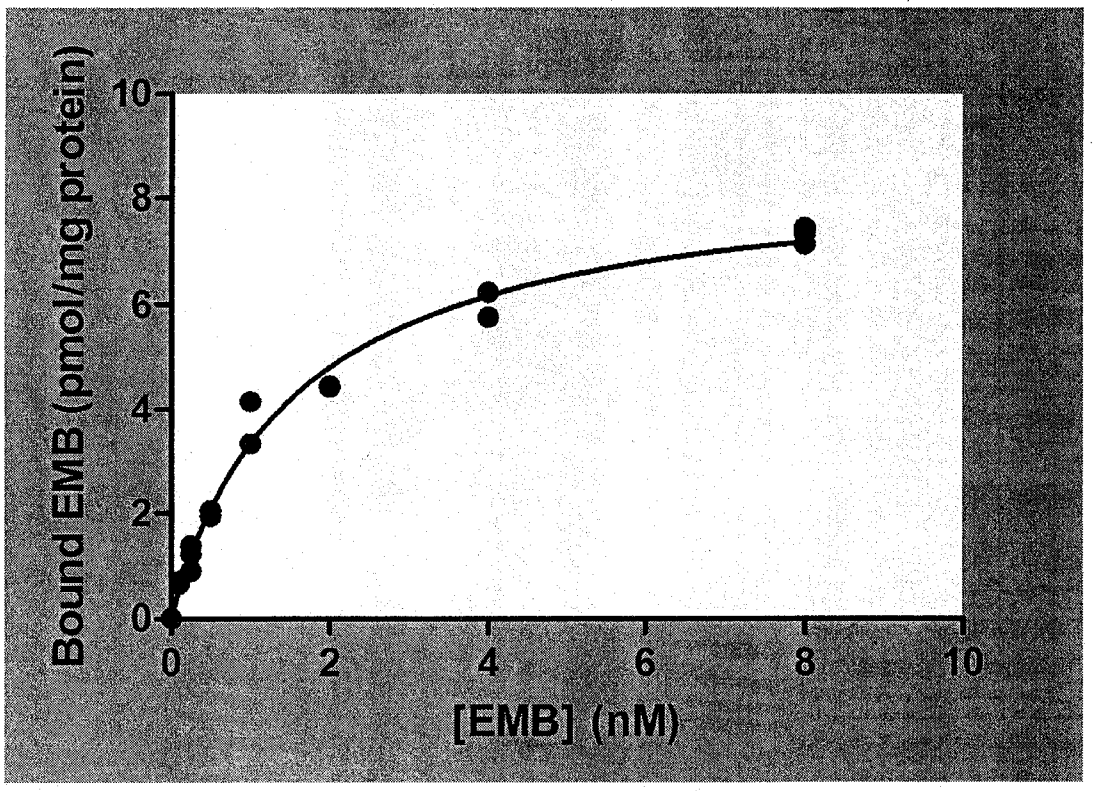

Figure 3.18. Binding of [^{3}H]EMB to membranes taken from sea lice at 20°C at various concentrations read by a liquid scintillation counter (20 μg of membrane/sample, n=3).

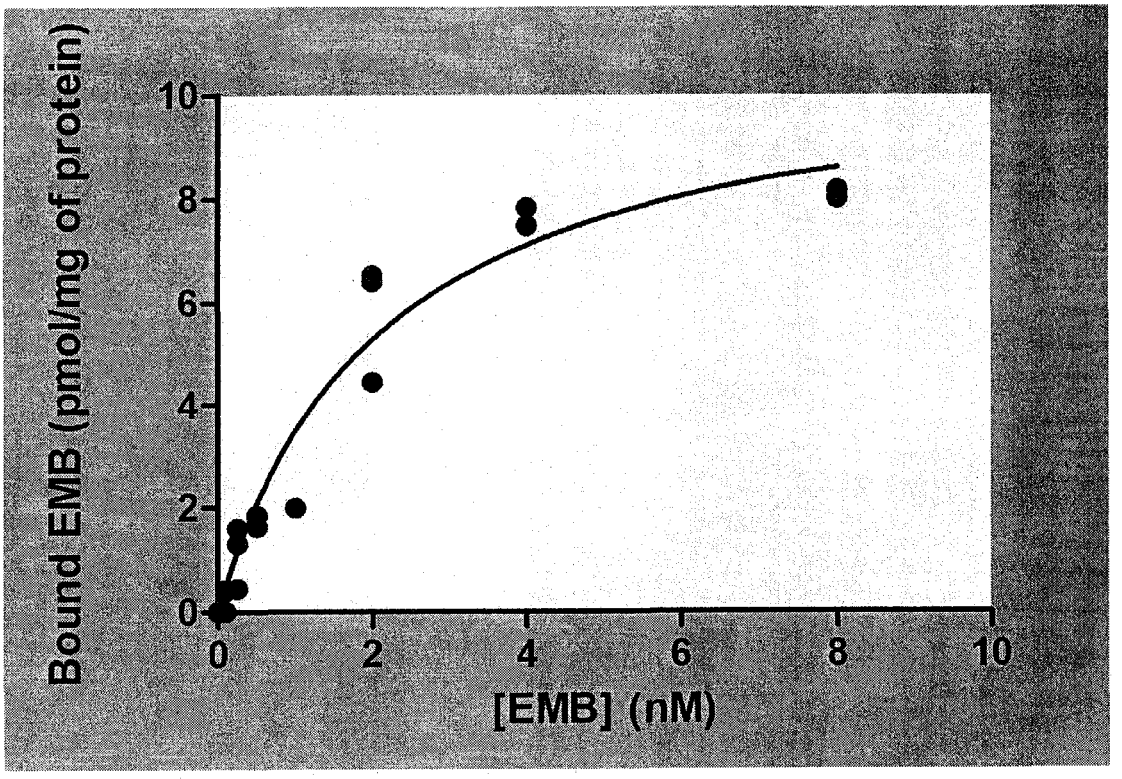

Figure 3.19. Binding of [^{3}H]EMB to membranes taken from sea lice at 5°C at various concentrations read by a liquid scintillation counter (20 μg of membrane/sample, n=3).

DISCUSSION

The glutamate-gated chloride channel is a major inhibitory ionotropic receptor located in nematodes and arthropods. This receptor is the primary target for the avermectins, which bind irreversibly to the receptor, thereby forcing it open. Currently, EMB is the most used drug in controlling sea lice infestations in salmonid aquaculture. While the efficacy of EMB against sea lice is known, no study has analyzed the receptor in detail.

The objective of this study was to investigate the binding characteristics of EMB to the GluClα subunit of *Lepeophtheirus salmonis*. Radio-ligand binding assays were used to determine the B_{MAX} and K_D of the drug in both membranes extracted from sea lice, as well as membranes of the cell line transfected with the putative GluClα gene. This study expands upon the work of Tribble *et al.* (2007a) who initially described a putative gene encoding for the GluClα subunit of *L. salmonis* as the potential target for the EMB mechanism of action. Improved knowledge of the interaction between EMB and the GluClα subunit will be useful in better understanding how the drug works in order to potentially improve therapeutic efficacy as well as developing a potential model of EMB-resistance development.

This study was intended to describe the interaction between EMB and the cellular membranes of *L. salmonis* in the context of ligand bound to a receptor. The results obtained were intended to provide a clear standard by which to judge the success of any developed model and provide a more detailed and precise characterization of the dynamics of the EMB-GluClα binding.

4.1. Binding Assays

The results of the saturation assay on the sea lice membranes suggest that the target for EMB is located on the cellular membrane. This corresponds with the known information about the action of avermectins in other organisms, as well as the proposed structure of the putative EMB target identified in Tribble *et al.* (2007a).

4.1.1. CHSE Cell Line

An aspect of this study was to isolate the putative gene for GluClα and to transfect in the CHSE-214 cell line so as to produce stable expression of the gene. The purpose of this was to provide confirmation that the gene did encode for the primary target for EMB, as well as to better characterize the interaction between EMB and the GluCl receptor with no uncontrolled factors. This required an isolation and purification of the gene encoding for GluClα, the development of a vector to be used in transfection, the transfection of a suitable cell line, and the detection and purification of membrane proteins to be used in the binding assay. If successful, this would provide a potential model in the affinity of EMB for GluCl that should be useful in identifying, or eliminating, the binding affinity as a possible avenue of resistance development. Unlike the membrane extracted from the lice, the concentration of receptor per mg of protein would have been higher in the cloned cell line, thereby reducing a major source of non-specific binding. This would have in turn reduced the variability, providing a much clearer result. This approach was modeled after the study on the GluClα subunit of *Haemonchus contortus* by Forrester *et al.* (2002).

Unfortunately, the results from the Western blotting indicated that there was no apparent expression of the GluClα subunit. The bands that were evident from the Western blot were also found in non-transfected cells and there were none at the expected molecular weight (~49 kDa). A binding assay was performed on the membrane extracted from the cell line confirmed a lack of specific binding. This was unexpected due to the expression of green fluorescence and the ability to grow in the presence of G418. PCR was performed on cDNA taken from the cell line in order to confirm the presence of the GluClα gene. The results showed that the supposed transcript for GluClα was present, indicating that the transfection was successful and stable. This leaves possible sources of error in either translation, resulting in no protein expression, or in post-translational processing, resulting in the protein being expressed in a non-functional state and not integrated into the membrane. However, it is worth noting that as there was no adequate positive control available that the product was actually present and the methods were incapable of detecting them.

Three possibilities for the lack of protein expression are either in the choice of the cell line, in the choice of the expression vector used, or that protein expression was present but too low to detect. Previous studies that have expressed GluClα in cell lines have used COS7 mammalian cells. CHSE-214 was chosen in this study as the incubation temperature of the cells more accurately represented the temperature at which the receptor would be exposed to in nature as an aquatic organism, rather than an intestinal parasite such as *H. contortus*. It is possible, however, that this cell type does not possess the ability to properly package or integrate the receptor into the membrane. It is also possible that the cells naturally process the mRNA to the point where it is not useful in translation. Spliced mRNA bands were present in the PCR product, however Forrester *et*

al. (2002) showed that truncated proteins also appeared with the full protein, indicating that this is not unusual. If potential splice sites disrupted expression, codon optimization of the gene may prevent this splicing from occurring. Due to the time and resource constraints of this study, COS7 was not used, however it may be beneficial to use in future attempts of cloning GluClα. The other possibility is a potential flaw in the vector design. The expression of green fluorescence protein and the resistance to G418 shows that genes from the vector were being expressed, pointing to a potential error in the gene insert. One possibility is that there may be a promoter sequence or motif necessary to facilitate expression of the insert that was not included, or that a frameshift mutation occurred during transfection resulting in a disruption of proper protein structure. The gene encoding for GFP itself may have been a contributing factor to the lack of protein expression, due to potential toxicity and disruption of normal cellular mechanisms as a result of the overexpression of GFP (Liu *et al.,* 1999). It is also possible that the protein was being expressed, but in concentrations too low to be detected either by Western blotting or the binding assay. This may still be tested using immunoprecipitation to increase the concentration of protein to detectable levels.

Further analysis on the transfected cell line may be needed in order to accurately determine the source of the error; a fresh cell line might be necessary. In order to better troubleshoot, a second attempt should probably use a cell line that has been used in previous studies involving GluCl.

4.1.2. Saturation Assays

This aspect of the study was designed to characterize the affinity of EMB to membranes extracted from sea lice. This provides a clearer picture of the action taken by

EMB against the lice, and presumably a more accurate depiction of real world usage than membranes taken from the cell line. While it is not as useful in terms of being a model of EMB-GluClα binding affinity, as there it cannot be shown that GluClα is the binding target, the results do provide a value which can be used to identify the range at which the affinity should be present in any potential models developed in the future.

The issue of the highly lipid-soluble nature of EMB became predominant during this aspect of the study. Due to logistical problems in extracting nervous tissue from the sea lice, whole cephalothoraxes were used. This resulted in a high lipid-receptor ratio in the binding assays and made it difficult to increase the concentration of protein to informative levels without increasing the levels of lipids to the point where there was high non-specific binding resulting in high variability within the results. In spite of these obstacles, however, the results of the assay do confirm the presence of a high affinity binding site for EMB in the cellular membranes of sea lice and that little to no binding occurs in the cytoplasm. This is in line with what has been previously shown in the interactions between avermectins and the receptors of other organisms.

However, the K_D observed in this study with EMB was calculated as being higher than for other avermectins studied using GluClα receptors taken from other organisms. Forrester *et al.* (2002) reported the K_D of ivermectin to the GluClα of *H. contortus* expressed in a cell line as being 0.11 nM. Other studies have reported the K_D to membranes taken directly from the *H. contortus* as ranging anywhere from 0.07 up to 0.6 nM (Rohrer *et al.,* 1994; Cheeseman *et al.*, 2001; Yates *et al.,* 2003). Schaeffer and Haines (1989) described the K_D of avermectins to the high affinity binding site of *C. elegans* as being 0.26 nM. Another study involving membranes extracted from the heads of *Drosophila* reported the K_D of ivermectin to be only 3 pM (Ludmerer *et al.,* 2002).

The results from this study suggest a lower affinity of EMB to the binding sites of *L. salmonis* due to a higher K_D than expected based upon previous literature. This may be due to the result of several factors. The first possibility is the physiochemistry of EMB results in a higher K_D than ivermectin, which was the drug tested in the previous literature. EMB has been shown to have a higher efficacy than ivermectin (Ramstad *et al.,* 2002), but this may be more in part due to improved ability to reach the target site, and a higher stability resulting in longer exposure times. The benzoate salt, while improving stability, may potentially have a negative impact on the overall affinity of the drug for the receptor.

Another possibility is the structure of the receptor itself. Tribble *et al.* (2007a) reported that the GluClα subunit of *L. salmonis* shows high genetic similarity to homologues from *Drosophila* (86 %), *H. contortus* (83 %), and *C. elegans* (82 %). It is possible that the differences in the structure of the receptor may affect the ability of EMB to bind due to conformational differences in the binding site. Further analysis of the similarities and differences in the EMB binding site may account for the higher K_D.

A third possibility may involve an unforeseen flaw in the study design. Ludmerer *et al.* (2002) used bacitracin in the assay binding buffer in order to prevent non-specific binding of ivermectin to the sides of the test tube that the membranes were incubated in. This study followed this protocol in an attempt to reduce non-specific binding, but had no mechanism by which to test the success of this strategy. Non-specific binding to the tube would result in a lower initial concentration of EMB in the solution, thereby resulting in a lower concentration of free EMB in the filtrate. In terms of affinity, the K_D would have been erroneously larger in the results proportional to the amount of EMB that bound non-specifically to the glass. As an example, if 50 % of the EMB had bound

to the sides of the tube and had been accounted for, the resulting calculations would have resulted in a K_D of approximately 0.75 nM at 15°C, which would be far more in line with previous literature. A binding assay where the filtrate was measured for yield quantity alongside the bound drug would test this possibility and potentially reduce variability, thereby giving more accurate results.

Finally, the high K_D may be in part due to the source material for the binding assays. In previous literature, assays were done on receptors isolated from cell lines or on nervous tissue extracted from the organism. The cell line assay was unsuccessful in this study and the difficulty involved in extracting nervous tissue resulted in whole cephalothoraxes being used. A previous study on *H. contortus* (Gill and Lacey, 1998) reported a second putative avermectin target with a K_D of 8.7 nM. The possibility of specific binding to lower-affinity but potentially more numerous receptors, such as GABA, may skew the results as having a higher K_D in the total organism than would have been for the GluClα alone. Therefore, the results obtained in this study may not be representative of the binding characteristics of EMB to GluClα, but more suggestive of the characteristics of EMB binding to the sea lice membrane as a whole. Typically, the results in this scenario would result in a saturation curve with a step-wise increase; however the low concentration of nervous tissue in the source for the membrane may prevent this from being detectable in the results. This was a known potential source of error in the design of this study, explaining why the results from the assay were to be compared to the results obtained from binding assay on a GluClα-expressing cell line.

As the binding assays performed on the cell line yielded no usable results, it is also possible that the binding detected in the sea lice membranes was not to the putative GluClα subunit previously isolated but some other protein expressed by a different gene

or other various constituents of the cellular membrane. Since EMB failed to bind to any protein that may or may not have been expressed by the gene the possibility must be considered that the gene may not express a functional protein, or that it is not actually the primary target of EMB in sea lice. Without binding to the protein in the cell line, it is impossible to determine whether or not that EMB binds to GluClα in sea lice at all, or if GluClα is even expressed.

Further exploration of developing a cell line-based model for EMB-GluClα binding would provide evidence towards whether or not the calculated K_D of this study is truly representative of the drug-receptor interactions and assist in explaining the differences in the results of this study and those in previous literature. Successfully demonstrating high affinity binding of EMB to the cell line will provide evidence of the putative GluClα acting as the primary target for EMB binding.

4.1.3. Temperature Assays

Westcott *et al.* (2008) suggested a possible seasonal or temperature variation associated with sea lice sensitivity to EMB. This portion of the study was designed to examine if this apparent trend was due to a potential difference in the level of binding of EMB to the membrane receptors. This was originally intended to have been done with the membranes taken from the cell line, but due to the lack of protein expression the membranes taken from the sea lice were used as a substitute. The affinity of EMB for the sea lice membranes was slightly lower at 20°C and 5°C than at the optimum temperature of 15°C, but well within the margin of error to suggest no significant trend. Interestingly enough, there was an apparent trend of increasing non-specific binding as the temperature decreased to the point where the results at 5°C had much more

variability than either 15°C or 20°C. This is possibly due to a lower solubility of the lipid membranes at lower temperatures, thereby decreasing the ability of EMB to dissociate from the membrane once bound, while having minimal effect on the drug-receptor binding characteristics.

This does not eliminate the possibility of the drug-receptor interactions being involved in temperature dependant sensitivity. This study focused on the effect of temperature on affinity; however temperature may also play a role in the rate at which steady-state binding is achieved. Further exploration into the role of temperature in EMB affinity should possibly examine the differences in binding rate at different temperatures, as lower temperatures have been known to reduce the time it takes for a drug-receptor complex to reach steady-state. This decreased rate coupled with increased potential for non-specific binding might result in decreased availability of the drug at the binding site level thereby explaining the lower sensitivity of the sea lice to EMB at lower temperatures.

4.1.4. Other Considerations

Several experiments could be performed to increase the strength of the data produced in this study. The second cell line should be established using either COS7 cells or the pCI-Neo vector shown to successfully express GluClα in order to assess the effectiveness of the methods used (Forrester *et al.*, 2002). Transfection protocols should be modified to allow for more passages in the presence of G418 to guarantee a stable transfection.

The results from the saturation assays could also be improved by reducing variability even further. The radioactivity of the glass tubes used for incubation and of

the filtrate should be measured in order to assess the amount of non-specific binding unaccounted for by the machine, and the total amount of drug available during incubation. This may reduce the K_D even further, possibly to a range that corresponds with the calculated affinity of avermectins to other species. In order to confirm that the drug is binding to a protein in the membrane, the membrane might be treated with a proteinase before a saturation assay is conducted to see if this reduces the amount of specific binding of EMB to the sea lice membrane.

To confirm the presence of the GluClα subunit in the sea lice membrane, an antibody should also be developed based upon the putative sequence obtained in Tribble *et al.* (2007a). This provide evidence in support of the GluClα subunit as the primary target of EMB in sea lice as well as provide a simpler means of detecting protein expression in the cell line without need of the FLAG sequence or anti-FLAG antibody.

These experiments were not done in this study due to time and resource constraints, but would provide a clearer picture and support of the ideas and data presented in this study.

4.2. Summary and Conclusion

This study is the first intended to look at the interaction between emamectin benzoate and the glutamate-gated chloride channels of *L. salmonis* on a molecular level. EMB is currently the primary chemotherapeutant used to control sea lice in salmon aquaculture and recent treatment failures have brought the potential for resistance development into consideration. The efficacy and selectivity of EMB continues to make it the desired drug of choice; this highlights the need for greater knowledge on the interaction between this drug and its target receptor. The GluCl receptor would act as a

useful target as it is unique to nematodes and arthropods and as the primary target it becomes a candidate as a potential mechanism of resistance development. Mutations in the putative GluCl gene may lower the affinity of EMB for the receptor, reducing binding and thereby conferring to the louse decreased sensitivity to the drug. In order to be able to detect this form of resistance, a model must be developed to act as a standard of non-resistant characteristics.

The GluClα receptor is an ionotropic receptor responsible for releasing Cl^- ions into neuronal cells to act as a neuronal inhibitor. Avermectins bind permanently to a site on this receptor, inducing a conformational change that causes a steady influx of Cl^- ions which leads to paralysis of the target organism. This study confirmed the presence of the EMB binding site in the membranes of *L. salmonis*, giving further evidence of a possible membrane receptor as the target for EMB based upon the sequence identified by Tribble *et al.* (2007a). Binding was observed to have a lower affinity for membranes of *L. salmonis* than other avermectins in studies involving the GluCl of other organisms. Temperature was shown to have minimal effect on the ability for EMB to bind to the membrane, but did not eliminate the possibility of a temperature-related effect on the overall efficacy of the drug.

Future studies involving the binding characteristics of EMB and GluClα should focus on isolating the gene and expressing the receptor protein in a controlled environment to further characterize the interaction. This could be used as a model and the results could be compared with receptors taken from potentially EMB-resistant *L. salmonis* in order to determine if a change in drug affinity for the receptor is a factor in resistance development. The cause of resistance will affect future development of

chemotherapeutant control of lice due to the potential loss of a therapeutic target and the need for either a new formulation or a drug with a different mechanism of action. The occurrence of treatment failures in Scotland and Chile highlight the need for this research to continue in order to further understand the mechanics of resistance development as well as provide a basis for determining the future of EMB usage as a sea lice control in salmon aquaculture.

4. REFERENCES

Banks B. J., Bishop B. F., Evans N. A., Gibson S. P., Goudie A. C., Gration K. A., Pacey M. S., Perry D. A., and Witty M. J. 2000. Avermectins and flea control: structure-activity relationships and the selection of selamectin for development as an endectocide for companion animals. Bioorg Med Chem 8; 2017-2025.

Barnes T. M., and Hekimi S. 1997. The *Caenorhabditis elegans* avermectin resistance and anesthetic response gene unc-9 encodes a member of a protein family implicated in electrical coupling of excitable cells. J Neurochem 69; 2251-2260.

Blackhall W. J., Prichard R. K., and Beech R. N. 2003. Selection at a γ-aminobutyric acid receptor gene in *Haemonchus contortus* resistant to avermectins/milbemycins. Mol Biochem Parasitol 131; 137–145.

Blackhall W. J., Pouliot J. F., Prichard R. K., and Beech R. N. 1998. *Haemonchus contortus*: selection at a glutamate-gated chloride channel gene in ivermectin- and moxidectin-selected strains. Exp Parasitol 90; 42-48.

Blagburn B. L., and Lindsay D. S. 1995. Ectoparasiticides. In Veterinary Pharmacology and Therapeutics, 7th edn. Ed. Adams, H.R. pp. 984 - 1003. Iowa State University Press, Ames, IA.

Bloomquist J. R. 2003. Chloride channels as tools for developing selective insecticides. Arch Insect Biochem Physiol 54; 145-156.

Boileau A. J., Evers A. R., Davis A. F., and Czajkowski C. 1999. Mapping the agonist binding site of the $GABA_A$ receptor: evidence for a β-strand. J Neurosci 19; 4847-4854.

Bjordal A. 1991. Wrasse as cleaner-fish for farmed salmon. Progr Underwat Sci 16; 17-28.

Burka J. F., Hammell K. L., Horsberg T. E., Johnson G. R., Rainnie D. J., and Speare D. J. 1997. Drugs in salmonid aquaculture - A review. J Vet Pharmacol Ther 20; 333-349.

Burridge L. E., Hamilton N., Waddy S. L., Haya K., Mercer S. M., Greenhalgh R., Tauber R., Radecki S. V. , Crouch L. S., Wislocki P. G., and Endris R. G. 2004. Acute toxicity of emamection benzoate (SLICE™) in fish feed to American lobster, *Homarus americanus*. Aqua Res 35; 713-722.

Cheeseman C. L., Delany N. S., Woods D. J., and Wolstenholme A. J. 2001. High-affinity ivermectin binding to recombinant subunits of the *Haemonchus contortus* glutamate-gated chloride channel. Mol Biochem Parasitol 114; 161-168.

Cully D. F., Paress P. S., Liu K. K., Schaeffer J. M., and Arena J. P. 1996. Identification of a *Drosophila melanogaster* glutamate-gated chloride channel sensitive to the antiparasitic agent avermectin. J Biol Chem 271; 20187-20191.

Cully D. F., Vassilatis D. K., Liu K. K., Paress P. S., Van der Ploeg L. H. T., Schaeffer J. M., and Arena J. P. 1994. Cloning of an avermectin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nature 371; 707-711.

Dent J. A., Smith M. M., Vassilatis D. K., and Avery L. 2000. The genetics of ivermectin resistance in *Caenorhabditis elegans*. Proc Natl Acad Sci USA 97; 2674-2679.

Egebjerg, J. 2003. The structure of ligand-gated ion channels. Textbook of Receptor Pharmacology 2nd ed. J. C. Foreman and T. Johansen. CRC Press. 111-130.

Fallang A., Denholm I., Horsberg T. E., and Williamson M. S. 2005. Novel point mutation in the sodium channel gene of pyrethroid-resistant sea lice *Lepeophtheirus salmonis* (Crustacea: Copepoda). Dis Aquat Org 65; 129-136.

Fallang A., Ramsay J. M., Sevatdal S., Burka J. F., Jewess P., Hammell K. L., and Horsberg T. E. 2004. Evidence for occurrence of an organophosphate-resistant type of acetylcholinesterase in strains of sea lice (*Lepeophtheirus salmonis* Krøyer). Pest Manag Sci 60; 1163-1170.

Fast M. D., Burka J. F., Johnson S. C., and Ross N. W. 2003. Enzymes released from *Lepeophtheirus salmonis* in response to mucus from different salmonids. J Parasitol 89; 7-13.

Feng X. P., Hayashi J., Beech R. N., and Prichard R. K. 2002. Study of the nematode putative GABA type-A receptor subunits: evidence for modulation by ivermectin. J Neurochem 83; 870-878.

Forrester S. G., Beech R. N., and Prichard R. K. 2004. Agonist enhancement of macrocyclic lactone activity at a glutamate-gated chloride channel subunit from *Haemonchus contortus*. Biochem Pharmacol 67; 1019-1024.

Forrester S. G., Prichard R. K., and Beech R. N. 2002. A glutamate-gated chloride channel subunit from *Haemonchus contortus*: expression in a mammalian cell line, ligand binding, and modulation of anthelmintic binding by glutamate. Biochem Pharmacol 63; 1061-1068.

Forrester S. G., Prichard R. K., Dent J. A., and Beech R. N. 2003. *Haemonchus contortus*: HcGluCla expressed in *Xenopus* oocytes forms a glutamate-gated ion channel that is activated by ibotenate and the antiparasitic drug ivermectin. Mol Biochem Parasitol 129; 115-121.

Georgiev P. G., Wolstenholme A. J., Pak W. L., and Semenov E. P. 2002. Differential responses to avermectins in ort mutants of *Drosophila melanogaster*. Pest Biochem Phys 72; 65-71.

Gill J. H., and Lacey E. 1998. Avermectin/milbemycin resistance in trichostrongyloid nematodes. Int J Parasitol 28; 863-877.

Glover K. A., Storset A., Nilsen F., and Skalla Ø. 2005. Sea lice – can we breed "resistant" salmon? Marine Research News. Havforskningsninstitutttet. NR. 8.

Gonzalez-Alanis P., Wright G. M., Johnson S. C., and Burka J. F. 2001. Frontal filament morphogenesis in the salmon louse *Lepeophtheirus salmonis*. J. Parasitol 87; 561-574.

Grant A. N. 2002. Medicines for sea lice. Pest Manag Sci 58; 521-527.

Grayson T. H., John R. J., Wadsworth S., Greaves K., Cox D., Roper J., Wrathmell A. B., Gilpin M. L., and Harris J. E. 1995. Immunization of Atlantic salmon against the salmon louse: identification of antigens and effects an louse fecundity. J Fish Biol 47S; 85-94.

Grontvedt O. M. 1997. Oral delousing – the effect of teflubenzuron on the shell of the salmon louse. Norsk Fiskeoppdrett. N R. 34-37.

Henderson J. E., Knipple D. C., and Soderlund D. M. 1994. PCR-based homology probing reveals a family of GABA receptor-like genes in *Drosophila melanogaster*. Insect Biochem Mol Biol 24; 363-371.

Hevrøy E. M., Boxaspen K., Oppedal F., Taranger G. L., and Holm J. C. 2003. The effect of artificial light treatment and depth on the infestation of the sea lice *Lepeophtheirus salmonis* on Atlantic salmon (*Salmo salar* L.) culture. Aquaculture 220; 1-14.

Hogans W. E. 1995. Infection dynamics of sea lice, *Lepeophtheirus salmonis* (Copepoda: Caligidae) parasitic on Atlantic Salmon (*Salmo salar*) cultured in marine water of the lower Bay of Fundy. Can Tech Rep Fish Aquat Sci 2067; 10.

Hotson I. K. 1982. The avermectins - a new family of antiparasitic agents. J S Afr Vet Assoc 53; 87-90.

Høy T., Horsberg E., and Nafstad I. 1990. The disposition of ivermectin in Atlantic salmon (*Salmo salar*). Pharmacol Toxicol 67; 307-312.

Hull M. Q., Pike A. W., Mordue A. J., and Rae G. H. 1998. Patterns of pair formation and mating in an ectoparasitic caligid copepod *Lepeophtheirus salmonis* (Krøyer 1837): implications for its sensory and mating biology. Philos T Roy Soc B 353; 753-764.

Jagannathan S., Laughton D. L., Critten C. L., Skinner T. M., Horoszok L., and Wolstenholme A. J. 1999. Ligand-gated chloride channel subunits encoded by the *Haemonchus contortus* and *Ascaris suum* orthologues of the *Caenorhabditis elegans gbr*-2 (*avr*-14) gene. Mol Biochem Parasitol 103; 129-140.

Johannessen A. 1978. Early stages of *Lepeophtheirus salmonis* (Copepoda, Caligidae). Sarsia 63; 169-176.

Johnson S. C., and Albright L. J. 1991. Development, growth, and survival of *Lepeophtheirus salmonis* (Copepoda, Caligidae) under laboratory conditions. J Mar Biol Assoc UK 71; 425-436.

Kane N. S., Hirschberg B., Qian S., Hunt D., Thomas B., Brochu R., Ludmerer S. W., Zheng Y., Smith M., Arena J. P., Cohen C. J., Schmatz D., Warmke J., and Cully D. F. 2000. Drug-resistant *Drosophila* indicate glutamate-gated chloride channels are targets for the antiparasitics nodulisporic acid and ivermectin. Proc Natl Acad Sci USA 97; 13949-13954.

Kim-Kang H., Bova A., Crouch L. S., Wislocki P. G., Robinson R. A., and Wu J. 2004. Tissue distribution, metabolism, and residue depletion study in Atlantic salmon following oral administration of [^{3}H]emamectin benzoate. J Agric Food Chem 52; 2108-2118.

Kvamme B. O., Skern R., Frost P., and Nilsen F. 2004. Molecular characterisation of five trypsin-like peptidase transcripts from the salmon louse (*Lepeophtheirus salmonis*) intestine. Int J Parasitol 34; 823-832.

Laughton D. L., Wheeler S. V., Lunt G. G., and Wolstenholme A. J. 1995. The β-subunit of *Caenorhabditis elegans* avermectin receptor responds to glycine and is encoded by chromosome 1. J Neurochem 64; 2354-2357.

Laughton D. L., Lunt G. G., and Wolstenholme A. J. 1997. Reporter gene constructs suggest that the *Caenorhabditis elegans* avermectin receptor β-subunit is expressed solely in the pharynx. J Exp Bio 200; 1509-1514.

Lees F., Baillie M., Gettinby G., and Revie C. W. 2008. The efficacy of emamectin benzoate against infestations of *Lepeophtheirus salmonis* on farmed Atlantic salmon (*Salmo salar* L) in Scotland, 2002-2006. PLoS ONE 3; 1-11 (2).

Liu H. S., Jan M. S., Chou C. K., Chen P. H., and Ke N. J. 1999. Is green fluorescent protein toxic to the living cells? Biochem Biophys Res Commun 260; 712-717.

Liu J., Dent J. A., Beech R. N., and Prichard R. K. 2004. Genomic organization of an avermectin receptor subunit from *Haemonchus contortus* and expression of its putative promoter region in *Caenorhabditis elegans*. Mol Biochem Parasitol 134; 267-274.

Ludmerer S. W., Warren V. A., Williams B. S., Zheng Y., Hunt D. C., Ayer M. B., Wallace M. A., Chaudhary A. G., Egan M. A., Meinke P. T., Dean D. C., Garcia M. L., Cully D. F., and Smith M. M. 2002. Ivermectin and nodulisporic acid receptors in *Drosophila melanogaster* contain both γ-aminobutyric acid-gated Rdl and glutamate-gated GluClα chloride channel subunits. Biochemistry 41; 6548-6560.

MacKinnon B. M. (1997) Sea lice: A review. World Aquacult 28; 5-10.

Narahashi T. 1971. Mode of action of pyrethroids. Bull World Health Organ 44; 337-345.

Njue A. I., Hayashi J., Kinne L., Feng X. P., and Prichard R. K. 2004. Mutations in the extracellular domains of glutamate-gated chloride channel α3 and β subunits from ivermectin-resistant *Cooperia oncophora* affect agonist sensitivity. J Neurochem 89; 1137-1147.

Njue A. I., and Prichard R. K. 2004. Genetic variability of glutamate-gated chloride channel genes in ivermectin-susceptible and -resistant strains of *Cooperia oncophora.* Parasitology 129; 741-751.

Ozoe Y., Akamatsu M., Higata T., Ikeda I., Mochida K., Koike K., Ohmoto T., and Nikaido T. 1998. Picrodendrin and related terpenoid antagonists reveal structural differences between ionotropic GABA receptors of mammals and insects. Bioorg Med Chem; 481-492.

Paiement J. P., Leger C., Ribeiro P., and Prichard R. K. 1999. *Haemonchus contortus*: effects of glutamate, ivermectin, and moxidectin on inulin uptake activity in unselected and ivermectin-selected adults. Exp Parasitol 92; 193-198.

Pemberton D. J., Franks C. J., Walker R. J., and Holden-Dye L. 2001. Characterization of glutamate-gated chloride channels in the pharynx of wild-type and mutant *Caenorhabditis elegans* delineates the role of the subunit GluCl-α2 in the function of the native receptor. Mol Pharmacol 59; 1037-1043.

Pike A. W., and Wadsworth S. L. 1999. Sealice on salmonids: their biology and control. Adv Parasitol 44; 233-337.

Pomes A., Kempner E., and Rohrer S. 1997. Target size analysis of an avermectin binding site from *Drosophila melanogaster*. BBA 1339; 233-238.

Ramstad A., Colquhoun D. J., Nordmo R., Sutherland I. H., and Simmons R. 2002. Field trials in Norway with SLICE® (0.2% emamectin benzoate) for the oral treatment of sea lice infestation in farmed Atlantic salmon *Salmo salar*. Dis Aquat Orgs 50; 29-33.

Raynard R. S., Bricknell I. R., Billingsley P. F., Nisbet A. J., Vigneau A., and Sommerville C. 2002. Development of vaccines against sea lice. Pest Manag Sci 58; 569-575.

Ritchie G. 2006. The host transfer ability of *Lepeophtheirus salmonis* (Copepoda, Caligidae) from farmed Atlantic salmon, *Salmo salar*. L. J Fish Dis 20; 153-157.

Rolland J. B., and Nylund A. 1998. Infectiousness of organic materials originating in ISA-infected fish and transmission of the disease via salmon lice (Lepeophtheirus salmonis). B Eur Assoc Fish Pat 18; 173.

Rohrer S. P., Jacobson E. B., Hayes E. C., Birzin E. T., and Schaeffer J. M. 1994. Immunoaffinity purification of avermectin-binding proteins from the free-living nematode *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster*. Biochem J 302; 339-345.

Roy W. J., Sutherland I. H., Rodger H. D., and Varma K. J. 2000. Tolerance of Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), to emamectin benzoate, a new orally administered treatment for sea lice. Aquaculture 184; 19-29.

Schaeffer J. M. and Haines H. W. 1989. Avermectin binding in *Caenorhabditis elegans*: A two state model for the avermectin binding site. Biochem Pharmacol 38; 2329-2338.

Schram T. A. (1994) Supplemental descriptions of the developmental stages of *Lepeophtheirus salmonis* (Krøyer, 1837) (Copepoda: Caligidae). In: Boxshall GA, Defaye D. Pathogens of wild and farmed fish: Sea lice. Ellis Harwood, Aberdeen; 30-80.

Sevatdal S., Magnusson A., Ingebrigtsen K., Haldorsen R., and Horsberg T. E. 2005. Distribution of emamection benzoate in Atlantic salmon (*Salmo salar* L.). J Vet Pharmacol Ther 28; 101-107.

Stephenson F. A. 1995. The $GABA_A$ receptors. Biochem J 310; 1-9.

Stone J., Roy W. J., Sutherland I. H., Ferguson H. W., Sommerville C., and Endris R. 2002. Safety and efficacy of emamectin benzoate administered in-feed to Atlantic salmon, *Salmo salar* L., smolts in freshwater, as a preventative treatment against infestations of sea lice, *Lepeophtheirus salmonis* (Krøyer). Aquaculture 210; 21-34.

Stone J., Sutherland I. H., Sommerville C., Richards R. H., and Varma K. J. 2000. Field trials to evaluate the efficacy of emamectin benzoate in the control of sea lice, *Lepeophtheirus salmonis* (Krøyer) and *Caligus elongatus* (Nordmann), infestations in Atlantic salmon *Salmo salar* L. Aquaculture 186; 205-219.

Stone J., Sutherland I. H., Sommerville C., Richards R. H., and Varma K. J. 1999. The efficacy of emamectin benzoate as an oral treatment of sea lice, *Lepeophtheirus salmonis* (Krøyer), infestations of Atlantic salmon, *Salmo salar* L. J Fish Dis 22; 261-270.

Treasurer J. W. 1994. Management of sea lice (Caligidae) with wrasse (Labridae) on Atlantic salmon (*Salmo salar* L.) farms. In: Boxshall GA, Defaye D. Pathogens of wild and farmed fish: Sea lice. Ellis Horwood, Chichester, UK; 335-345.

Treasurer J. W. 2002. A review of potential pathogens of sea lice and the application of cleaner fish in biological control. Pest Manag Sci 58; 546-558.

Tribble N. D. 2007. An investigation into potential emamectin benzoate resistance mechanisms in sea lice, *Lepeophtheirus salmonis*. Ph. D. Thesis, UPEI, Charlottetown, PEI.

Tribble N. D., Burka J. F., and Kibenge F. S. B. 2007a. 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*). J Vet Pharmacol Therap 30; 163-167.

Tribble N. D., Burka J. F., and Kibenge F. S. B. 2007b. Evidence for changes in the transcription levels of two putative P-glycoprotein genes in sea lice (*Leopeophtheirus salmonis*) in response to emamectin benzoate exposure. Mol Biochem Parasitol 153; 59-65.

Tully O. 1989. The succession of generations and growth of the caligid copepod *Caligus elongatus* and *Lepeophtheirus salmonis* parasitising farmed Atlantic salmon smolts (*Salmo salar* L.). J Mar Biol Assoc UK 69; 279-287.

Vassilatis D. K., Elliston K. O., Paress P. S., Hamelin M., Arena J. P., Schaeffer J. M., Van der Ploeg L. H. T., and Cully D. F. 1997a. Evolutionary relationship of the ligand-gated ion channels and the avermectin-sensitive, glutamate-gated chloride channels. J Mol Evol 44; 501-508.

Vassilatis D. L., Arena J. P., Plasterk R. H., Wilkinson H. A., Schaeffer J. M., Cully D. F., and Van der Ploeg L. H. T. 1997b. Genetic and biochemical evidence for a novel avermectin-sensitive chloride channel in *Caenorhabditis elegans*. J Biol Chem 272; 33167-33174.

Westcott J. D., Hammell K. L., and Burka J. F. 2004. Sea lice treatments, management practices, and sea lice sampling methods on Atlantic salmon farms in the Bay of Fundy, New Brunswick, Canada. Aquacul Res 35; 784-492.

Westcott J. D., Stryhn H., Burka J. F., and Hammell K. L. 2008. Optimization and field use of a bioassay to monitor sea lice *Lepeophtheirus salmonis* sensitivity to emamectin benzoate. Dis Aquat Org 79; 119-131.

Wolstenholme A. J., and Rogers A. R. 2005. Glutamate-gated chloride channels and the mode of action of the avermectin / milbemycin anthelmintics. Parasitol 131; S85-S95.

Wolstenholme A. J., Fairweather I., Prichard R., von Samson-Himmelstjerna G., and Sangster N. C. 2004. Drug resistance in veterinary helminths. Trends Parasitol 20; 469-476.

Wootten R., Smith J. W., and Needham E. A. 1982. Aspects of the biology of the parasitic copepods *Lepeophtheirus salmonis* and *Caligus elongatus* on farmed salmonids and their treatment. Proc R Soc Edin B 81; 185-197.

Yates D. M., Portillo V., and Wolstenholme A. J. 2003. The avermectin receptors of *Haemonchus contortus* and *Caenorhabditis elegans*. Int J Parasit 33; 1183-1193.

Zeng Z., Andrew N. W., Woda J. M., Halley B. A., Crouch L. S., and Wang R. W. 1996. Role of cytochrome P450 isoforms in the metabolism of abamectin and ivermectin in rats. J Agri Food Chem 44; 3374-3378.

APPENDIX A.

BLAST results of Gene Sequence in Vector vs DQ916037 comparing the vector sequence used in transfection to GluClα sequence cited in Tribble *et al.* (2007a).

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Query  75    CTTTTTCTTCTCGTGTATTTTAACTCAACAGCCCTCCAAAATAGACGAGATAAGATAGAA  134
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     CTTTTTCTTCTCGTGTATTTTAACTCAACAGCCCTCCAAAATAGACGAGATAAGATAGAT  60

Query  135   TATCGTTTGAAGGAAAAACAAATCCTAGACCATGTTTTAGGGCCACTCCGATATGACAAA  194
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    TATCGTTTGAAGGAAAAACAAATCCTAGACCATGTTTTAGGGCCACTCCGATATGACAAA  120

Query  195   AGGATTCGACCACCTGGATCCGGCAATTTGACAGGCCCATCTCCTACAGTTGTCTCAATA  254
             |||||||||||||||||||| |||||||||||||||||||||||||||||||| |||||
Sbjct  121   AGGATTCGACCACCTGGATCCAGCAATTTGACAGGCCCATCTCCTACAGTTGTCACAATA  180

Query  255   AACACGTATCTCCGTGCTATAGATCGCATAGATGATTATAAAATGGAGTACAGTGTACAA  314
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   AACACGTATCTCCGTGCTATAGATCGCATAGATGATTATAAAATGGAGTACAGTGTACAA  240

Query  315   TTAACATTCAGAGAAAATTGGATGGATTCGCGTTTAATGTTTAACGATTTGAACGGTAAA  374
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   TTAACATTCAGAGAAAATTGGATGGATTCGCGTTTAATGTTTAACGATTTGAACGGTAAA  300

Query  375   ATAAAATACTTAACATTGACGGATGCTGAAAAAGTTTGGATGCCTGACACttttttCAA  434
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   ATAAAATACTTAACATTGACGGATGCTGAAAAAGTTTGGATGCCTGACACTTTTTTTCAA  360

Query  435   AATGAGAAACTTGGACATTTCCATAACATTATTGTACCCAATGTTTATGTCCGAATATTT  494
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   AATGAGAAACTTGGACATTTCCATAACATTATTGTACCCAATGTTTATGTCCGAATATTT  420

Query  495   CCAACAGGAAGTGTTTTATATAGTATAAGGATTTCCTTGACATTAGCTTGTCCAATGGAT  554
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   CCAACAGGAAGTGTTTTATATAGTATAAGGATTTCCTTGACATTAGCTTGTCCAATGGAT  480

Query  555   CTGAAGCTATATCCTTTAGATCGTCAAGTCTGTGAGATGAGAATAGCTAGTTATGGATGG  614
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   CTGAAGCTATATCCTTTAGATCGTCAAGTCTGTGAGATGAGAATAGCTAGTTATGGATGG  540

Query  615   ACAACGGATGACTTAGTCTATAGATGGAAAAGCAAGGATCCAGTGCAATTCGTTCAGGAT  674
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   ACAACGGATGACTTAATCTATAGATGGAAAAGCAAGGATCCAGTGCAATTCGTTCAGGAT  600

Query  675   TTAAATCTTCCTCGATTCAAACTCGAGAGCTTTAGTACATCGTATTGTAATTCAAAAACT  734
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   TTAAATCTTCCTCGATTCAAACTCGAGAGCTTTAGTACATCGTATTGTAATTCAAAAACT  660

Query  735   AACACAGGGGAGTACAGCTGTTTGAAAATCAATCTAGTTTTCAAAAGAGAATTTTCCTAC  794
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   AACACAGGGGAGTACAGCTGTTTGAAAATCAATCTAGTTTTCAAAAGAGAATTTTCCTAC  720

Query  795   TATCTACTGACCATTTATGTACCTTCCTGTATGCTTGTTATCATTTCATGGGTGAGTTTC  854
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   TATCTACTGACCATTTATGTACCTTCCTGTATGCTTGTTATCATTTCATGGGTGAGTTTC  780

Query  855   TGGTTAGACTCGAAATCGGTTCCTGCACGGGTAGCTTTAGGTGTAACAACACTTCTTACT  914
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   TGGTTAGACTCGAAATCGGTTCCTGCACGGGTAGCTTTAGGTGTAACAACACTTCTTACT  840
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Query  915   ATGTCTACCCAAACAGCTGGTGTAAATAGATCACTACCTCCAGTAGCTTACACAAAAGCA  974
             ||||||||  ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   ATGTCTACTCAAACAGCTGGTGTAAATAGATCACTACCTCCAGTAGCTTACACAAAAGCA  900

Query  975   ATAGATGTGTGGAGT-GGTGCATGTGTTATTTTTGTATTTAGTGCCTTATTAGAATTTGC  1033
             |||||  ||  ||||  |  ||  ||||||||||||||||||||||||||||||||||||
Sbjct  901   ATAGACGTATGGA-TCGGCGCATGTGTTATTTTTGTATTTAGTGCCTTATTAGAATTTGC  959

Query  1034  ATTTGTTAACTATGCTTCACGACATGATCGAAGAAAGGGAAGGAAATCTAGATCTGCAAT  1093
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  960   ATTTGTTAACTATGCTTCACGACATGATCGAAGAAAGGGAAGGAAATCTAGATCTGCAAT  1019

Query  1094  GAATTATAACATGGATGACGATGAAATAGATTATGATCAAGGTCTTGATTGCACCTCACG  1153
             |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  1020  GAATTATAACATGGATGACGATGAAATAGATTATGATCAAGGTCTTGATTGCACTTCACG  1079

Query  1154  AATTCGTTCTCATTATGGAAAACGAGGATCCTTTAACTACGGAGAGAACGGTAATATCCT  1213
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1080  AATTCGTTCTCATTATGGAAAACGAGGATCCTTTAACTACGGAGAGAACGGTAATATCCT  1139

Query  1214  TCTGAGCTATATTGCTGGAGAGGGCGTGACGACGACTaaaaaaa-GTCCTGGCTTGCTGA  1272
             |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  1140  TCTGAGCTATATTGCTGGAGAGGGCGTGACGACGACTAAAAAAAAGTCCTGGCTTGCTGA  1199

Query  1273  AAGATTTCCAAGGAGATCAAAAAGAATTGATGTAGTAGCTCGTATTTTATTTCCGGGCAT  1332
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1200  AAGATTTCCAAGGAGATCAAAAAGAATTGATGTAGTAGCTCGTATTTTATTTCCGGGCAT  1259

Query  1333  ATTTGCCATCTTTAATTTTAGCTATTGGTTGTATTATTTGTCCGCAGAACATGAATCAAG  1392
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1260  ATTTGCCATCTTTAATTTTAGCTATTGGTTGTATTATTTGTCCGCAGAACATGAATCAAG  1319

Query  1393  GATTTACAAA  1402
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Sbjct  1320  GATTTACAAA  1329
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APPENDIX B.

Chemical composition of buffers and solutions used.

RT Buffer (Invitrogen) - 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$]

Binding buffer (Roche) - 3 M guanidine-thiocyanate, 10 mM Tris-HCl, 5% ethanol , pH 6.6

Washing buffer (Roche) - 20 mM NaCl, 2 mM Tris-HCl, pH 7.5

Tango Buffer (Fermentas) - 33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA

Ligase Reaction Buffer (Invitrogen) - 250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000

Resuspension solution (Sigma) - Cat. R1149 - Proprietary

Lysis solution (Sigma) - Cat. L1912 - Proprietary

Neutralization/Binding solution (Sigma) - Cat. N5158 - Proprietary

Column Preparation solution (Sigma) - Cat. C2112 - Proprietary

Wash Solution (Sigma) - Cat. W3886 - Proprietary