

THE POTENTIAL ROLE OF ABC TRANSPORTERS
AS FACTORS INFLUENCING DRUG
SUSCEPTIBILITY IN THE SALMON LOUSE,
LEPEOPHTHEIRUS SALMONIS (KRØYER, 1837)

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DECLARATION

This thesis is the result of my own work, except where specifically indicated in the text. It has not been previously submitted, in part or completely, to any university or institution in order to obtain any other qualification.

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ABSTRACT

Efficient control of sea lice is a major challenge for the sustainable production of farmed Atlantic salmon (*Salmo salar* (Linnaeus, 1758)). These marine ectoparasites feed on mucus, skin and blood of their hosts, thereby reducing the salmon's growth rate and overall health. In the northern hemisphere, the most prevalent species is *Lepeophtheirus salmonis* (Krøyer, 1837). In 2006, global costs of sea lice infections are estimated to have exceeded €300 million, with the majority spent on a limited number of chemical delousing agents. Emamectin benzoate (EMB; SLICE®), an avermectin, has been widely used since its introduction in 2000, due to its convenient administration as an in-feed medication and its high efficacy against all parasitic stages of *L. salmonis*.

However, over-reliance on a single or limited range of medicines favours the emergence of drug resistance and, as a result, the efficacy of this compound in treating *L. salmonis* has decreased in recent years, as reported from *e.g.* Chile, Norway, Scotland and Canada. Declining efficacy underlines the need for an improved understanding of the molecular mechanisms underlying EMB drug resistance in *L. salmonis*. Elucidation of these mechanisms would allow for improved monitoring tools, earlier detection of developing resistance, extended usability of current delousing agents and development of new parasiticides.

The work described in this thesis sets out to examine the molecular mechanisms underlying EMB resistance in *L. salmonis*. In earlier studies, research in nematodes and arthropods has linked drug efflux transporters belonging to the family of ATP-binding cassette (ABC) transporters to ivermectin (IVM) resistance, a parasiticide with high chemical similarity to EMB. ABC transporters such as permeability glycoprotein (P-gp), transport a wide range of substrates, including drugs, and have been suggested to provide a potential molecular mechanism through which EMB resistance might be mediated in sea lice. As an example of such mechanisms, increased expression of P-gp is one of the causative factors for drug resistance in human cancer cells and avermectin resistance in nematode parasites such as *Caenorhabditis elegans* or *Haemonchus contortus*.

Initial research involved screening for novel salmon lice P-gps that might contribute to EMB resistance. A novel P-gp, SL-PGY1, was discovered using a combined bioinformatic and molecular biological approach. The expression was compared in two well-characterised *L. salmonis* strains differing in their susceptibility to EMB (S = susceptible, R = resistant). Prior to EMB exposure, mRNA levels did not differ from each other, while, after 24 h exposure, a 2.9-fold increase in SL-PGY1 mRNA expression was observed in the R strain. SL-PGY1 appears not to be a major factor contributing to reduced EMB susceptibility, although it could play a role, as expression levels increased upon exposure to EMB. A further four additional drug transporters (ABC C subfamily) were also discovered showing high homology to multidrug-resistance proteins (MRP). The relative expression levels of each MRP was compared in the strains S and R, before and after exposure to EMB. No significant changes were found in their expression patterns.

If ABC drug transporters mediate the efflux of EMB and thereby reduce the intracellular concentrations of the drug in exposed animals, the inhibition of those ABC drug transporters was expected to lead to higher intracellular levels of EMB. This could result in an enhanced toxic effect when EMB is co-administered with an inhibitor. Two known inhibitors of human P-gps and MRPs, cyclosporin A (CSA) and verapamil (VER), were co-administered with EMB. CSA increased the toxic effect of EMB in both tested strains, implying that the targets of CSA are expressed at comparable levels and that they may be part of the mechanism conferring EMB resistance. VER increased the toxic effect of EMB in the R strain, but had no significant effects on the S strain. This implies that the expression of factors inhibited by VER differs between the two *L. salmonis* strains. It is hypothesised that a number of ABC transporters with distinct, yet overlapping patterns of inhibitor specificity are affected by those inhibitors.

The search for drug-resistance conferring genes was complemented with a systematic, genome-wide survey of ABC transporters in *L. salmonis* to find additional members of this important gene family. Next-generation high-throughput RNA sequencing (RNA-seq) was employed to assemble a reference transcriptome from pooled total RNA of salmon lice at different development stages. The transcriptome was assembled against the *L. salmonis* genome and

annotated. Thirty-nine putative ABC transporters were found. Of further interest were transcripts of the subfamily B, C and G, as they contain drug-transporting ABC proteins. For the ABC B subfamily, one full (SL-PGY1) and three half transporter transcripts were found. Only full transporters are known to transport drugs and SL-PGY1 is apparently not a major factor contributing to EMB resistance. Fourteen ABCC sequences were found – 11 MRPs and 3 homologues to sulfonylurea receptors. Of interest are MRPs, as they contribute to drug detoxification in humans and invertebrates. Four MRPs had been identified previously and their expression ratios did not differ between S and R strain parasites. Seven sequences belonging to ABCG subfamily were found. However, none of the *L. salmonis* ABCG transcripts identified showed sufficient homology to known drug transporters in other species.

With the currently limited understanding of the mechanisms conferring EMB resistance, monitoring the susceptibility of *L. salmonis* subpopulations is essential. Dose-response bioassays are currently widely used. Tests with pre-adult II or adult parasites requires relatively large numbers of parasites (~150) to conduct this type of bioassay, which may not always be available. Addressing this issue, we tested the feasibility of a single-dose bioassay (requiring fewer test animals than dose-response bioassays) to discriminate between *L. salmonis* strains with differing EMB susceptibility. This alternative approach uses time-course toxicity analysis, where the toxic effect of EMB is monitored over time. After clearly defining the effect criteria, we found that it is possible to discriminate between those *L. salmonis* strains. However, while requiring fewer test animals, time course toxicity analysis is more labour-intensive, but the alternative design can be suitable under certain circumstances.

The work reported here has provided new knowledge concerning the mechanisms of EMB resistance in sea lice. Several novel putative drug transporters have been identified, an important first step toward unravelling the complex interactions of genes involved in EMB resistance in this commercially important parasite.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABC – ATP-binding cassette

ATP – adenosine triphosphate

BBB – blood-brain barrier

DNA – deoxyribonucleic acid

EMB – emamectin benzoate (4'-deoxy-4' epimethylaminoavermectin B1)

EST – expressed sequence tag

GO – gene ontology

IVM – ivermectin (22,23-dihydroavermectin B_{1a} + 22,23-dihydroavermectin B_{1b})

MRL – maximum residue level

MRP – multidrug resistance-associated protein

mRNA – messenger RNA

OP – organophosphate

PCR – polymerase chain reaction

P-gp – P-glycoprotein

PEG – polyethylene glycol

R – resistant

RNA – ribonucleic acid

RNAi – RNA interference

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RT-PCR – real-time PCR

S – susceptible

SL-PGY1 – salmon lice P-glycoprotein 1

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1 INTRODUCTION

Atlantic salmon (*Salmo salar*) has become a highly valued and intensively farmed fish in recent years due to increasing worldwide demand for fish-derived protein and associated health benefits. Major production centres of farmed salmon are currently found in Norway, Chile, Scotland, Canada and Ireland (Lees 2008). Global production was 1.84 million tonnes of farmed salmon in 2013 (Marine Harvest 2014), with Norway being the largest producer with 1.165 million tonnes (Norwegian Fish Directorate 2013), while in Scotland 163,000 tonnes were produced (Marine Science Scotland 2013). Output on such an industrial scale requires large farming operation, for example, Norwegian farms can accommodate up to 2 million fish (equals 10,000 tonnes) for the 18 to 20 months of seawater the production phase.

However, as the marine phase of salmon farming takes place in open cages exposed to the ocean, there is a continuous chance for infection with sea-borne diseases. Commercial salmon farming is particularly affected by ectoparasites commonly known as sea lice. First outbreaks occurred on Norwegian farms in the 1960s (Pike & Wadsworth 1999). Infection with sea lice can affect the growth rate of salmon and reduce their feed conversion ratio (Mustafa et al., 2001). In 2006, global costs due to sea lice infections were estimated to exceed €300 million (approximately £240 million in 2014), a figure including treatment costs and economic losses due to impaired growth (Costello 2009). Furthermore, apart from direct costs associated with sea lice infections, the salmon farming industry incurs a negative perception by consumers, especially during major outbreaks (Hansen & Onozaka 2011).

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Infections of farmed and wild Atlantic salmon are mostly caused by two species - *Lepeophtheirus salmonis* (Krøyer, 1837) and *Caligus elongates* (Johnson et al., 2004; Costello 2006). Integrated pest management (IPM) strategies have been implemented in all production areas to keep infection levels below pre-defined thresholds. The intention is to avoid serious economic and environmental impact from sea lice infections, while improving profitability and sustainability of salmon farming. IPM combines various approaches to reducing infection levels, including biological control with predators such as wrasse (Deady et al., 1995), following (Bron et al., 1993), vaccine development (Carpio et al., 2011) and treatment with anti-parasitic drugs (Denholm et al., 2002).

Amongst different chemical substance classes used for salmon delousing, the latest generation of drugs belongs to the group of avermectins, which are macrocyclic lactones (ML). Avermectins exert their toxic effects in nematodes and arthropods by binding to and activating neuronal glutamate-gated chloride channels, resulting in disrupted neurotransmission, followed by paralysis and death. Avermectins are usually administered orally to fish as feed additives, thereby avoiding immersion bath treatments, which are potentially stressful for the fish and can pose logistical problems under adverse weather conditions (Stone et al., 2000).

The first avermectin derivative used for salmon louse control was ivermectin (IVM); however, it was not used widely due to its narrow therapeutic index (Davies & Rodger 2000). Another avermectin drug, emamectin benzoate (EMB), displays a wider therapeutic index (Stone et al., 1999; Horsberg 2012) and is the active ingredient of the salmon delousing agent SLICE®, marketed by Merck Animal Health. EMB is well tolerated by the fish and highly effective against all stages of sea lice at different water temperatures (Armstrong et al., 2000). EMB confers protection against lice for up to nine weeks after administration of a one-week course of 50 µg EMB per kg fish biomass (Stone et al., 1999). The convenient application method, high

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efficiency and the low environmental risk exerted by EMB are factors that have contributed to this drug becoming one of the most commonly used salmon delousing agents since the early 2000s (Lees et al., 2008). However, the overreliance on a single product can favour the formation of drug resistance in the parasite. After several years of EMB use, decreased efficacy of treatment was observed with *Caligus rogercresseyi* (Bravo et al., 2010) in Chile and with *L. salmonis* in Europe (Hjelmervik et al., 2010) and Canada (Westcott et al., 2010). These reports of shifts in parasite susceptibility to the drug are likely early signs of resistance to EMB.

1.1 Sea lice and salmon lice

“Sea lice” is the common name for marine ectoparasites of the family *Caligidae* (Subclass: Copepoda; Order: Siphonostomatoida). Different *Caligidae* species have become a problem for the global salmon industry: *Caligus elongates* Nordmann for fish farmers in the Northern Atlantic, *Lepeophtheirus salmonis* (Kroyer, 1837) for salmonid farming in the Northern Atlantic and Pacific region and *Caligus clemensi* for salmon farms located in the Northern Pacific region. In the Southern Pacific, *Caligus rogercresseyi* is a major problem for salmon farmers in Chile (Bravo et al., 2013). *L. salmonis*, also called salmon louse, has the greatest economic impact on intensive aquaculture as this parasite infects commercially important salmonids of the genera *Oncorhynchus*, *Salmo* and *Salvelinus*, (Costello et al., 2004).

Salmon lice are capable of finding and attaching themselves to their host, where they scrape away layers of mucus and skin to feed on. Eventually they can also feed on blood, when the continuous abrasions expose deeper lying tissues (Bron et al., 1993b). This is a stressor for the infected host (Pike & Wadsworth 1999; Tully & Nolan 2002), which then has a higher potential

for secondary infections by bacteria or fungi and increased osmoregulatory problems (Boxaspen 2006). The result is a diminished growth rate of infected fish, a reduction of their food conversion ratio and compromised immune status.

While *L. salmonis* is found in the Atlantic as well as the Pacific, recent studies demonstrate there are genetic differences within the species. Comparison of microsatellite DNA at six loci found distinct differences between the Atlantic and Pacific variety (Todd et al., 2004) and a study of expressed sequence tags (ESTs) and mitochondrial DNA by (Yazawa et al., 2008; Messmer et al., 2011) found a 3.2% difference for nuclear genes and 7.1% difference for the mitochondrial genome of Atlantic and Pacific salmon lice. This is in agreement with the genetic divergence of their salmonid hosts, which occurred at least 20 million years ago (McKay et al., 1996). It might also provide a clue to understanding the wider range of hosts for Pacific *L. salmonis*, which includes the three-spined stickleback (*Gasterosteus aculeatus*) (Jones & Prosperi-Porta 2011).

1.1.1 Life cycle

Salmon lice go through several developmental stages and infection of a host is required early on in order to progress through the full cycle of eight developmental stages:

After hatching from eggs dispersed in the water column, there are two planktonic non-feeding nauplius larval stages, which are pelagic and disperse in the water column. The next stage is the infective copepodid stage, which requires finding a host in order to survive and continue development (Johnson & Albright 1991). During these first stages, the larva feeds either on other plankton (planktotrophic) or relies on its lipid reserves in the yolk sac (lecithotrophic)

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(Fast 2014) but can only survive for up to seven days, with an average survival of 40 to 50 degree days (Tucker et al., 2000). Temperature (Heuch et al., 2000), salinity (Bricknell et al., 2006), light intensity and host velocity (Genna et al., 2005) as well as local water currents (Bron et al., 1991) affect survival and settlement chance of copepodids. For example, lower settlement rates were observed at 7°C when compared to rates at 12°C and for a salinity of 24 ‰ compared to 34 ‰ (Tucker et al., 2000).

A number of behavioural traits assist sea lice in the process of host-finding. These include positive rheotaxis, allowing the parasite to detect currents caused by the host, positive semiotaxis, whereby a gradient of kairomones excreted by the host is detected (Ingvarsdóttir et al., 2002), and positive phototaxis, allowing the sea louse larva to migrate closer to the surface in a daytime-dependent manner (reviewed by Mordue et al., 2009).

The majority of copepodids initially attach to the fins and gills, but they can be found anywhere on the host (Bron et al., 1991). Here, the parasite moults into the first chalimus stage. While older studies suggest there are four chalimus stages separated by ecdysis (Johnson & Albright 1991; Pike & Wadsworth 1999), a recent publication observed only two molts for chalimus stages I and II, thereby reconciling *L. salmonis*' number of developmental stages with that of other copepodids (Hamre et al., 2013). The chalimus larval stages are characterised by a prominent frontal filament attaching the parasite to the host skin and rendering it immobile (Johnson & Albright 1991). The development of salmon lice then proceeds through two pre-adult stages (I and II), which can move freely over the host's skin, followed by a final moult to mobile adults. For reproduction, adult male salmon lice engage in a pre-copula with virgin pre-adult II females. Copulation occurs directly after the female moults to the adult stage and involves cementing spermatophores to the copulatory pores of the ventromedial side of the female's genital complex, followed by extensive mate guarding by the male (up to 48 h) (Pike

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& Wadsworth 1999). Still, this does not fully prevent polyandry from occurring and while relatively rare, genetic contributions from several males have been found in fertilised eggs (Todd et al., 2005). The stored spermatophores allow female *L. salmonis* to fertilise several batches of eggs and to produce six to eleven pairs of uniseriate egg strings over her lifetime, containing on average 285 eggs/string (Heuch et al., 2000). Eggs are released from egg strings into the water column and the next generation of nauplii hatches.

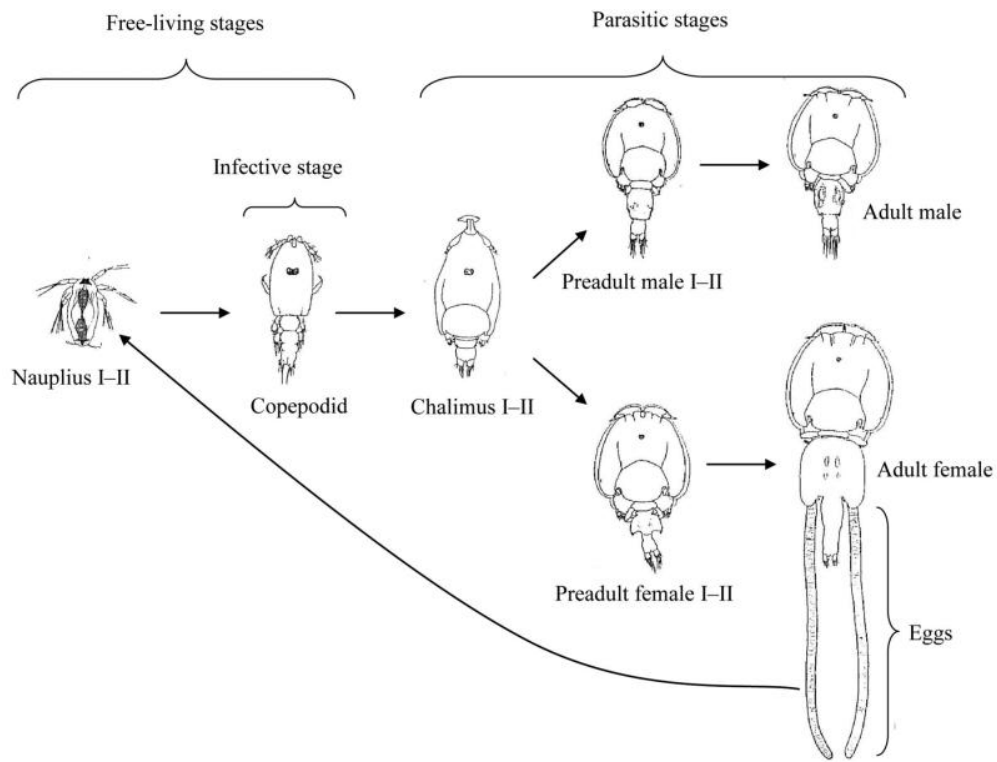


Figure 1: Life cycle of *Lepeophtheirus salmonis* (not to scale, from Igboeli et al., 2014)

1.1.2 Host-lice interactions

Once infected, fish hosts will display a number of physiological and pathological signs, depending on the intensity of the infection and the developmental stage of the attached parasite. They can range from stress and depressed appetite to death (Boxaspen 2006). In general, the damage caused increases with parasite development; earlier stages are less damaging than later ones. Recently attached copepodids cause a localised inflammatory response, visible as a dark area around the attachment site. Similarly, the sessile chalimus stages cause only limited and localised inflammation (Jones et al., 1990). However, once the parasite has moulted into pre-adults and adults, their increased feeding activity can further erode the upper skin layers and, in severe cases, expose underlying skeletal muscle. The consequences are osmoregulatory problems for the host (Wagner et al., 2003) and an increased susceptibility to secondary infections, including salmon anaemia virus (Finstad et al., 2000; Barker et al., 2009; Nylund et al., 1994). Furthermore, negative effects result from increased stress levels related to infection, leading to physiological changes like altered electrolyte and blood glucose levels, reduced red blood cell count, as well as behavioural changes. These include reduced appetite, increased irritability, flashing (fish attempts to remove the parasites by rubbing against surfaces or occasionally leaping out of the water) and poor swimming performance (Wagner et al., 2008). Ultimately, biomass production is reduced, which translates into direct financial losses for the salmon farmer (Costello 2009).

The severity of *L. salmonis* infections varies when comparing the range of their Pacific salmon hosts (*Oncorhynchus* spp.). Pink salmon (*O. gorbuscha*) and coho salmon (*O. kisutch*) have a higher capacity to purge lice from their skin, while Chinook salmon (*O. tshawytscha*) and chum salmon (*O. keta*) are more susceptible in comparison (Johnson & Albright 1992; Jones et al.,

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2007). Coho salmon reacts with rapid inflammatory infiltration a few days after infection, leading to epithelial hyperplasia and parasite encapsulation, effectively shedding more than 90 per cent of the lice within 14 days (Johnson & Albright 1992). Furthermore, when comparing Pacific coho salmon and rainbow trout (*O. mykiss*) with Atlantic salmon, Fast et al. (2002) observed that Atlantic salmon has the lowest activity of low molecular weight proteases investigated, lowest mucous lysozyme levels and overall the thinnest epidermal layers of the three species. In addition, lice matured faster on Atlantic salmon and were generally not rejected. Different mucus biochemistry could be a factor in why Atlantic salmon is more susceptible to salmon lice infections (Johnson & Albright 1992; Fast et al., 2003).

A comparison of the metabolic response of Atlantic, pink and chum salmon yielded a similar result. Sutherland et al. (2014) co-habited these three species when carrying out infection trials with *L. salmonis*. Post-infection blood cortisol concentration and haematocrit were recorded and samples of the blood, anterior kidney and skin were collected and analysed for their transcriptomic response. Chum salmon showed the highest susceptibility, followed by Atlantic salmon and pink salmon, with the lowest susceptibility. These differences in susceptibility were observed as early as three days post infection and were paralleled by transcriptional changes. They included altered expression patterns of acute phase proteins, cellular protection mechanisms and parts of the immune system of the host. Furthermore, a localised increase in pro-inflammatory cytokines interleukin-1 beta and tumour necrosis factor alpha were observed at infection sites of pink salmon, but not in Atlantic or chum salmon.

Knowledge about host-parasite interaction is still fragmentary. While a limited number of genetic markers has been published for *L. salmonis* (SNPs and microsatellite markers for less than 30 loci) (Glover et al., 2011), there is no comprehensive genetic map available for this parasite. Additional insight could be derived from screening the parasite's full genome. *L.*

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salmonis' genome contains approximately 600 Mbp and has been sequenced to a significant degree of coverage at the Institute of Marine Research, Bergen, Norway, with the publication of the full assembly in progress (as of 2014, <http://sealouse.imr.no/>). This will most likely allow researchers to improve their understanding of the underlying genetic differences between different host species and their susceptibility to salmon lice.

1.2 Control and treatment strategies

Several different approaches are used to reduce the number of sea lice on salmon farms: They include adherence to best management practices, which comprises area management and coordinated fallowing, stocking with single-year classes, removal of sick and dead fish (Bron et al., 1993), supported by continuous reporting and tracking of infection levels (North Atlantic Salmon Conservation Organization 2010). The co-habitation of farmed salmon with natural predators (e.g. wrasse) of salmon lice (Treasurer 2002) and administration of chemical delousing agents (Roth et al., 1996; Stone et al., 2000) is also important to control the infection levels.

Wrasse (family Labridae) can be added to salmon cages, where they prey on ectoparasites and dead skin from other fish. Currently, there are five species used in aquaculture, and they can provide effective control of sea lice (Groner et al., 2013). However, this strategy, while being cost effective and having little environmental impact, has disadvantages: Some wrasse are lost during winter (Sayer et al., 1996; Treasurer 2002) or tend to prefer fouling on cages and salmon food over *L. salmonis*. Selective breeding intends to exploit the different susceptibilities to sea lice infections within Atlantic salmon families (Glover et al., 2004; Glover et al., 2005), which

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could produce hosts with a reduced susceptibility to infection (Gjerde et al., 2011). However, it can be a challenging and time-consuming task (Glover et al., 2004) as many factors related to salmon's susceptibility to infection with *L. salmonis* in the wild, on fish farms or under laboratory conditions, are not fully understood yet (Glover et al., 2004; Hamre & Nilsen 2011). These include geographical (e.g. Europe or Canada), environmental (hydrology of sites, temperature) or farm management differences. Furthermore, differences in parasite strains and host families have to be taken into account, too.

Another attempt for sea louse control was a change in salinity. *L. salmonis*' settlement success and development was found to be reduced at lower salinity (Tucker et al., 2000), however, immersing salmon in fresh water for three hours did not reduce infection levels (Stone et al., 2002). Most likely, it would require a longer period of low salinity to substantially affect salmon lice; however, this is not feasible, as the host will suffer from reduced oxygen uptake. Also, the use of common disinfectants does not prevent *L. salmonis* eggs from hatching and developing (Pietrak & Opitz 2004). At the moment, the most common and effective measure is chemical delousing.

1.2.1 Chemotherapeutants

Since the beginning of large-scale salmon farming in the 1970s, veterinary drugs have been crucial in controlling salmon lice infections. As salmon lice reproduce all year round, effective control intends to decimate juvenile and pre-adult lice, ultimately preventing the appearance of gravid females (Treasurer & Grant 1997).

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The earliest attempts to control sea louse infections employed formaldehyde baths, but these did not show the required efficacy (Johannessen 1974). The first generation of effective anti-salmon louse drugs contained organophosphates (OPs), which have been used since the 1970s as bath treatments. Early examples of fish medicines of this drug class are dichlorvos in Scotland and metrifonate in Norway (Rae 1979; Bruno et al., 1990). However, resistance to OPs developed during the 1980s and was widespread by the early 1990s (Jones et al., 1992; Fallang et al., 2004). In turn, hydrogen peroxide was used as an alternative bath treatment (Treasurer & Grant 1997). However, the narrow safety margin and temperature dependence of its effects make this treatment difficult to control. Similarly cumbersome were natural pyrethrins, extracted from chrysanthemum flowers, which were administered as a top dressing oil on the surface of cages (Jakobsen & Holm 1990). The range of antiparasitic drugs tested for suitability as anti-sea louse agents further included the macrocyclic lactone, ivermectin (Johnson et al., 1993), which had sufficient and long-lasting effects on salmon lice, but also presented a rather narrow safety margin (Palmer et al., 1987). In contrast, benzoylphenylureas such as diflubenzuron (Horsberg & Hoy 1991) and teflubenzuron (Branson et al., 2000) were found to have a wide safety margin and be suitable for in-feed administration. However, a disadvantage of benzoylphenylureas is related to the action of these compounds as chitin synthesis inhibitors, which means they are only effective against moulting stages of salmon lice and ineffective against adult parasites. The mid 1990s saw the introduction of pyrethroids, synthetic derivatives of the pyrethrins (e.g. cypermethrin and deltamethrin) (Hart et al., 1997). These compounds have a sufficient safety margin, are effective against all developmental stages of salmon lice, but have to be applied as bath treatments, which is labour-intensive.

The last major release of a delousing agent was in 1999, when EMB was introduced (Stone et al., 2000). This macrocyclic lactone can be orally delivered, which is less labour-intensive, and

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has a wider safety margin than ivermectin. As it is effective against all developmental stages and confers protection of up to ten weeks (Stone et al., 2000), it became the first choice of fish farmers.

1.2.1.1 Modes of action

The majority of veterinary drugs used to control salmon lice numbers disrupt neuronal signal transmission in the parasite (Roberts & Hudson 1999).

OPs (e.g. dichlorvos, azamethiphos) are inhibitors of the enzyme, acetylcholinesterase. This is a carboxylesterase found at cholinergic synapses and neuromuscular junctions, where it hydrolyses the neurotransmitter acetylcholine, thereby terminating synaptic transmission. Organophosphates prevent the enzyme from cleaving acetylcholine, which leads to an accumulation of acetylcholine in the synaptic cleft, leading to paralysis and death (Corbett 1974). The effect of OPs can be observed within hours on pre-adults and adults, but they are less effective against chalimus stages (Wootten et al., 1982).

Pyrethroids, such as deltamethrin and cypermethrin (Hart et al., 1997), prevent neuronal voltage-gated sodium channels from closing, thereby stimulating permanent excitation, which leads to muscle paralysis, starvation and death.

Hydrogen peroxide is a strong oxidiser, which peroxidises lipids, damages enzymes, disrupts the parasite's membrane integrity, and provokes gas bubbles to form within their bodies. While treatment results are quick to observe, bath treatments clear about 85 to 100 per cent of pre-adult and adult lice, but are much less effective against chalimus stages (Thomassen 1993).

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Benzoylphenylureas (like diflubenzuron and teflubenzuron) are insect growth inhibitors acting as chitin synthesis inhibitors. As chitin is a main component of the exoskeleton in arthropods, exoskeleton formation in treated parasites will be defective, leading to death. While benzoylphenylureas are effective against eggs, larval and all immature parasite stages, adult sea lice do not undergo further moults and, therefore, remain unaffected (Branson et al., 2000).

Avermectins are neurotoxins, which bind with high affinity to the neuronal ligand-gated chloride channels of arthropods and nematodes and their target sites include channels gated by glutamate and γ -amino butyric acid (GABA) (Cully et al., 1994). Drug binding leads to the opening of these channels, resulting in hyperpolarisation of the postsynaptic membrane. As a result, neurotransmission is disrupted, leading to paralysis, starvation, and death (Arena et al., 1995). The avermectin emamectin benzoate (EMB) can be added to fish food and the ingested drug will disperse throughout the host's body. EMB will build up to concentrations in the mucous of the skin (Stone et al., 2000) sufficient to affect parasitizing sea lice. While EMB is very effective against all parasite stages, it takes two to three weeks for the effect of the treatment to emerge (Stone et al., 2000).

1.2.1.2 Treatment methods

The delivery method and the type of chemotherapeutant used can influence treatment outcome.

1.2.1.2.1 Immersion bath treatments

Bath treatment involves immersing infected fish in a solution containing the delousing agent (e.g. organophosphates, pyrethroids, hydrogen peroxide) for a pre-defined period. Advantageous is that all parasites can be exposed at the same time to a similar concentration of delousing agent, especially when well boats are used. Less efficient is skirting of sea cages with tarpaulin and subsequent drug administration to the enclosed volume, as the drug concentration will rapidly decrease due to water exchange with the surrounding ocean (Corner et al., 2011). Furthermore, bath treatments are labour-intensive and thereby costly (Costello 2009). In addition, they cannot be applied in bad weather, which could interfere with an effective application regime (Robbins et al., 2010) and only one cage can be treated at a time. Therefore, as a farm with multiple cages cannot clear the infection at once, an increased risk of reinfection exists for recently treated cages. In fact, modelling of salmon lice spread indicates that two thirds of the *L. salmonis* abundance originates from within the same farm (Aldrin et al., 2013), which highlights the limitations of bath treatments.

1.2.1.2.2 Treatment using in-feed drugs

In-feed treatments make use of fish food that is medicated with delousing agents. Following oral uptake by the fish, the drug will accumulate in tissues and mucus, and sea lice feeding on skin and mucus will be exposed by ingesting the drug. However, as fortified feed has to be consumed by the host, failure to reach therapeutic levels can occur, as fish appetite is difficult to predict and depends on factors like health status or hierarchy of salmon within the cage (Martins et al., 2012). Sick individuals will suffer from depressed appetite, while those at the lower end

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of the hierarchical order will feed less due to social intimidation (Martins et al., 2012). Furthermore, drug residues in tissue have to be below Maximum Residue Limits (MRL). In Europe, Annex I recommends MRLs of 100 µg/kg for salmonids (European Communities 1990). To ensure MRL are not exceeded, only one treatment with EMB within 60 days prior to harvest is recommended (MSD Animal Health 2013). Still, in-feed administration offers the advantages of reducing handling and occupational health risk for farm workers, being less labour-intensive than bath treatments and not requiring expensive equipment such as well boats (Ramstad et al., 2002). Furthermore, compared to bath treatments, the in-feed administration of drugs is less stressful for the fish (Stone et al., 2000). Moreover, in-feed treatments can be applied regardless of weather conditions and be delivered to all cages at the same time, allowing for the farm-wide and even management area-wide simultaneous treatment, thereby reducing risk of reinfection from within the farm or from nearby farms (Stone et al., 1999).

1.2.1.3 Avermectins and emamectin benzoate

In 1978, Japanese researchers from the Kitasato Institute in Tokyo, isolated a new actinomycete from a soil sample taken from Itou City, Shizuoka Prefecture. Researchers at the Merck Sharp and Dohme Research Laboratory, New Jersey, USA, found that extracts of the microbe had long-lasting anthelmintic properties (Burg et al., 1979). The actinomycete was identified as *Streptomyces avermitilis* (later renamed *Streptomyces avermectinius* (Takahashi et al., 2002)) and the antibiotic-acting compounds identified as polyketides, which later became known as avermectins, a group of closely related hydrophobic macrocyclic lactones (Burg et al., 1979).

By the 1980s, avermectins were used in human and veterinary medicine. A wide range of gastrointestinal nematodes, lungworms, as well as important ectoparasites such as lice, mites,

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ticks and crop-damaging insects can be treated with avermectins (Leibee et al., 1995). For example, the avermectin, ivermectin (IVM), is used to treat the canine heartworm, *Dirofilaria immitis* (Geary et al., 2011) or the filarial worm, *Onchocerca volvulus*, the causative agent of onchocerciasis (river blindness) (Green et al., 1989).

IVM (22,23-dihydroavermectin B1a + 22,23-dihydroavermectin B1b), was tested as a delousing agent (Johnson et al., 1993) and used for some time on salmon farms in Scotland, Ireland, Canada and Chile. However, IVM is poorly absorbed by the fish and, due to a high toxicity in salmon, displays a low safety margin (Johnson et al., 1993).

Emamectin is a semi-synthetic derivate of the macrocyclic lactone abamectin. It is a mixture of the two homologous compounds 4''-deoxy-4''-epimethylaminoavermectin B1a and B1b, they differ on the C25 side chain by a single methylene group (Figure 1); B1a carries a *sec*-butyl group, while B1b has an isopropyl group. For commercial applications, emamectin is usually prepared as the benzoic acid salt emamectin benzoate (EMB), where the specified ratio of compound B1a to B1b is 90:10 (w/w) (Velde-Koerts 2011).

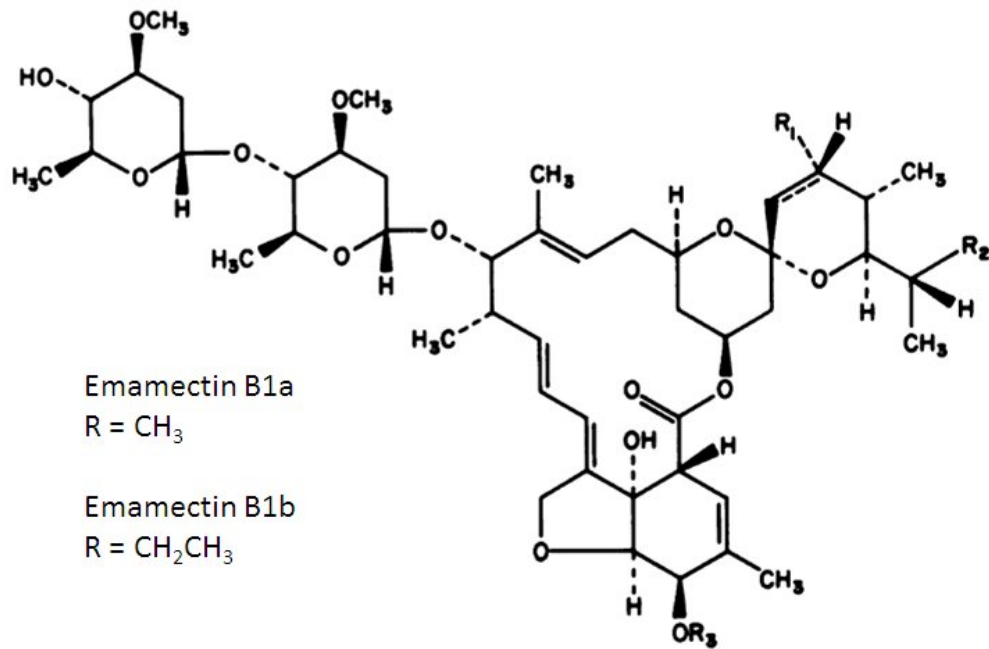


Figure 2: Chemical structure of the macrocyclic lactone emamectin. Emamectin is a mixture of the two homologous compounds 4'-deoxy-4'-epimethylaminoavermectin B1a and B1b (modified from (Burg et al., 1979))

EMB has a wider therapeutic margin than IVM and has proven to be very effective for salmon lice control (Stone et al., 1999; Stone et al., 2000; Armstrong et al., 2000). Stone et al. (1999) recommend a therapeutic dose of 50 µg of the drug per kg fish biomass and day to be orally administered over the course of seven days. Subsequent field studies on commercial farms showed that treatment with EMB is very efficient in clearing salmon lice. Thirty-five days after EMB administration, treated fish had up to 89 per cent fewer parasites than untreated fish and it

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was found that EMB conferred protection against sea louse re-infection for up to nine weeks (Stone et al., 2000). Due to emamectin benzoate's convenient application in-feed, relatively low occupational health hazard, high efficacy against all developmental stages of salmon lice and long-term protection after treatment, it is now widely used on salmon farms in Scotland, Ireland, Norway, Canada and Chile (Saksida et al., 2010).

1.2.1.3.1 Pharmacological considerations of avermectin treatments

Avermectins are lipophilic and will accumulate in fatty tissues, from where they are slowly released to become systemically available, leading to long-lasting biologically active concentrations. Following a one week course of in-feed treatment, EMB remains in the host's system for up to nine weeks (Sevatdal et al., 2005), conferring prolonged protection against salmon louse infections (Stone et al., 1999).

SLICE® is a commercially available premix distributed by Merck Animal Health, Whitehouse Station, New Jersey, USA. It contains 0.2 per cent EMB per gram (w/w) and is used to coat standard salmon food prior to treatment. The drug-fortified food is then consumed and digested by the fish. EMB is absorbed from the gut via diffusion due to high lipophilicity. It gets widely distributed throughout the host's body, with high concentrations found in blood, skin, kidney and liver, while lower concentrations are found in muscle tissue (Sevatdal et al., 2005). As the skin cells secrete mucus, EMB reaches significant concentrations in the mucus layer as well, and is eventually ingested by feeding salmon lice. While ingestion is the main route of exposure to EMB, cuticular absorption due to contact with EMB-infused mucus could also be a way for the drug to enter the parasite, although to a lesser extent. Very limited metabolism of EMB (Kim-Kang et al., 2004) and enrichment in fatty tissue ensures high persistence in the host's

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body, and this is beneficial for long-term protection. However, as medicated salmon are intended for human consumption, EMB treatment must be timed appropriately to ensure MRLs remain below the recommended 100 µg/kg (European Communities 1990). Therefore, it is suggested to use SLICE® only once within a 60-day period before salmon is harvested (MSD Animal Health 2013).

EMB pharmacokinetics depend also on the water temperature. At 6°C, the EMB concentration in salmon muscle tissue fell below the detection limit within 77 days since the end of oral SLICE® administration, but at a water temperature of 15°C, EMB was undetectable after 49 days (Roy et al., 2006). This is in concordance with observed seasonal variations in EMB efficacy (Lees et al., 2008).

1.2.1.4 Avermectin toxicity

Blood-tissue barriers such as the blood-brain barrier (BBB) or the placenta, are highly selective barriers, restricting free movement of substances between the bloodstream and the brain's extracellular fluid. The barrier is formed by the capillary endothelia being connected by tight junctions, thereby becoming a barrier virtually impenetrable to fluid. Matter exchange is facilitated via selective transporters, which supply molecules required for brain function such as glucose, amino acids and hormones. However, the BBB permits passage of gases and lipophilic substances by passive diffusion.

To prevent lipophilic and potentially neurotoxic substances from entering the brain, an array of drug efflux transporters, like P-glycoprotein (P-gp), multidrug resistance proteins (MRPs) or organic anion transporting polypeptides (OATPs) are located at the apical side of endothelial

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cell membranes (Loscher & Potschka 2005). Their role is to prevent a wide range of lipophilic and chemically very different molecules from entering the brain by actively extruding them back into the bloodstream. Avermectins, as lipophilic molecules, can enter the plasma membrane of endothelial cells, where they are quickly sequestered and removed by drug transporters, thereby protecting the central nervous system. For example, mice lacking either the *mdr1a* or combined *mdr1a/b* genes (equivalent to human *ABCB1/P-gp*), display enhanced brain accumulation of P-gp substrates, among them IVM (Schinkel et al., 1995). Also, certain dogs of the Collie breed are deficient in P-gp and show hypersensitivity when treated with IVM (Roulet et al., 2003). Here, toxicity arises when IVM accumulates in the brain, where it interferes with GABA-gated chloride channels (Kiki-mvouaka et al., 2010).

In Atlantic salmon, IVM is used to control infections with sea lice. It has a narrow therapeutic margin; single oral doses of 0.2 mg IVM per kg fish did not cause treatment-related mortality, while a single 0.4 mg per kg dose caused significant mortalities (Palmer et al., 1987). It accumulates in the central nervous system of salmon, which indicates that the BBB of *S. salar* is less efficient in preventing neurotoxic IVM from accumulating than mammalian systems (Hoy et al., 1990). The chemically similar compound EMB has a wider therapeutic margin. Roy et al. (2000) fed different doses of EMB to Atlantic salmon to study potential toxic effects. At the highest dose of 500 µg per kg fish, signs of EMB intoxication (skin discolouration, reduced appetite, poor swimming performance and lethargy), but no mortality were found (Roy et al., 2000). The recommended dose for treating salmon is much lower, at 50 µg EMB per kg fish (Stone et al., 2000).

1.2.1.5 Environmental concerns regarding EMB

EMB is administered under veterinary prescription to treat salmon lice infections. However, the compound is ultimately released into the environment through uneaten fish food and salmon excreta (Nash 2003). Accordingly, potential effects of EMB on non-target organisms are of concern (BurrIDGE et al., 2010).

EMB has a low solubility in water and a relatively high octane-water partitioning coefficient ($K_{ow} = 5.0$), therefore it is more likely to bind to sediments, where it is retained for prolonged periods. The half-life of EMB in the environment is estimated to be 193.4 days under aerobic conditions and 427 days under anaerobic conditions (McHenery & Mackie 1999). Adverse effects of EMB have been reported for a number of non-target species, among them American lobster (*Homarus americanus*) (Waddy et al., 2002) and four common marine copepods, *Acartia clausi*, *Pseudocalanus elongatus*, *Temora longicornis* and *Oithona similis* (Willis & Ling 2003). However, negative effects were only observed at much higher doses than predicted environmental concentrations around treated fish farms (Willis & Ling 2003). In a field study, for one year, Telfer et al. (2006) monitored, for one year, large mobile fauna, sediment-dwelling and sentinel animals on a commercial salmon farm on the West coast of Scotland regularly administering EMB as an in-feed treatment. The authors found a maximum concentration of 2.73 µg EMB/kg wet weight in sediments collected four months after treatment. At this concentration and 10 metres distance from sea cages, they did not observe any acute toxic effects on indigenous species like common whelks (*Buccinum undatum*), hermit crab (*Pagurus* spp.), common shore crab (*Carcinus maenas*) and brown swimming crabs (*Liocarcinus depurator*).

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With the current usage practice of EMB in the salmon farming industry, there is no evidence for negative environmental impacts of the pesticide. However, due to its long half-life and tight association with sediments (McHenry & Mackie 1999), it is possible that EMB might accumulate near fish farms. Hence, it is imperative to use EMB sparingly and with tight monitoring, as the drug could possess the potential to accumulate to higher concentrations, which could subsequently pose a risk to non-target marine organisms.

1.2.2 Antiparasitics resistance in sea lice

The term resistance describes the heritable ability of a parasite to survive treatment with anti-parasitic drugs at concentrations that would normally kill parasites of the same species and developmental stage (Sangster 2001; Prichard et al., 1980). Cross-resistance describes the loss of efficacy of anti-parasitic drugs with different modes of action (Prichard et al., 1980), while multiple resistance is used to describe the situation, when a parasite population becomes insensitive to several anti-parasitic agents with different modes of action (Wrigley et al., 2006).

Subpopulations of *L. salmonis* have become resistant to several classes of anti-parasitics. In the early 1990s, Norwegian and Scottish lice populations became resistant to organophosphates (Jones et al., 1992; Fallang et al., 2004), hydrogen peroxide (Treasurer et al., 2000) and later to pyrethroids (Fallang et al., 2005; Sevatdal et al., 2005). Subsequently, their use declined, and salmon farmers switched to other delousing agents. Introduced in 2000, EMB, due to high efficacy and convenient administration, became widely used in Scotland (Lees et al., 2008) and elsewhere (Westcott et al., 2008). In fact, over-reliance by salmon farmers on EMB quickly became a concern (Westcott et al., 2004).

1.2.3 Salmon louse resistance to EMB

Resistance to the antiparasitic avermectins has developed not only in salmon lice (Denholm et al., 2002), but also in parasites of ruminants like sheep (Swan et al., 1994; Sangster et al., 1999) and cattle (West et al., 1994; Condi et al., 2009) or the causative agent of onchocerciasis in humans, the parasitic nematode *O. volvulus* (Osei-Atweneboana et al., 2007). It reflects that antiparasitic drugs often lose their capacity to control parasites after a certain period of use (Prichard et al., 1980).

A limited variety of treatment options has exacerbated the resistance problem in salmon lice (Denholm et al., 2002). Over-reliance on a single antiparasitic agent needs to be avoided (Wolstenholme et al., 2004), yet this tendency still exists when it comes to delousing agents for salmon farms. This could be related to difficulties in developing and licencing new drugs (Denholm et al., 2002) and that farmers are more likely to use tried and tested treatments.

For several years, it was a major concern that resistance to EMB would emerge and bioassays were developed to monitor sensitivity of sea lice to EMB treatment (Sevatdal & Horsberg 2003; Westcott et al., 2008). Resistance to EMB did develop and in the late 2000s, European (Hjelmervik et al., 2010; Lees et al., 2008) and Canadian salmon farms (Westcott et al., 2010; Jones et al., 2013) reported treatment failure of *L. salmonis* infections, while Chilean farmers observed reduced efficacy of EMB against *C. rogercresseyi* (Bravo et al., 2008).

The observed decreased efficiency of EMB treatments highlights the need for continuous and reliable monitoring of EMB susceptibility in *L. salmonis* (Westcott et al., 2008; Treasurer & Pope 2000). For example, a study examining the trends of EMB efficacy used to treat salmon lice infections in New Brunswick between 2004 and 2008 found decreased treatment efficacy (Jones et al., 2013). The authors found that EMB susceptibility varied between farms, but

overall decreased at a faster rate compared with Scottish farms between 2002 and 2006 (Lees et al., 2008). In Chile, IVM was used for ten years prior to the introduction of EMB in 2000 and until 2007, EMB was the only approved delousing agent (Bravo et al., 2013). Chilean farmers also exceeded the recommended EMB doses in an attempt to counter declining treatment efficiency (Bravo et al., 2008). Therefore, over-reliance on avermectins for more than 15 years is the most likely factor which has contributed to the observed reduced EMB susceptibility of *C. rogercresseyi* (Bravo et al., 2008).

1.2.3.1 Resistance mechanisms

Intimate knowledge of resistance mechanisms and markers linked to resistance is beneficial for tracking resistance emergence and development in parasites, particularly at a molecular and receptor level (Blackhall et al., 1998; Prichard & Roulet 2007). It would allow for a better understanding of drug interaction with their targets and would be useful for developing diagnostic tools. Subsequently, this would the use of antiparasitics with different modes of action, thereby possibly extending their shelf life (Geary et al., 1999). Drug resistance is usually associated with drug-target interaction and drug concentration at target site.

Target site

Mutations of the drug target can alter the ability of drugs to bind, e.g. steric changes, alteration of important side chains for binding or electrostatic conditions. Modification of a single amino acid changed the spatial properties of acetylcholinesterase, the target site of organophosphates,

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in salmon lice, thereby rendering those parasites resistant to treatment (Jones et al., 1992; Fallang et al., 2004). Alternatively, reduced expression of the drug target was associated with avermectin resistance in *H. contortus* (Wolstenholme et al., 2004). Knockdown resistance has been observed for deltamethrin, where point mutations modify the pyrethroid's target, para-type sodium channels of nerve membranes (Fallang et al., 2005); and cross-resistance to cypermethrin, which is structurally similar, is likely to occur (Denholm et al., 2002). Increased metabolism, e.g. higher activity of cytochrome P450 monooxygenases, results in an increased detoxification capacity for drugs, which was observed in pyrethroid-resistant *L. salmonis* (Sevatdal et al., 2005).

Numerous studies regarding avermectin resistance in parasites have been carried out with a focus on nematodes infecting humans or economically important animals such as sheep and cattle (Wolstenholme & Kaplan 2012). Two biochemical mechanisms are believed to be involved in avermectin resistance – altered ligand-gated chloride channels and increased expression of drug transporters belonging to the ABC transporter superfamily.

The neurotoxic effect of avermectins is believed to be associated with their capacity to bind and block glutamate-gated (GluCl) and GABA-gated chloride channels, found in the invertebrate nervous system (McCavera et al., 2007; Wolstenholme & Rogers 2005). Glutamate-gated chloride channels have also been suggested as a target for IVM in free-living and parasitic nematodes (Dent et al., 2000; Njue et al., 2004). However, it appears to require multiple mutations in different genes coding for GluCl channels before IVM resistance is observed (Njue et al. 2004). There is not much evidence linking genetic alterations of GluCl channels to IVM resistance (El-Abdellati et al., 2011; Williamson et al., 2011).

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Hence, attention was given to another potential resistance mechanism - drug extruding ABC proteins such as P-glycoprotein (P-gp) (Lespine et al., 2012). These proteins are involved in the rapid removal of drugs, thereby reducing intracellular concentrations (Prichard & Roulet 2007).

ATP-binding cassette (ABC) transporters

ABC transporters belong to one of the largest protein superfamilies, with members found in all organisms, from bacteria to humans (Dassa & Bouige 2001). While prototypical ABC proteins transport molecules by coupling ATP hydrolysis with substrate translocation across biological membranes, the ABC superfamily also contains ion channels, regulators of ion channels, receptors, and proteins with roles in ribosome assembly and translation (Dean et al., 2001). In ABC transporters, at least two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) are required to constitute a functional pump. In full transporters, all required domains are found in a single polypeptide, while half transporters possess only one TMD and one NBD, and thereby require homo- or heterodimerisation for functionality (Dassa & Bouige 2001). Metazoan ABC transporters are currently divided into eight subfamilies (A-H) and drug transport is facilitated by members of the subfamilies B, C and G (Dean et al., 2001; Sheps et al., 2004; Sturm et al., 2009)

Permeability-glycoprotein (P-gp)

P-glycoprotein (P-gp, MDR1 or ABCB1) is a full transporter of the ABCB subfamily and was first observed in tumour cells showing increased expression of this protein and resistance to

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several cytotoxic agents, a phenomenon known as multidrug resistance (MDR) (Dano 1973). P-gp is a major contributor to this clinically important phenomenon, as it interacts and transports a large array of structurally and functionally unrelated substrates. P-gp's substrates are usually hydrophobic compounds, for example cytotoxic drugs, steroid hormones, immunosuppressants, calcium channel blockers and cardio-active glycosides. Increased expression of P-gp has been observed in MDR cancer cells (Chen et al., 1986). Thereby, P-gp's broad substrate spectrum, combined with increased expression, has the potential to limit bioavailability and retention time of drugs (Varma 2003), which subsequently can exacerbate MDR, resulting in chemotherapy becoming less effective (Bansal et al., 2009).

In non-cancerous tissues, P-gp improves the barrier function of protective tissues such as the placenta and BBB (Ma et al., 2010) by rapidly extruding toxic compounds (Fortuna et al., 2011). Furthermore, it plays an important role in the secretion of xenobiotics and metabolites into bile, urine or the gut lumen and in the biochemical defence against toxicants in general (Schinkel et al., 1994).

Ivermectin is a high-affinity substrates of P-gp and is transported by this protein to the outer layer of the cell membrane (Lespine et al., 2007; Bain & LeBlanc 1996). Mice lacking expression of this transporter in the gut and the brain, as the result of targeted gene disruption, are hypersensitive to IVM (Schinkel et al., 1994). While no data are available for emamectin, IVM and five other avermectins affected MDR P-glycoprotein-dependent ATPase activity at low micromolar concentrations, suggesting that they may also be P-gp substrates (Lespine et al., 2009). Accordingly, P-glycoprotein has been suggested as a potential factor contributing to avermectin resistance in a number of parasites. For example, IVM-selected strains of the gastrointestinal nematode *Haemonchus contortus* (Rudolphi 1803) showed a higher mRNA expression of P-gp, which coincided with an enrichment of a certain allele at the locus coding

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for the transporter (Xu et al., 1998; Blackhall et al., 1998). Furthermore, evidence for possible genetic selection around a P-glycoprotein locus also exists in the human parasitic nematode, *O. volvulus* (Ardelli et al., 2006).

For marine invertebrates, P-gp has been implicated as an important factor protecting them from harmful xenobiotics, such as pollutants (Smital & Kurelec 1998).

Multidrug resistance-associated proteins (MRP)

MRPs are full transporters belonging to the ABCC subfamily. They act as organic anion transporters with broad substrate-specificity and can translocate a wide range of substances, including sulphate, glucuronyl and glutathione conjugates of steroid hormones and bile salts, cysteinyl leukotrienes (Kruh & Belinsky 2003) and the mycotoxin aflatoxin B1 (Lorico et al., 2002). MRPs are another contributing factor to MDR observed in tumours (Borst et al., 1999).

Breast Cancer Resistance Protein (BCRP/ABCG2)

This half-transporter is a member of the ABCG subfamily and cells expressing this transporter show a drug resistance profile that is similar, yet not identical, to P-gp-expressing cells (Doyle & Ross 2003). BCRP is found alongside P-gp in barrier tissues like the blood-brain barrier, placenta and intestine (Vlaming et al., 2009; Prouillac & Lecoeur 2010). During lactation, it is also expressed by mammary glands, where BCRP secretes its substrates into the milk (Jonker et al., 2005).

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These drug transporters are an important component of the hosts' biochemical defence system against toxins and contaminants; the tenacity and complexity of the system is rooted in a) the ubiquitous presence of multiple transporters being expressed at various locations and b) in the considerable overlap of their substrate spectra (Jones et al., 2009). This redundancy provides an efficient barrier, which protects organisms from toxic effects of xenobiotics, but also limits the internal concentration of drugs and therefore their efficacy (Marquez & Van Bambeke 2011). Furthermore, the activity of drug transporters plays a clinically relevant role in drug interactions when several drugs are co-administered (Sarkadi et al., 2006)

1.2.3.2 Reduced drug uptake

Efficient efflux of toxic drugs prevents their accumulation inside the cell, thereby those drugs will not reach therapeutic levels and hence their intended effect will not be observed. As drug efflux is a major contribution to antineoplastic resistance in cancer cells, research is focussing on those transport proteins with the intention of selectively influencing their activity (Modok et al., 2006). Various strategies have been employed to counteract drug efflux transporter activity in cancer cells with the purpose of increasing cytotoxic drug uptake and retention times. These strategies include attempts to bypass transporters with targeted drug delivery systems (peptide conjugates (Mazel et al., 2001), liposomes (Goren et al., 2000)) as well as the use of inhibitors (Lespine et al., 2008; Bartley et al., 2009). Particularly inhibitors hold great potential for altering pharmacokinetics of drugs and enhance their bioavailability (Varma 2003).

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P-gp, the first drug transporter discovered, can be inhibited in several ways: a) competitive/non-competitive or allosteric blockage of the drug binding site; b) decreasing activity by interfering with ATP hydrolysis (Shapiro & Ling 1997) or c) changing cell membrane fluidity (Drori et al., 1995; Werle 2008).

Enzyme inhibitors are molecules able to bind to enzymes and thereby decrease their activity, resulting in lower turnover or even complete stoppage.

Types of inhibitors

Reversible inhibitors are interacting temporarily with their target enzymes (Gillet & Gottesman 2010). Non-covalent bonds like hydrophobic interactions, hydrogen or ionic bonds facilitate the association. Multiple weak bonds between the inhibitor and the target site allow for sufficient binding strength and specificity. If the inhibitor has an affinity for the active site, it is termed competitive inhibition as the inhibitor and the substrate compete for access to the same space on the enzyme, whereby less substrate can be processed (Alberts et al., 2007). If the inhibitor requires the substrate-enzyme complex to be formed, it is a case of non-competitive inhibition. Here, the binding of the inhibitor decreases the overall turnover rate of the enzyme (Alberts et al., 2007). Allosteric inhibitors bind to a different site of the enzyme, thereby inducing a conformational shift, which will decrease affinity of the substrate towards the active site, resulting in a reduced turnover rate (Berg et al., 2002). Non-competitive inhibitors will bind to the enzyme and reduce overall reaction rate without decreasing substrate affinity to the active site (Alberts et al., 2007).

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Most irreversible inhibitors covalently modify their target enzymes and the effect is permanent (Berg et al., 2002). Often, this involves electrophilic functional groups of the inhibitors to react with amino acid side chains forming covalent bonds. This will modify the active site, reducing or even preventing access for the substrate, while the enzyme's functionality remains intact (Alberts et al., 2007).

Inhibitor generations

Since the discovery of drug transporting proteins like P-gp, the number of inhibitors has grown and it is possible to distinguish different generations.

The first inhibitor of P-gp was discovered in 1981. The calcium channel blocker, verapamil, was shown to reverse MDR in vincristine-resistant P388 leukaemia cells (Tsuruo et al., 1981). It was part of the "first generation" P-gp inhibitors like cyclosporin A and quinidine, which were not specifically developed to inhibit P-gp (Amin 2013). These exhibited other pharmacological activities and had relatively low affinities for P-gp. Therefore, their usefulness was limited as high serum concentrations were necessary to achieve relevant P-gp inhibition, these were often toxic (Lomovskaya & Bostian 2006).

Second generation inhibitors, like dexverapamil and valsopodar, were more potent, but had a number of unwanted side effects (Pusztai et al., 2005). They often interacted with several ABC transporters, not only P-gp, or with CYP3A4, a crucial enzyme for drug metabolism. This often led to complicated pharmacokinetic interactions, which were undesirable and often toxic (Malingré et al., 2001; Pusztai et al., 2005).

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Improved knowledge and better understanding of structure-affinity relationships between inhibitors and P-gp allowed the development of third generation inhibitors. They display higher *in vitro* potency, increased selectivity at nanomolar concentrations and generally lower toxicity (Kwak et al., 2010). However, some inhibitors still showed cross-reactivity with BCRP, another ABC transporter (Ahmed-Belkacem et al., 2005) and, in one case, CYP3A4 (Dantzig et al., 1999). Furthermore, several third-generation inhibitors did not increase therapeutic efficacy as anticipated (Bardelmeijer et al., 2004). Hence, alternative strategies are sought for designing fourth generation inhibitors, such as investigation of natural compounds, design of dual activity ligands or peptidomimetics (Palmeira et al., 2012). For example piperine, an alkaloid responsible for the pungency of black pepper, is a known P-gp inhibitor (Singh et al., 2013). It competes with ATP/ADP for the same position at the NBD site and could be used as starting point to develop novel P-gp inhibitors with fewer negative side effects (Singh et al., 2013).

1.2.3.3 Inhibitor-mediated reversal of drug resistance

Resistance to avermectins has been reduced in *H. contortus* using inhibitors such as verapamil (Molento & Prichard 2001), thereby confirming the role of ABC drug transporters in resistance to IVM and moxidectin. Decreasing the transport activity of ABC transporters has the potential to enhance the bioavailability of avermectins (Bartley et al., 2009). Yet, possible toxic side effects, differences in pharmacokinetics of avermectins and inhibitors, potential withdrawal periods and costs of the respective inhibitors have to be considered as well (Lespine et al., 2008).

However, as IVM has been shown to be transported by ABC transporters in invertebrates (Xu et al., 1998; Sangster et al., 1999), it would be reasonable to use inhibitors to investigate the role

of ABC transporters identified in the course of this study with regard to their potential role in EMB resistance.

1.2.3.4 Metabolic changes and resistance

Drug resistance can also be related to enhanced detoxification systems (Denholm et al., 2002), such as cytochrome P450 monooxygenase-mediated resistance to pyrethroids in salmon lice (Sevatdal et al., 2005). Whether there is significant metabolism of EMB in *L. salmonis*, it is currently unknown. Very limited metabolic breakdown occurs in Atlantic salmon (Kim-Kang et al., 2004), so if one would assume a similar metabolic turnover for salmon lice, it is unlikely that changes in EMB metabolism would be sufficient to overcome EMB toxicity. Furthermore, concentration of EMB within Atlantic salmon declines slowly due to sustained enterohepatic recirculation (Kim-Kang et al., 2004), therefore it is unlikely that changes to EMB metabolism in salmon would significantly affect the drug concentration of tissue ingested by the parasite.

1.2.4 Drug resistance monitoring

Detecting resistance to parasiticides in nematodes infecting livestock involves *in vivo* (e.g. reduction of egg numbers in faeces) and *in vitro* methods (e.g. monitoring of hatching success or larval development) (Coles et al., 1992). These methods were also employed to study avermectin efficacy when treating river blindness caused by human filarial nematode *O. volvulus* (Churcher & Basáñez 2009) and ruminants infected with parasitic nematodes (Coles et

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al., 2006). There is evidence for avermectin resistance in *O. volvulus* (Osei-Atweneboana et al., 2007) and nematodes parasitizing ruminants (Kaplan 2004; Wolstenholme et al., 2004).

Constant monitoring is important to detect reduced sensitivity to a parasiticide, which will help to ascertain when treatment should be continued with a different class of chemicals, thereby delaying the development of resistance (Zhao et al., 2006). Monitoring of salmon lice is conducted with the same intention: Assessing the optimal treatment regime for salmon production areas, while taking local differences into consideration (Brooks 2009), as the emergence of resistance to currently used delousing agents is a constant threat to the farmer's ability to control salmon lice infections. Therefore, it is vital to constantly collect information regarding treatment success and efficacy of the chemicals employed.

To obtain this information, bioassays are widely used to assess the sensitivity of a salmon louse test population to a parasiticide such as EMB, deltamethrin and cypermethrin (Sevatdal & Horsberg 2003; Bravo et al., 2008; Westcott et al., 2008). Bioassays can also be employed to investigate the effects of toxicants like EMB with regard to gene expression in surviving parasites (Tribble et al., 2007; Heumann et al., 2012; Heumann et al., 2014). However, the current bioassay design is labour-intensive, does not allow for high-throughput and lacks simplicity (Westcott et al., 2008). It requires testing pre-adult or adult salmon lice, which have been removed from their hosts. While bioassays are useful for monitoring the susceptibility of salmon lice to parasiticides, they suffer from limitations: Firstly, the collected parasites have to be used quickly, usually within 2 days after removal from their natural environment, because their vitality gradually declines. Secondly, to ensure consistency and comparability of bioassays, it is important to define endpoints and effects. This can be difficult, particularly when assessing sublethal effects of delousing agents, as the parasite can remain moribund for prolonged periods (Denholm et al., 2002). Thirdly, the currently used dose-response protocol

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suggests to test at least five different concentrations of toxicant. This should include concentrations which were previously effective (Westcott et al., 2008), but it may not always be possible to choose useful concentrations and a second bioassay, with adjusted concentrations, would be necessary.

Monitoring of drug resistance in salmon lice should be efficient and quick. The bioassays used for monitoring should be simple, robust, sufficiently sensitive to detect changes in susceptibility and easy to replicate, with clearly defined endpoints. However, these requirements are hard to meet in practice. In addition, the use of different chemicals makes it even harder to develop a suitable bioassay suitable for all circumstances. The difficulty is rooted in the parasiticides' different life stage specificity, speed of effect and their general physical and chemical properties (Denholm et al., 2002).

Once the biochemical causes for drug resistance have been discovered, *in vitro* tests of the respective resistance mechanisms could be developed, which would lead to easier, faster and potentially cheaper resistance monitoring (Denholm et al., 2002; von Samson-Himmelstjerna & Blackhall 2005). With only a limited number of delousing agents licenced for treatment and the capacity of salmon lice to develop resistance to delousing agents, it is important to better understand their response to parasiticides (Walsh et al., 2007). Knowledge of the molecular basis of the respective resistance mechanism could help to develop biochemical assays measuring the quantity of resistance-conferring enzymes or molecular (e.g. RT-PCR-based) methods to detect qualitative differences in drug resistant salmon lice (von Samson-Himmelstjerna & Blackhall 2005). Development of additional tools to monitor drug resistance would contribute to the overall success in controlling salmon lice (Denholm et al., 2002).

Changes detected in the expression pattern and the structure of P-gp (Sangster et al., 1999) or β -tubulin (Eng et al., 2006) could be the first step towards the development of genetic markers

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allowing for the monitoring of resistance, which can develop in parasites continuously selected with avermectins (Eng & Prichard 2005). Changes in β -tubulin have been studied as potential markers for IVM and moxidectin resistance in nematodes, but additional research is required (Prichard & Roulet 2007). Prolonged use of IVM to treat river blindness appears to have reduced the genetic diversity of a P-gp homologue in *O. volvulus* (Ardelli et al., 2006). Tracking this decrease could be useful for monitoring IVM resistance (Bourguinat et al., 2008).

Analysing differential gene expression in *L. salmonis* could also contribute to drug resistance monitoring. Molecular biological methods such as real-time quantitative PCR could be employed to investigate how genes of interest are regulated and if there are changes in expression as a reaction to parasiticide exposure. Expression is compared to constitutive genes required for maintenance of metabolism and other fundamental cellular processes, which are unlikely to be regulated in response to drug exposure (Frost & Nilsen 2003). Frost and Nilsen (2003) tested the suitability of several reference genes, which could be used to identify transcription changes that occur during the development of *L. salmonis*. They found that structural ribosomal protein S20 (RPS20) and the eukaryotic elongation factor 1 alpha (eEF1a) showed less than two fold variation in their transcription levels in samples covering the whole life cycle of *L. salmonis* (Frost & Nilsen 2003). Therefore, these two genes could be used as reference genes when investigating transcriptional changes in salmon lice triggered by EMB exposure (Frost & Nilsen 2003).

Over the years the costs of molecular biological analyses has fallen, while their sensitivity, reliability and potential for automation has increased (Bass et al., 2004). Once the mechanisms for drug resistance are better understood and the implicated genes identified, it is likely that molecular biological assays will become an important tool for resistance detection and salmon louse management (Sangster 2001; von Samson-Himmelstjerna 2006). This could be

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particularly useful if the resistance mechanisms involve changes to the drug's target site, as seen for drug-resistant cat fleas, *Ctenocephalis felis* (Bass et al., 2004). Molecular biological tools will also become essential to study host-parasite interactions. This would contribute towards developing new therapeutic or prophylactic strategies aimed at decreasing salmon louse infections.

1.3 Aims and objectives

EMB is the last of a number of delousing agents, where loss of efficacy has occurred (Torrissen et al., 2013). This highlights the urgency for a better understanding of EMB resistance mechanisms, particularly at a molecular level. This could pave the way for developing diagnostic markers capable to reliably predict the susceptibility of subpopulations, a more timely rotation of parasiticide classes and possibly could prolong the shelf life of EMB. The overall aim of this project is to expand our knowledge of potential factors contributing to EMB resistance in *L. salmonis*.

Drug resistance can be caused by qualitative or quantitative changes to drug detoxification mechanisms or target sites. As the salmon louse genome was not fully sequenced at the inception of this project, we planned to use a bioinformatics approach to screen publicly available ESTs of salmon lice for candidate genes putatively involved in drug resistance. Of particular interest were ABC transporters, a gene family that contains members functioning as drug efflux pumps. Increased expression of ABC transporters has been linked to drug resistance in humans (Cole et al., 1992), agricultural parasites (Sangster et al., 1999; Blackhall et al.,

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2008), and marine invertebrates (Luckenbach & Epel 2008). We intended to test the hypothesis that ABC transporters are major factors in EMB resistance in *L. salmonis*. Available genomic resources were searched and candidate genes for ABC drug transporters isolated. The sequences were incomplete; therefore, for selected candidates, full-length coding sequences were to be established. Real-time quantitative PCR was used to measure the relative expression ratios of the candidate genes in two well-characterised salmon lice strains differing in their susceptibility to EMB. These strains were reared at the University of Stirling for several years and their susceptibility to EMB has remained relatively constant (personal communication Dr A Tildesley). Expression was to be observed before and after aqueous exposure to EMB in bioassays. Furthermore, bioassays were performed during the course of this project, with the intention to monitoring EMB resistance in both strains; acquiring genetic material before and after exposure to the pesticide and for improving the current bioassay protocol.

Bioassays are used to monitor changes of a parasite subpopulation exposed to different toxicants (French-Constant & Roush 1990). In the standard bioassay for monitoring EMB resistance, multiple concentrations are used for 24 h aqueous exposure, after which the parasite's response is recorded (SEARCH Consortium 2006; Sevatdal et al., 2005). However, a large number of parasites of the same developmental stage (~150 animals) is required and the standard bioassay cannot be replicated in rapid succession; it lacks simplicity and results can differ substantially depending on the season (Westcott et al., 2008). This means that the current standard bioassay might not be suitable at all times of the year for assessing resistance, given varying circumstances like developmental stage of the parasite (Westcott et al., 2008; Heumann et al., 2012) and speed of the drug action (Denholm et al., 2002). We intend to test the feasibility of an alternative bioassay design, which could be more suitable under some circumstances, as it requires only half the animals needed for a standard bioassay. The response of animals exposed

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to EMB will be monitored over time. We intended in confirming that it is possible to differentiate between susceptible and resistant lice using time course toxicity analysis.

Furthermore, bioassays require labour-intensive detachment of salmon lice, resulting in low throughput and require human assessment of the parasite's condition. This is disadvantageous for large-scale testing and automation. Furthermore, lice must be used soon after collection, as prolonged separation from the host will induce additional stress; hence, bioassays must commence within a relatively short period. In addition, it can be challenging to define endpoints and which observed behaviour represents an effect caused by treatment (Denholm et al., 2002). These are inherent disadvantages of the aqueous bioassay system. Alternative methods for monitoring resistance in salmon lice should be developed; these should be simpler, cheaper, allow for higher throughput and possibly partial automation. Molecular biological methods like real-time quantitative PCR could be a welcome addition to the toolbox for resistance monitoring. Better understanding of resistance mechanisms would allow for the identification of resistance-associated genes. Their expression could be monitored and developing resistance might be discovered earlier, ideally before resistance in many individuals of a subpopulation appears (Eng & Prichard 2005). For example, it was shown that avermectins can induce overexpression of resistance-associated genes such as P-gp and other ABC transporters in *C. elegans* (James & Davey 2009). Therefore, we identified several putative ABC drug transporters from the salmon louse and measured their expression levels before and after EMB treatment. Expression changes of resistance-related genes could be used to detect EMB resistance developing in *L. salmonis* subpopulations on salmon farms, with the development of on-site monitoring tools as a long-term objective.

Identifying ABC transporters in *L. salmonis* and their potential role in EMB resistance was the main purpose of this project.

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2 MOLECULAR CLONING AND CHARACTERISATION OF A NOVEL P-GLYCOPROTEIN IN THE SALMON LOUSE, *LEPEOPHTHEIRUS SALMONIS* (KRØYER, 1837)

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(J. Heumann designed and carried out the experiments, analysed the results, prepared the graphics and wrote the manuscript, S. Carmichael conducted the experiment with moderately resistant salmon lice, A. Tildesley provided training and practical assistance, while A. Sturm and J. Bron supervised the work and helped with analysis and writing.)

Abstract

The salmon louse, *Lepeophtheirus salmonis*, is a crustacean ectoparasite of salmonids. At present, sea louse control on salmon farms relies heavily upon chemical treatments. Drug efflux transport, mediated by ABC transporters such as P-glycoprotein (Pgp), represents a major mechanism for drug resistance in parasites. We report here the molecular cloning of a new Pgp from the salmon louse, called SL-PGY1. A partial *Pgp* sequence was obtained by searching sea louse ESTs, and extended by rapid amplification of cDNA ends (RACE). The open reading frame of *SL-PGY1* encodes a protein of 1438 amino acids that possesses typical structural traits of P-glycoproteins, and shows a high degree of sequence homology to invertebrate and vertebrate P-glycoproteins. In the absence of drug exposure, *SL-PGY1* mRNA expression levels did not differ between a drug-susceptible strain of *L. salmonis* and a strain showing a ~7-fold decrease in sensitivity against emamectin benzoate, the active component of the in-feed sea louse treatment SLICE® (Merck Animal Health). Aqueous exposure of the hyposensitive salmon louse strain to emamectin benzoate (24 h, 410 µg/L) provoked a 2.9-fold up-regulation of SL-PGY1. Adult male lice of both strains showed a greater abundance of *SL-PGY1* mRNA than adult females.

2.1 Introduction

Caligid copepods, also called sea lice, are common ectoparasites on wild and farmed marine fish (Costello, 2006). With the expansion of marine and brackish water aquaculture, sea lice have become increasingly important as disease-causing agents (Johnson et al., 2004; Pike & Wadsworth, 1999). The species most problematic for farmed salmonids are the salmon louse *Lepeophtheirus salmonis* (Krøyer, 1837) and *Caligus elongatus* (Nordmann, 1832), which occur principally on salmonid hosts in the Northern hemisphere, and *Caligus rogercresseyi*, which has been reported on farmed salmon in the Southern hemisphere (Costello, 2006). While heavy sea lice infections have the potential to cause high levels of mortality on salmon farms (Pike & Wadsworth, 1999), treatment and management strategies in place today have greatly reduced infection levels (Costello, 2006). The annual costs of sea louse infections to the global salmon industry have been estimated to exceed 300 million euros, which includes the costs of treatments as well as economic losses due to impaired growth and carcass downgrading (Costello, 2009).

As part of integrated pest management strategies and in the absence of effective vaccination procedures, chemical treatments currently have a central role in sea louse control. At present, one of the most common anti-sea louse treatments is the in-feed fish medicine SLICE® (Merck Animal Health), which contains the avermectin drug, emamectin benzoate (4''-deoxy-4'' epimethylaminoavermectin B1) (Stone et al., 1999). Advantages of SLICE are the convenient in-feed administration, the effectiveness against all host-attached life stages of sea lice, the prolonged protection achieved by a standard one-week treatment, and a favourable environmental profile (Stone et al., 1999; Telfer et al., 2006). However, the continued reliance

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on few licensed anti-sea louse medicines potentially promotes the risk of development of drug resistance in the parasite. Decreased treatment efficacies have been reported for a number of drugs used to treat *L. salmonis* infections (e.g. organophosphates (Jones et al., 1992), pyrethroids (Sevatdal & Horsberg, 2003), H₂O₂ (Treasurer et al., 2000)) and *C. rogercresseyi* (emamectin benzoate (Bravo et al., 2010)), while for other compounds used as anti-sea louse agents, resistance formation has been reported in insect pests (Kristensen & Jespersen, 2003; Oppenoorth & Van der Pas, 1977).

Drug resistance in parasites can result from a number of different molecular mechanisms, including changes in the molecular target of the drug that disrupt the drug's action, changes in drug metabolism that inactivate the drug or prevent its activation, changes of drug distribution in the parasite, and amplification of targets to overcome drug action (Wolstenholme et al., 2004). Avermectins are also used to control helminths, and resistance against this class of drugs has been reported in a number of parasitic gastrointestinal nematodes of livestock (Kaplan, 2004). Two main biochemical mechanisms are currently thought to provide the basis of avermectin resistance in parasitic nematodes; changes in ligand-gated chloride channels that constitute the molecular target of the drug (Wolstenholme & Rogers, 2005), and increased expression of drug transporters of the large class of ABC (ATP-binding cassette) proteins, which are thought to decrease internal exposure of the parasite to the drug by efflux pumping activity (Prichard & Roulet, 2007).

ABC proteins constitute one of the largest protein superfamilies, with members in all organisms from bacteria to humans (Dassa & Bouige, 2001). While prototypical ABC proteins are

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transporters coupling ATP hydrolysis with substrate translocation across biological membranes, the ABC superfamily also contains ion channels, regulators of ion channels, receptors, and proteins with roles in ribosome assembly and translation (Dean et al., 2001). In ABC transporters, two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) are required to constitute a functional pump. In full transporters, all required domains are combined in a single polypeptide, while half transporters possess only one TMD and one NBD, and require homo- or heterodimerisation for function (Dassa & Bouige, 2001). Metazoan ABC transporters are currently divided into eight subfamilies (A-H), of which subfamilies B, C and G contain pumps capable of mediating drug transport (Dean et al., 2001; Sheps et al., 2004; Sturm et al., 2009). MDR (multidrug resistance) P-glycoprotein (ABCB1) is a full transporter capable of mediating the cellular efflux of a large array of structurally and functionally unrelated drugs. Expression of ABCB1 can cause MDR in cancer (Chen et al., 1986). In normal tissues, ABCB1 has important roles in the biochemical defence against toxicants (Schinkel et al., 1994). The avermectin drug, ivermectin, is transported by ABCB1 (Bain & LeBlanc, 1996), and mice lacking expression of this transporter in the gut and the brain as the result of targeted gene disruption are markedly hypersensitive to ivermectin (Schinkel et al., 1994). While no data are available for emamectin, ivermectin and five other avermectins affected MDR P-glycoprotein-dependent ATPase activity at low micromolar concentrations, suggesting that they may also be substrates (Lespine et al., 2009). Accordingly, P-glycoprotein has been investigated as a potential factor in the resistance against avermectins in a number of parasites. Ivermectin-selected strains of the nematode helminth, *Haemonchus contortus* (Rudolphi 1803), showed a higher mRNA expression of P-glycoprotein, which coincided with an enrichment of a certain allele at the locus coding for the transporter (Blackhall et al., 1998; Xu et al., 1998). Evidence for a possible genetic selection around a P-glycoprotein locus also exists in the human parasite, *Onchocerca volvulus* (Bickel, 1982) (Ardelli et al., 2006).

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The aim of this study was to isolate cDNA sequences coding for P-glycoprotein in the salmon louse, *Lepeophtheirus salmonis*, and to explore the usefulness of this gene as a potential molecular marker for drug resistance. While the free-living model nematode, *C. elegans*, possesses at least 15 P-glycoproteins (Sheps et al., 2004; Zhao et al., 2007) and the parasitic model nematode, *H. contortus*, possesses ten P-gps (Laing et al., 2013), only two P-glycoprotein sequences were found in the genome of the crustacean, *Daphnia pulex* (Linnaeus 1758) (Sturm et al., 2009). Using the conserved nucleotide binding domains of *Daphnia* ABC transporters as queries, we searched publicly available EST databases for sequences encoding sea louse ABC transporters. By this approach, one partial cDNA coding for a salmon louse P-glycoprotein was isolated, which was called SL-PGY1, and the entire open reading frame (ORF) of the transporter was obtained by rapid amplification of cDNA ends (RACE). The constitutive expression of SL-PGY1 and effects of emamectin benzoate exposure were examined in drug susceptible salmon lice, and lice populations showing a decreased susceptibility to emamectin benzoate. The present study is the first report of an authentic P-glycoprotein sequence in sea lice.

2.2 Material and methods

2.2.1 Salmon lice

A susceptible salmon louse (*L. salmonis*) strain S was established eight years ago in our laboratory, originating from a farm isolate obtained from the West coast of Scotland, where no control agents had been used except for hydrogen peroxide. The sea louse strain R, which showed decreased susceptibility to emamectin benzoate, was established in December 2008 from animals obtained from another salmon farming site of the West coast of Scotland with reports of variable clearance rates following treatment. Following isolation, both strains have been maintained in the laboratory under identical conditions in the absence of exposure to any sea louse treatments. To maintain the lines, gravid female lice are removed from fish under anaesthesia (2-phenoxy ethanol), egg strings are hatched and incubated to the infective copepodid stage, which are then used to re-infect Atlantic salmon (*Salmo salar*). All laboratory infections were carried out under UK Home Office licence and appropriate veterinary supervision. Host fish typically had an initial mean weight of 500-1000 g, and were held in circular tanks provided with a continuous supply of fresh seawater at ambient temperature and at flow rate of 0.5-5 L min⁻¹ per kg fish, and with a photoperiod corresponding to natural day length and adjusted weekly. During copepodid infection challenges, water flows were suspended and copepodids were introduced to the tanks from the incubation vessels. When water flows were restored, fish were inspected for lice settlement and infection rates were maintained at levels that were unlikely to compromise the welfare of the fish. Fish were then inspected regularly to ensure that no re-infections occurred due to the parasite cycling in the tanks.

2.2.2 Emamectin benzoate bioassay

Technical grade emamectin benzoate was donated by Merck Animal Health®. The susceptibility of sea lice to emamectin benzoate was determined in bioassays using 24 hour aqueous exposures (Sevatdal et al., 2005). Salmon lice were collected by anaesthetising host fish with 2-phenoxy ethanol and removing lice using fine forceps. Test solutions were prepared using aerated filtered sea water equilibrated to a temperature of 12°C, the temperature being maintained throughout the assay. PEG300 was used to solubilise the emamectin benzoate (maximum final concentration 0.01%). Seven concentrations of emamectin benzoate and a PEG300 control were tested, using three replicate beakers, each containing 450 mL of testing solution and ten sea lice per treatment. Labelling of test containers was coded to ensure unbiased rating of sea louse motility at the end of exposures, when lice were recorded as normally motile or immotile upon visual examination and stimulation with a fine brush. To confirm exposure concentrations, water samples from selected bioassays were sent to a commercial laboratory for emamectin benzoate residue analysis (Eclipse Scientific, Chatteris, UK). Samples were extracted with acetonitrile before being subjected to liquid chromatography with detection by MS-MS.

2.2.3 On-host exposure of sea lice to emamectin benzoate

An experiment was carried out to assess potential effects of emamectin benzoate on gene expression in sea lice following administration of the drug by treatment of host fish with SLICE. In late May 2010, *L. salmonis* egg strings were obtained from a Scottish salmon

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production site with reports of decreased treatment efficacies, and incubated in seawater until development reached the infective copepodid stage. Fifty naïve Atlantic salmon were infected with copepodids and maintained for four weeks, which allowed lice to develop to the adult stage. The obtained louse population, from here on called SF, was removed from anaesthetised fish, and male adults used for performing an emamectin bioassay. Remaining lice of both sexes were used to infect another batch of naïve fish, which were then randomly allocated to two tanks (20 fish per tank). From day 13 to day 19 post infection, one group of fish was treated with SLICE-containing feed, while the other group continued to be fed a control diet. Uneaten pellets were collected and the estimated average emamectin benzoate delivery was 52.1 µg/kg fish biomass/day for the treated tank. Fish were maintained for a further three weeks until day 40 post infection, when they were anaesthetised and lice counted and preserved pending RNA extraction. Individual lice were sampled into tubes containing RNAlater® and stored at -20°C.

2.2.4 Molecular cloning of sea louse P-glycoprotein

Total RNA was isolated from adult salmon lice of both sexes using a commercial reagent (TRIzol™, Invitrogen). Prior to extraction, lice were ground in liquid nitrogen using a pestle and mortar. The initial protocol (used in all cloning experiments and expression analyses in control lice) used this homogenisation step only. In an improved protocol (used in experiments on the effects of drug exposure on SL-PGY1 expression), the sample was further homogenised for 10 seconds in an Ultra-Turrax® device after addition to the TRIzol® reagent. RNA integrity was confirmed by agarose gel electrophoresis and ethidium bromide staining. RNA concentration and purity were assessed by UV spectroscopy (NanoDrop 1000 spectrophotometer, Thermo Scientific).

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Salmon louse (*L. salmonis*) ESTs available in the GenBank database were searched for sequences showing homology to nucleotide binding domains (NBDs) of *Daphnia pulex* P-glycoproteins (Sturm et al., 2009) using the TBLASTN algorithm. From the hits obtained in this search, a minimum set of contiguous sequences (contigs) was obtained using the Lasergene SeqMan version II software (Lasergene, Madison, U.S.). The contigs were allocated to ABC subfamilies according to their best BLAST hit. One contig, based on two ESTs (GenBank accession numbers: **EX483668**, **EX483669** (Yazawa et al., 2008)), showed a high similarity to MDR P-glycoproteins in other species, but did not resemble published salmon louse P-glycoprotein sequences (Tribble et al., 2008). To obtain the complete open-reading frame of this novel salmon louse P-glycoprotein, called SL-PGY1, rapid amplification of cDNA ends (RACE) was used. 5' and 3' RACE reactions were performed using commercial kits (5'RACE System, Invitrogen or SMART RACE, Clontech) and high quality reverse transcriptase (SuperScript II, Invitrogen) and DNA polymerase (Expand Long Template PCR System, Roche Applied Science). RACE products were subcloned using a commercial T-vector (Promega) and chemo-competent *Escherichia coli* (DH5alpha strain). Plasmids were sequenced with a CEQ 8800 sequencer (Beckman Coulter). To confirm that the obtained RACE products and the contig derived from ESTs corresponded to the same cDNA, the total cDNA sequence of SL-PGY1 was amplified in one PCR (primers: TGGATCCACAAATCATCATCA and TTTTATGATGCGAACAATTGAAT), and subcloned. The sequence reported here was derived from at least three plasmid clones containing the total SL-PGY1 sequence, which were from independent RT-PCRs using different total RNA samples.

2.2.5 Quantitative real-time RT-PCR

The mRNA expression of SL-PGY1 was compared between the two aforementioned salmon louse strains S and R, differing in emamectin benzoate susceptibility. Adult males and females were considered. For each combination of strain and sex, total RNA was obtained from 4-7 pools of five lice each. Subsequent experiments investigating the effects of emamectin benzoate exposure on SL-PGY1 expression were restricted to adult male lice, as they are less susceptible to EMB (Westcott et al., 2008). In a first experiment, animals were pooled prior to RNA extraction as above. Two further experiments performed total RNA extraction and subsequent analyses on single adult lice, taking advantage of an improved extraction protocol (see above). SL-PGY1 mRNA levels were assessed by quantitative real time RT-PCR, using the SYBR Green method and relative quantification (Pfaffl, 2001). Ribosomal protein S 20 (RPS20) and eukaryotic elongation factor 1 alpha (eEF1 α) were used as reference genes, as these have been validated in a previous study (Frost & Nilsen, 2003). 4,000 ng salmon louse total RNA were reverse transcribed with SuperScript II (Invitrogen) using oligo dT primers in a total volume of 20 μ l. Quantitative PCRs were carried out using a commercial kit (ABsolute QPCR SYBR Green Mix, Thermo) and a Quanta Real Time Thermal Cycler (Techne). The product of the reverse transcription was diluted 1:20, and 5 μ l of this dilution was used per PCR reaction (final volume of 20 μ L). Primers were used at 70 nM. Each run comprised of an initial 15 min activation cycle at 95°C, followed by 40 cycles of 15 sec denaturation at 95°C, 30 sec annealing at 60°C and 30 sec elongation at 72°C. Fluorescence was read during the 72°C elongation step. A melting curve analysis was performed following the completion of the PCR programme to confirm that single products had been amplified. Crossing points were analysed using the PCR cycler software. Internal controls included in each run comprised samples in which either the

reverse transcriptase or the cDNA template were omitted. Standard curves were derived from serial dilutions made from a reference cDNA pool. Each measurement was performed in triplicate. Relative expression ratios (RER) (Pfaffl, 2001) were calculated using the following formula:

$$\text{RER} = \frac{(\text{E target})^{\Delta\text{CP target (control - sample)}}}{(\text{E reference})^{\Delta\text{CP reference (control - sample)}}$$

2.2.6 Bioinformatic analyses

The cloned sea louse P-glycoprotein sequence was examined for the presence of conserved domains using InterProScan version 4.6 with standard settings, as implemented on the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (<http://expasy.org>). Further ExPASy programs used in this study were ScanProsite, which was employed to elucidate the extension of NBD (prosite profile PS50893) in different ABC transporters, and TopPred, which served to predict the topology of sea louse P-gp. Evolutionary analyses were carried out using the software package MEGA 4 (Tamura et al., 2007), using the neighbour-joining algorithm and employing 5,000 bootstrapped replicates to derive phylogenies (Tamura et al., 2007).

2.2.7 Statistics

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Median effective concentrations (EC_{50} s) describing the toxicity of emamectin benzoate were derived by probit analysis using the statistical software minitab 15.1 (Minitab Inc). Relative expression ratios of SL-PGY1 mRNA were compared between different groups of lice by one-way ANOVA followed by *post-hoc* comparisons with the Tukey-Kramer test using the program InStat 3.05 (GraphPad Inc.). Data were log-transformed prior to ANOVA, and the homogeneity of variances after transformation was confirmed by Bartlett's test. In all tests, the significance level was set at 0.05.

2.3 Results

To isolate cDNA sequences coding for P-glycoproteins in the salmon louse, *L. salmonis*, we used sequences of conserved NBDs of *D. pulex* P-glycoproteins (Sturm et al., 2009) as the query statement in TBLASTN searches of *L. salmonis* ESTs available in the GenBank database. The obtained *L. salmonis* ESTs were assembled into contigs and assigned to ABC subfamilies according to their best BLASTX hit. Three contigs fell into the ABCB subfamily (Table 1). Within the ABC superfamily, the ABCB subfamily is unique in that it contains both full transporters targeted to the plasma membrane called P-glycoproteins, and half transporters locating to intracellular membranes (Dean et al., 2001). Contig 1 was highly homologous to metazoan P-glycoproteins and named SL-PGY1, while contigs 2 and 3 showed a high homology to mitochondrial ABCB subfamily half transporters (Table 1). Contig 3 contained a region corresponding to SL-Pgp1, a partial *L. salmonis* cDNA previously described as a P-glycoprotein (Tribble et al., 2007), with 97.8% sequence identity in the shared region. A further sequence previously annotated as sea louse P-glycoprotein, named SL0525, showed a high homology to ABCF subfamily proteins in BLASTX searches (Table 1).

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Table 1: Sea louse (*L. salmonis*) ABCB proteins isolated in this study and sequences previously published as putative sea louse P-glycoproteins. cDNA sequences were subjected to BLASTX searches at the National Center of Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>) using default settings.

Sequence	Length (bp)	ESTs (GenBank accession nos.)	Best blastx hit ¹	E-value
Contig 1 (SL-MDR1)	921	EX483668 EX483669	Multixenobiotic resistance protein [<i>Crassostrea virginica</i>]	5 e-76
Contig 2	1080	EX480766 EX480767 FK903290 FK919647 FK919648	ATP-binding cassette sub-family B member 7, mitochondrial [<i>Camponotus floridanus</i>]	1 e-73
Contig 3	1245	FK917819 FK933430 FK933431	ATP-binding cassette sub-family B member 8, mitochondrial precursor [<i>Salmo salar</i>]	1 e-134
SL-Pgp1 ²	642	N/A	ATP-binding cassette, sub-family B (MDR/TAP), member 8 [<i>Xenopus tropicalis</i>]	1e-76
SL0525 ²	717	N/A	ATP-binding cassette sub-family F member 1 [<i>Culex quinquefasciatus</i>]	9e-103

1 Excluding inconclusive databank entries such as “hypothetical protein” etc.

2 From Tribble et al., 2007; GenBank accession numbers **EF093796** and **DQ458787**.

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The evolutionary relationship of sea louse sequences SL-PGY1 and SL-Pgp1 to ABCB subfamily transporters from other organisms was elucidated in a phylogenetic analysis (Figure 3). The sea louse sequence isolated in this study, named SL-PGY1, grouped in one well-supported clade (bootstrap value of 90%) with *Drosophila*, *Daphnia* and human P-glycoproteins (Figure 3). In contrast, SL-Pgp1, an *L. salmonis* cDNA previously described as a P-glycoprotein (Tribble et al., 2007), clustered together with hABCB8, a human mitochondrial transporter, and similar *Drosophila* and *Daphnia* proteins (Figure 3).

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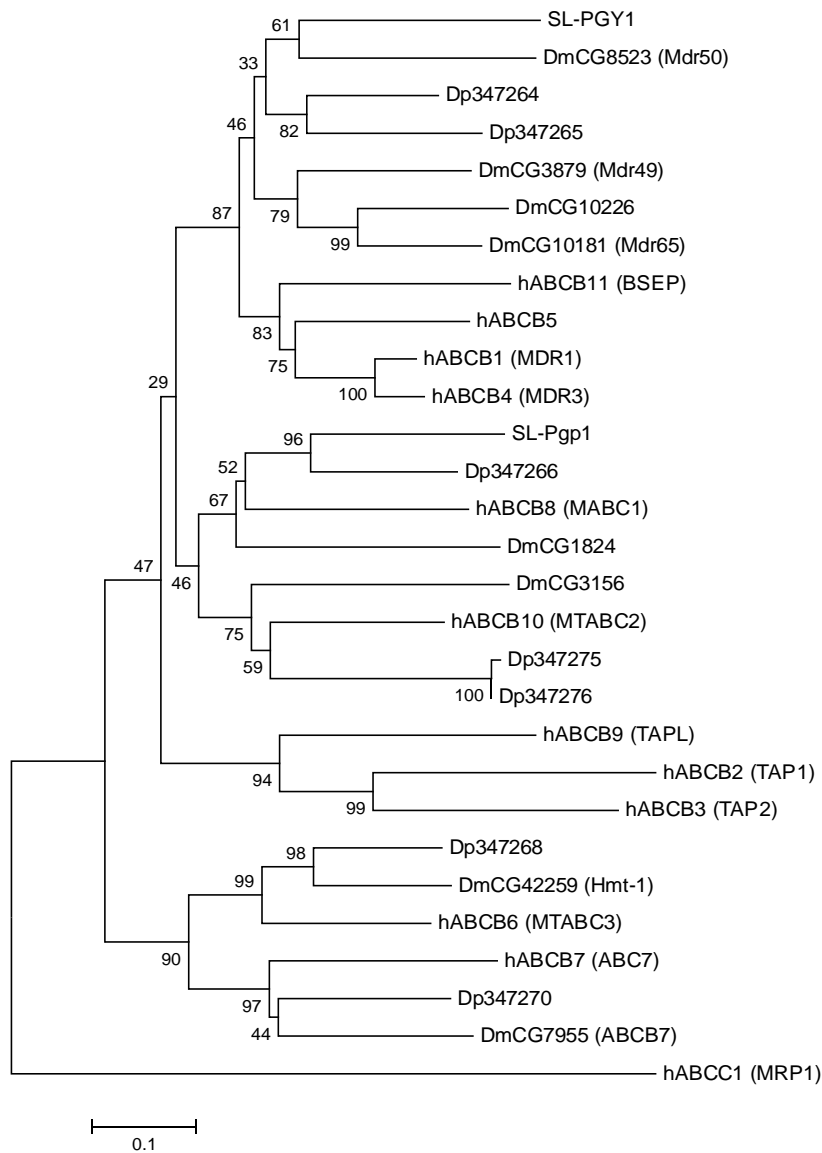


Figure 3: Phylogenetic tree of ABCB transporters. The analysis took into account available sequences from the sea louse *L. salmonis* (SL), the water flea *Daphnia pulex* (dp), the fruit fly *Drosophila melanogaster* (dm) and human (h). Human ABCC was included to root the tree. Predicted amino acid sequences of C-terminal nucleotide binding domains were aligned using ClustalX, and a phylogenetic tree generated using the neighbour joining method (Tamura et al., 2007). Bootstrapping was used to determine the relative support of the various branches (5,000 replicates, support expressed as percent).

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The partial SL-PGY1 cDNA sequence obtained from EST database entries was expanded in 5'- and 3'-RACE reactions using total RNA from adult sea lice, which yielded further sequences that together with the sequence identified *in silico* covered the putative full open reading frame of a SL-PGY1 mRNA (GenBank accession no. HQ684737), with a predicted protein product of 1438 amino acids (Figure 4).

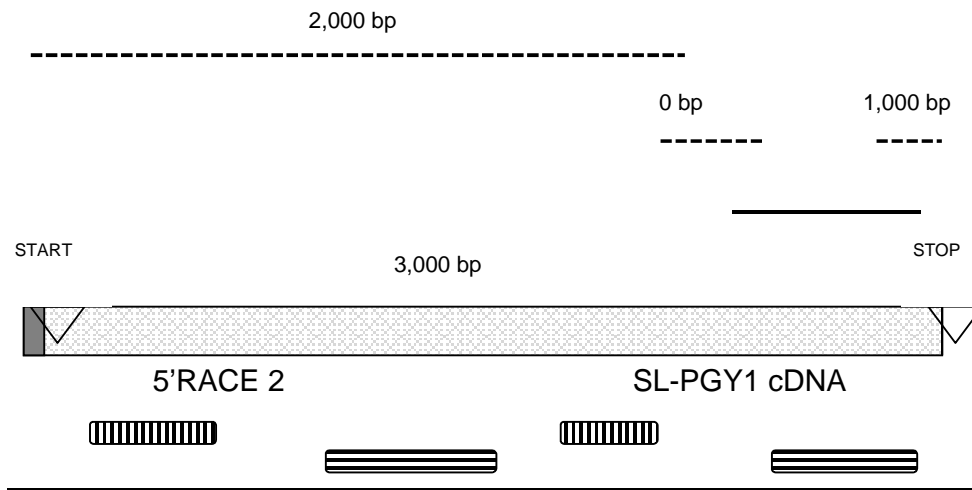


Figure 4: Cloning strategy to obtain *L. salmonis* P-glycoprotein (SL-PGY1). The SL-PGY1 cDNA sequence is represented in the middle, with light grey (coding) and dark grey (non-coding) regions. Lines indicate the relative position of fragments obtained by TBLASTN searches (EST, solid) or rapid amplification of cDNA ends (RACE, dashed). Bars below the SL-PGY1 sequence indicate the position of regions encoding transmembrane (TMD) and nucleotide binding domains (NBD).

The conceptual translation of the SL-PGY1 cDNA sequence was examined for the presence of conserved domains using InterProScan. This analysis detected ABC-transporter transmembrane domains (TMDs) and nucleotide binding domains (NBD), arranged in an N-terminal to C-

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terminal order of TMD-NBD-TMD-NBD, the typical domain architecture of ABC full transporters (Figure 4). The program TopPred was used to predict the topology of SL-PGY1 (Figure 5).

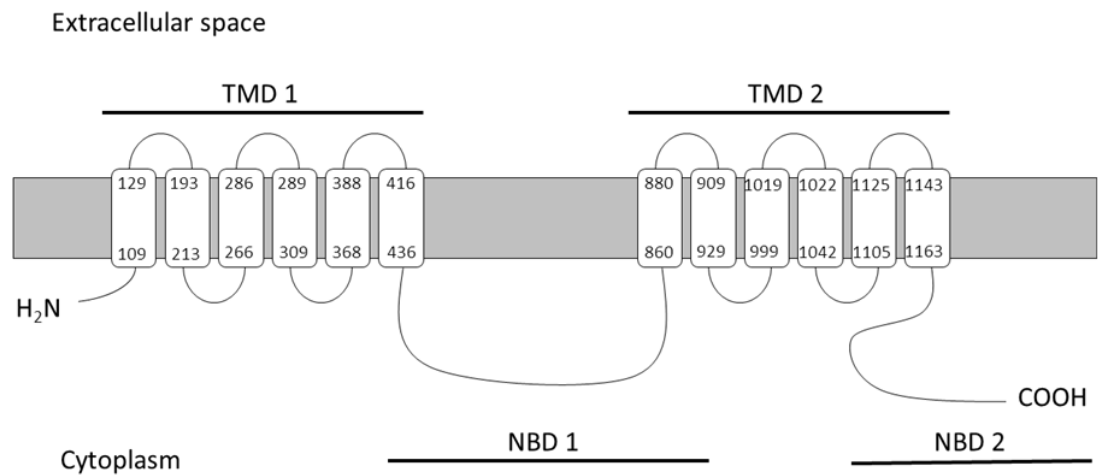


Figure 5: Predicted topology of *L. salmonis* P-glycoprotein (SL-PGY1). Diagram showing the topology of SL-PGY1 predicted by the TopPred algorithm. The grey bar represents the plasma membrane, while membrane-spanning α -helices are shown as white boxes. Numbers represent the first and last amino acid of the respective α -helix. Similar to human MDR1 P-glycoprotein (hABCB1), SL-PGY1 is predicted to have two nucleotide binding domains (NBD) and two transmembrane domains (TMD) consisting of six membrane spanning helices each.

In accordance with current models of P-glycoprotein, the prediction found two TMDs with six transmembrane helices each, and two cytosolic domains.

Different mechanisms are conceivable, by which a putative drug transporter such as SL-PGY1 could cause differences in emamectin benzoate susceptibility between sea louse populations. Less susceptible populations might show increased constitutive SL-PGY1 expression levels, or

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a greater ability to upregulate the transporter upon exposure to the drug. Alternatively, populations with lower susceptibilities may be enriched in allelic polymorphisms of SL-PGY affecting the transporter's affinity for substrates and thus increasing its efficacy to function as a drug pump. To investigate these possibilities, experiments were carried out with two laboratory strains of *L. salmonis* differing in emamectin benzoate susceptibility (Table 2).

Table 2: Susceptibility of two *L. salmonis* strains to emamectin benzoate. Adult females and males were used in 24 h bioassays with motility as the readout.

Strain	Stage	EC ₅₀ (µg/L)	(95% confidence limits)
S	Adult female	82	(63-108)
S	Adult male	145	(106-207)
R	Adult female	524	(360-813)
R	Adult male	1211	(766-2442)
SF	Adult male	615	(502-754)

Compared to a susceptible strain S, which had been isolated before the onset of chemotherapeutant use and maintained in the laboratory since, susceptibility to emamectin benzoate was decreased in the more recently isolated R approximately 7- fold (Table 2). We first compared SL-PGY1 mRNA expression levels between the S and R strains in the absence of emamectin, using quantitative real time PCR and employing either eEF1α or RPS20 as reference genes (Figure 6).

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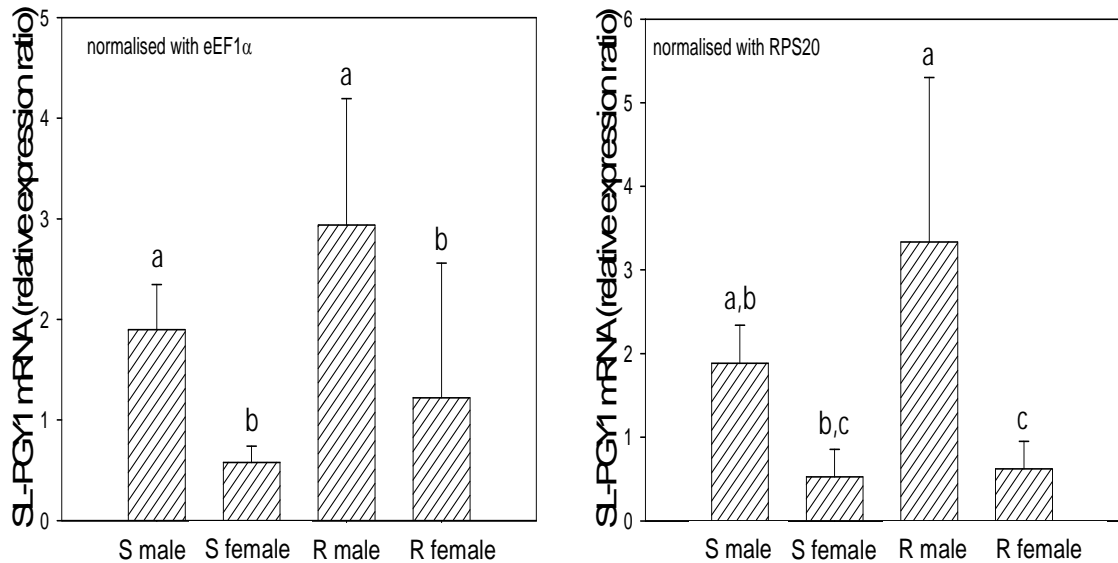


Figure 6: SL-PGY1 mRNA expression in two salmon louse strains. Quantitative real-time RT-PCR was used to analyse mRNA levels of SL-PGY1 by relative quantification using eEF1 α or RPS20 as reference genes (Frost & Nilsen, 2003). Columns show the median and standard deviation of relative expression ratios determined in pools (n = 4-7) of 5 adult salmon lice of both sexes (M = male, F = female) from two strains (S = susceptible; R = 6.4 to 8.4-fold reduced sensitivity to emamectin benzoate). Groups did not differ significantly when columns share a common letter, with the significance level set at 0.05.

Relative mRNA expression levels of SL-PGY1 tended to be higher in male than female lice; however, this difference was not significant for the drug susceptible S strain when eEF1 α was used for normalisation. Regardless of the reference gene used, SL-PGY1 mRNA relative expression levels did not differ significantly between the two strains within males or females (Figure 6). Next, we investigated the effects of acute aqueous exposure of sea lice to emamectin

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benzoate on SL-PGY1 mRNA expression. In a first experiment, adult male sea lice of the S and R strains were exposed for 3 h to a nominal concentration of 200 µg/L of emamectin benzoate (measured concentration at the end of the assay: 99.5 ± 5.2 µg/L). In both louse strains, emamectin benzoate exposure had no effect on SL-PGY1 mRNA levels determined using eEF1 α (Figure 7A) or RPS20 (data not shown) as a reference gene.

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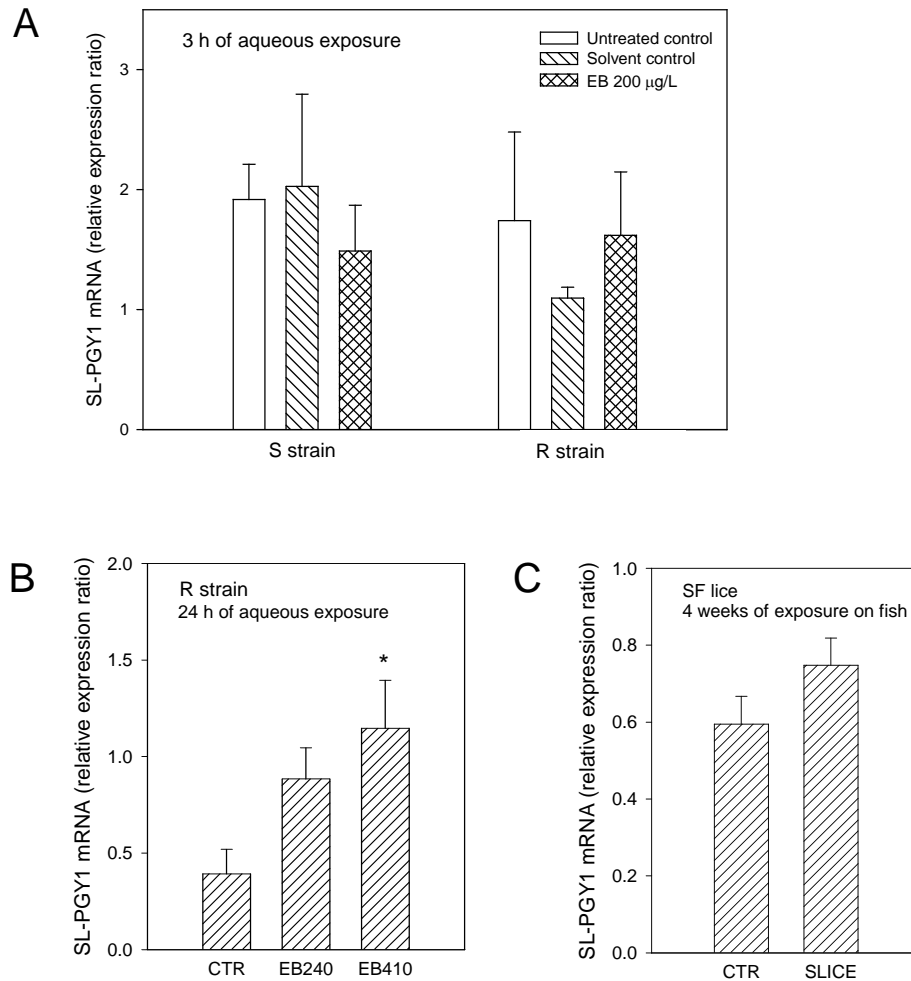


Figure 7: Effect of emamectin benzoate exposure on SL-PGY1 mRNA expression. A, B. Adult male salmon lice of two strains (S, R) differing in emamectin benzoate susceptibility (Table 2) were subjected to waterborne EMB exposure. PEG 300 was used to solubilise EMB (final concentration 0.01%). C. Salmon louse egg strings were obtained from a salmon farm (SF) with reports of decreased SLICE treatment efficiencies, and allowed to develop to the adult stage in the laboratory. Salmon were infected with adult lice, maintained under standard conditions for 12 days, and then given a feed containing SLICE or a control diet for 7 days, followed by a further three weeks of maintenance under standard conditions. Male lice were then collected for gene expression analysis. SL-PGY1 expression was analysed by RT-qPCR using eEF1 α as a reference gene. Columns show the average and standard error of relative expression ratios determined in pools of 4 lice (A: n = 3) or in single lice (B: n=4-6; C: n=12). Asterisk indicates that expression differed significantly from the appropriate control ($P < 0.05$).

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In a second experiment, SL-PGY1 mRNA expression in the R strain was assessed following 24 h of exposure to emamectin benzoate at concentrations of 240 µg/L and 410 µg/L, i.e. a more severe, but still sub-lethal exposure scenario. The mRNA expression of SL-PGY1 (eEF1α as reference) was significantly enhanced by 2.9-fold following exposure to 410 µg/L emamectin benzoate (Figure 7B). However, when RPS20 was used for normalisation, in the same treatment a 1.7-fold increase in expression levels was not statistically significant (data not shown).

Potential drug effects on SL-PGY1 expression might depend on the exposure route. Therefore, a further experiment investigated SL-PGY mRNA expression in sea lice exposed to emamectin benzoate through treatment of their salmon hosts with the oral fish medicine SLICE, using salmon lice grown in the laboratory from egg strings of a salmon farm (SF), where decreased treatment efficacies with SLICE had been reported. SF salmon lice showed a moderate decrease in emamectin benzoate susceptibility in the bioassay (Table 2). Atlantic salmon were infected with adult SF lice. Fish were then randomly assigned into two groups, one untreated control group and one experimental group which was treated with SLICE from day 12 to 18 after infection, mimicking the recommended use of the medicine SLICE as a seven-day treatment course. This is known to result in sustained emamectin benzoate concentrations in the fish and thus protection against sea lice for several weeks. Sea lice were maintained on the fish for a further three weeks after the end of SLICE treatment, before lice were collected and subjected to analyses. SLICE treated fish had 38.4% fewer lice than control fish. No differences in SL-PGY1 mRNA levels were found between lice from untreated and SLICE treated hosts (Figure 7C).

Finally, we explored the possibility that the two sea louse strains S and R might show different haplotype frequencies of polymorphisms of SL-PGY1. RT-PCR was used to amplify cDNAs comprising the entire open reading frame of the transporter from four male lice of each strain, and following the subcloning of the amplicon into a suitable T-vector, we sequenced the inserts

of 2-3 plasmid clones per individual. A number of nucleotide polymorphisms was observed, but with none of the polymorphisms was there a clear difference in haplotype frequencies between the two strains (data not shown).

2.4 Discussion

We report here the molecular cloning of a P-glycoprotein from the sea louse, *L. salmonis*, called SL-PGY1 (GenBank accession number **HQ684737**), and demonstrate that two sequences previously published (Tribble et al., 2007) and deposited in databases as salmon louse P-glycoproteins (GenBank accession numbers **EF093796** and **DQ458787**) are misannotations of ABC transporters unrelated to P-glycoprotein. The high homology of SL-PGY1 to vertebrate and invertebrate P-glycoproteins suggests a role for the transporter in the detoxification of xenobiotics. Two sea louse strains differing in susceptibility to emamectin benzoate did not show distinct mRNA expression levels of SL-PGY1 in the absence of exposure to the drug. Exposure to emamectin benzoate had variable effects on mRNA expression in sea lice, depending on intensity and route of exposure and louse strain. An approximately 3-fold induction of SL-PGY1 mRNA expression was observed in the R strain of sea lice following water-borne exposure to a relatively high emamectin benzoate concentration (410 µg/L). No sequence polymorphisms of SL-PGY1 correlating with emamectin benzoate susceptibility could be identified in this study. Together, the data suggest that SL-PGY1 is probably not the only causative factor responsible for decreased emamectin benzoate susceptibility in the studied sea louse strains, even though upregulation of the pump during acute emamectin benzoate exposure could contribute to the low susceptibility of the R strain. Adult male sea lice showed

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significantly greater levels of SL-PGY1 mRNA than adult females. This could point to potential additional physiological functions of the transporter.

SL-PGY1 isolated in this study shows all the hallmarks of a P-glycoprotein, i.e. it encodes an ABCB subfamily full transporter having two TMDs and two NBDs. In contrast, SL-Pgp previously reported from *L. salmonis* (Tribble et al., 2007) has only one TMD and one NBD, suggesting it corresponds to an ABCB subfamily half transporter. Indeed, SL-Pgp1 grouped together with human and fruit fly mitochondrial ABC proteins in phylogenetic analyses, and showed a greater homology to these proteins than to P-glycoproteins (data not shown). ABCB half transporters are peptide pumps targeted to intracellular membranes (Herget & Tampé, 2007), and have so far not been linked to drug transport. A further sea louse “putative P-glycoprotein sequence” reported by the same laboratory (Tribble et al., 2007, 2008) was found to be an ABCF subfamily protein. ABCF subfamily members lack transmembrane domains and are not transporters, but are factors associated with the ribosome and are involved in the initiation of transcription (Kerr, 2004).

Mammalian MDR1 P-glycoprotein (ABCB1) is a well-known broad spectrum cellular efflux pump for hydrophobic organic chemicals. While originally discovered as a factor associated with multidrug resistance in tumours (Gottesman et al., 2002), the normal physiological role of ABCB1 is to contribute to the biochemical defence against endogenous and foreign toxicants (Leslie et al., 2005). However, further mammalian ABCB subfamily full transporters are unrelated to drug transport, and these include the bile salt efflux pump BSEP/ABCB11 (Gerloff, 1998), the biliary phospholipid transporter MDR3/ABCB4 (Smith et al. 1994) and a protein of unknown function called ABCB5 (Annilo et al., 2006).

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The analysis of ABC transporter phylogeny between the vertebrate and invertebrate genomes revealed numerous lineage-specific gene duplications complicating the inference of function (Dean et al., 2001; Sheps et al., 2004). In the case of P-glycoproteins, however, available data seem to support the notion that the evolutionarily ancient role of these ABC transporters is that of broad spectrum toxicant efflux pumps (Epel et al., 2008). Among non-drug transporting mammalian ABCB proteins, ABCB4 and ABCB5 have arisen within the mammalian lineage and are lacking in birds and fish (Annilo et al., 2006), which strongly suggests they are also absent in invertebrates. While the bile salt efflux pump ABCB11 is found in all vertebrates (Annilo et al., 2006), evidence for bile salt transport by invertebrate P-glycoproteins is missing. In contrast, numerous studies demonstrate in invertebrates the presence of ABCB1-like P-glycoproteins capable of drug transport. The presence of xenobiotic-transporting P-glycoproteins in marine bivalves and sponges was demonstrated in early studies by the group of Kurelec (Kurelec & Pivcević, 1991; Smital & Kurelec, 1998), this being recently confirmed using molecular approaches (Goldstone et al., 2006; Luckenbach & Epel, 2008). In parasitic nematodes, indirect evidence for drug transport by P-glycoproteins is provided by data showing an increased expression of P-glycoproteins in an ivermectin-resistant strain of *Haemonchus contortus* (Xu et al., 1998), and by the fact that inhibitors of P-glycoprotein reverse ivermectin resistance in isolates of *Teladorsagia circumcincta* (Stadelman, 1894) and *H. contortus* (Bartley et al., 2009). In the free-living nematode, *Caenorhabditis elegans* Maupas, 1900, there are at least 15 P-glycoproteins (Sheps et al., 2004; Zhao et al., 2007), of which three (pgp-3, pgp-14 and C44C10.3) were upregulated by pesticide exposure (Lewis et al., 2009), while pgp-1 had a protective role against toxic metals (Broeks et al., 1996) and showed increased expression in an ivermectin resistant strain (James & Davey, 2009). Similarly, in the fruit fly, P-glycoprotein

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mdr49 is induced upon exposure to environmental toxicants (Tapadia & Lakhota, 2005; Vache et al., 2007).

In the present study, the upregulation of SL-PGY1 mRNA in the R sea lice strain in response to 24 h of aqueous exposure to 410 µg/L emamectin benzoate is in accordance with a hypothetical role for the transporter in the biochemical defence against toxicants. However, SL-PGY1 mRNA was not upregulated in R sea lice exposed to 240 µg/L emamectin benzoate, a concentration which would cause a 100% paralytic response in the S strain. Similarly, SF lice showed no upregulation of SL-PGY1 mRNA despite showing only a partial responsiveness to a SLICE treatment. Together this demonstrates that a phenotype of decreased emamectin benzoate susceptibility can be observed in the absence of SL-PGY1 mRNA upregulation. While we cannot exclude the possibility that SL-PGY1 protein levels were changed in exposed lice despite the absence of apparent regulation on the mRNA level, it appears likely that factors other than SL-PGY1 are involved in determining sea louse susceptibility to emamectin benzoate.

Using a strategy that does not make prior assumptions regarding the number of Pgps in *L. salmonis*, we isolated one sequence called *SL-PGY1* from this species. Similarly, only two P-glycoprotein sequences were found in the genome of the crustacean *D. pulex* (Sturm et al., 2009). However, among further ABC proteins that could potentially function as drug efflux transporters, *Daphnia* shows at least six ABCC proteins and 23 ABCG proteins (Sturm et al., 2009). Accordingly, a promising direction for future research in sea lice will be to extend the search for potential markers of drug resistance to other ABC families.

2.5 Acknowledgements

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3 ISOLATION AND CHARACTERISATION OF FOUR PARTIAL cDNA SEQUENCES ENCODING MULTIDRUG RESISTANCE- ASSOCIATED PROTEINS (MRPs) IN THE SALMON LOUSE *LEPEOPHTHEIRUS SALMONIS* (KRØYER, 1837)

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Abstract

The salmon louse *Lepeophtheirus salmonis* (Krøyer, 1837) is an ectoparasite of salmonid fish severely affecting cultured salmon production in the North Atlantic. Salmon louse control on farms currently relies in part upon the use of veterinary drugs, however, as only a limited number of salmon delousing agents is available, there are concerns about development of drug resistance in salmon lice. The common anti-salmon louse drug SLICE® (Merck Animal Health) contains the avermectin, emamectin benzoate (EMB). Members of this large gene superfamily of ABC (ATP-binding cassette) transporters have been identified as potential avermectin resistance factors in parasitic nematodes. In salmon lice, only three ABC transporters have been cloned and studied to date. We report here upon the isolation of four novel *L. salmonis* ABC transporters, and employ an inhibitor-based approach to assess the role of *L. salmonis* ABC transporters in the toxicology of EMB. To isolate salmon louse ABC transporters, publicly available *L. salmonis* expressed sequence tags (ESTs) were subjected to homology searches, and the retrieved ESTs assembled into contiguous sequences and annotated using the BLASTx algorithm. Potential ABC drug transporters isolated by this approach comprised four multidrug resistance-associated proteins (MRPs). In addition, five ABC proteins having likely roles unrelated to drug resistance were also obtained. Quantitative real time PCR (RT-qPCR) was used to analyse mRNA levels of the MRPs in *L. salmonis* strains differing in EMB susceptibility. In the absence of their EMB, all studied MRPs showed similar transcript levels in the drug-susceptible strain S and the moderately EMB-resistant strain PT. Moreover, mRNA expression of the four studied MRPs remained unaffected by exposure to EMB. Taken together, the results of RT-qPCR analyses did not provide evidence for roles of the studied MRPs as

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factors affecting EMB susceptibility. Further experiments used an inhibitor-based approach to investigate the roles of ABC transporters in EMB toxicity. In immotility bioassays, salmon lice of the two strains were exposed to EMB, provided alone or in combination with cyclosporin A or verapamil, which are known inhibitors of P-glycoprotein and MRPs, respectively. Cyclosporin A increased EMB toxicity to a similar degree in both strains, suggesting the biochemical factors affected by this inhibitor show similar levels in both strains. In contrast, verapamil increased EMB effects only in the PT strain. This result could be indicative of enhanced expression of hitherto unknown ABC transporters in the moderately EMB-resistant PT strain. More research is required to identify the target of verapamil in salmon lice.

Keywords: ABC transporter, MRP, drug resistance, emamectin benzoate, sea lice

3.1 Introduction

Sea lice (Copepoda: Caligidae) are marine fish ectoparasites feeding on their host's skin, mucus and blood. *Lepeophtheirus salmonis* (Krøyer, 1837), the salmon louse, infects farmed and wild Atlantic salmon (*Salmo salar* L.), and can cause stress, lead to reduced growth and promote secondary infections with water-borne viruses, bacteria and fungi in the host (Pike & Wadsworth 2000). In addition to farm management strategies, the use of veterinary drugs constitutes a key component of sea louse control on fish farms. Globally, the cost of sea lice infections of farmed salmon is estimated to exceed €300 million (Costello 2009).

The avermectin compound emamectin benzoate (EMB, 4'-deoxy-4'' epimethylaminoavermectin B₁) is the active component of the delousing agent SLICE® (Merck Animal Health) (Stone et al., 2000). SLICE is conveniently administered through feed and confers up to nine weeks of protection to salmon ingesting the medicated feed (Stone et al., 2000). However, losses of EMB efficacy in *L. salmonis* have been recently reported from Scotland and New Brunswick (Lees et al., 2008; Jones et al. 2013), and this could be indicative of the early stages of drug resistance. Similarly, reductions in EMB sensitivity have been reported for the Chilean sea louse species *Caligus rogercresseyi* (Bravo et al., 2008; Bravo et al., 2010).

Drug resistance in parasites can be based on several molecular mechanisms including structural changes of the molecular drug target, as well as the up-regulation of detoxification pathways involving drug metabolising enzymes and drug transporters (Wolstenholme et al., 2004). Drug transporters of the ABC gene family mediate the cellular efflux of a diverse range of substrates through an ATP-dependent mechanism (Shimabuku et al., 1992). Among the eight known metazoan ABC subfamilies (Dean et al., 2001), subfamilies B, C and G contain drug

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transporters including P-glycoprotein (P-gp/MDR1/ABCB1), the multidrug resistance-associated proteins MRP1-3 (ABCC1-3) and the breast cancer resistance protein (BCRP/ABCG2) (Leslie et al., 2005). In mammals, avermectins are transported by P-gp (Bain and LeBlanc, 1996; Igboeli et al., 2012) and MRPs (Lespine et al., 2006). ABC transporters have been proposed as factors potentially associated with avermectin resistance in nematodes. In ivermectin-resistant nematodes, an increased expression of P-gp has been reported for the parasite *H. contortus* (Xu et al., 1998), while an up-regulation of both P-gps and MRPs was found in free-living *C. elegans* (James & Davey, 2009).

In a previous study, we have reported the cloning and characterisation of the P-gp homologue SL-PGY1 in *L. salmonis* (Heumann et al., 2012). However, constitutive SL-PGY1 mRNA levels were similar between a moderately EMB-resistant and a drug-susceptible strain of salmon lice, suggesting that other factors may be involved in emamectin hyposensitivity (Heumann et al., 2012). In the present study, we set out to isolate further ABC drug transporters from *L. salmonis* and analyse their mRNA expression in two salmon louse strains differing in EMB susceptibility. In addition, potential roles of ABC transporters as biochemical factors modulating the effects of EMB in salmon lice were investigated using inhibitors of ABC protein function.

3.2 Material and Methods

3.2.1 Bioinformatics

Nucleotide binding domains (NBD) of *Daphnia pulex* ABC transporters (Sturm et al., 2009) were isolated using the software tool ScanProsite (profile PS50893), as provided by ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (<http://expasy.org>, last accessed 28th May 2013). NBDs were used as queries to search ESTs available in the GenBank database for homologous sequences in *L. salmonis* using the TBLASTn algorithm. Hits were parsed and assembled into contiguous sequences (contigs) using Lasergene® SeqMan™ software version 2 (LaserGene®, Madison, USA). To confirm contig identities, contigs were used to query non-redundant protein sequences using the BLASTx algorithm as implemented on the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). Phylogenetic analysis compared NBDs from *Homo sapiens* and *L. salmonis*. Sequences were aligned with ClustalX and subjected to phylogenetic analyses with the programme Molecular Evolutionary Genetics Analysis (MEGA) version 4 (Tamura et al., 2007), using the neighbour-joining algorithm and limiting bootstrapping to 5,000 replicates.

3.2.2 Salmon louse laboratory strains

Salmon louse laboratory strains used in this study have been described previously (Heumann et al., 2012). In brief, the strain S is susceptible to all current anti-sea louse treatments and has been in culture since 2003, when it was isolated from a Scottish salmon production site at which no delousing agents were used, except for hydrogen peroxide. The strain PT shows a 7-fold decrease in susceptibility to EMB (Heumann et al., 2012). It was obtained from egg strings collected in 2008 on another production site observing variable results for EMB treatments. The SF strain displays a 4-fold decrease in EMB sensitivity (Heumann et al., 2012), and was collected in 2010 from another fish farm also reporting variable clearances following EMB application.

The maintenance of salmon louse strains was carried out under standardised laboratory conditions in the absence of exposure to delousing agents, as described in detail before (Heumann et al., 2012). In brief, host salmon (weight 500-1,000 g) were kept in circular tanks with continuous seawater flow-through ($0.5-5 \text{ L min}^{-1}$ according to stocking density) at ambient temperature and photoperiod. In order to propagate the populations, louse-bearing salmon were anaesthetised with 2-phenoxy-ethanol and gravid females carefully removed with forceps. Egg-strings were detached and allowed to hatch in aerated seawater. When larvae had developed into infective copepodids, they were used to re-infect salmon. For infection, water flow to the tanks was temporarily suspended and a limited number of copepodids was released into the tank which would lead to louse settlement rates unlikely to compromise the host's health. After the water flow was restored, the fish were checked regularly for the number of settled lice and general welfare. All handling and experimental infections of host fish took place under veterinary supervision and in accordance with UK Home Office regulations.

3.2.3 On-fish exposure of salmon lice to emamectin benzoate

SF strain lice were used to infect a batch of naïve Atlantic salmon, which were then randomly allocated to two tanks (20 fish per tank). From day 13 to day 19 post-infection, fish in one of the tanks were treated with SLICE®-containing feed, while fish in the other tank continued to be fed a control diet. The average emamectin benzoate delivery was estimated at 52.1 µg per kg fish biomass and per day for the treated tank, based on the amount of food given and the number of uneaten pellets collected. This dose is close to the recommended dose of 50 µg per kg fish biomass and per day (Stone et al., 2000). Both groups of fish were maintained on a control diet from day 20 to day 40 post infection. EMB shows a relatively long half-life in fish, and following a standard 7-day treatment with SLICE®, EMB will be detectable in mucus for several weeks (Sevatdal et al., 2005). At day 40, host fish were anaesthetised with 2-phenoxy-ethanol, and salmon lice were carefully collected using forceps and stored in RNAlater® (Sigma-Aldrich, Dorset) for subsequent RNA extraction.

3.2.4 Salmon louse immotility bioassays

Seawater immotility bioassays were used to assess the susceptibility of adult male salmon lice to EMB (Westcott et al. 2008). Salmon lice were carefully collected from host fish anaesthetised with 2-phenoxy-ethanol and allowed to recover for 2 h in filtered (55 µm), aerated seawater (referred to as 'seawater' from here on) at 12°C. Emamectin benzoate (EMB technical grade

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donated by Merck Animal Health) was solubilised in PEG300 and a dilution series prepared in seawater directly before the bioassay. The final PEG300 concentration was 0.01% (v/v) for all EMB concentrations. In addition to seawater and solvent controls lacking toxicant (0.01 % PEG300), a serial dilution series of five EMB concentrations was included in each test, with the selection of concentrations depending on the sensitivity of the louse strain tested and whether inhibitors of ABC transport activity were included (see below). Ten adult male lice were used per 90 mm petri dish containing 60 mL of testing or control solution. Depending on the availability of salmon lice, experimental treatments were run in duplicate or triplicate, while controls were run either as single replicate or duplicate. After 24 hours of incubation at 12°C in the dark, salmon lice were rated as normal or immotile (*i.e.* not swimming away when gently touched with a soft brush) (Westcott et al., 2008).

3.2.5 Seawater exposure of salmon lice to EMB

To investigate short-term effects of EMB on transporter mRNA expression, adult male salmon lice of strains S and PT were subjected to acute sub-lethal exposures to EMB. A first experiment involved exposure to 200 µg L⁻¹ EMB, seawater, or solvent (0.01% PEG300, v/v) for 3 h. A second experiment used 24 h of exposure time and higher EMB (240 and 410 µg L⁻¹) levels with adult male salmon lice of the PT strain. Seawater and solvent controls were also included. In both experiments, no effects on salmon louse motility were observed. At the end of exposures, animals were collected in RNAlater® (Sigma-Aldrich, Dorset, UK) and stored at -20 °C pending total RNA extraction.

3.2.6 Co-administration of EMB and ABC transporter inhibitors

To investigate potential roles of ABC transporters in the toxicology of EMB, a motility bioassay with EMB was carried out in the presence or absence of the functional inhibitors of ABC transporters cyclosporin A (CSA, 10 μ M, VETRANAL® analytical standard) and verapamil (VER, 20 μ M, (\pm)-verapamil hydrochloride, pharmaceutical standard, Sigma-Aldrich, Dorset, UK). Immotility bioassays were carried out in the presence of one or other inhibitor as described above. While VER solutions were prepared in seawater, the solvent DMSO (0.01% v/v) was used as a vehicle for CSA. Seawater controls and appropriate solvent controls were included in all assays.

3.2.7 Total RNA extraction

Individual frozen adult male salmon lice were removed from RNAlater®, transferred to a pre-chilled mortar and ground with a pestle while covered with a small amount of liquid nitrogen. TRIzol® (Life Technologies, Paisley, UK) was added to the ground sample, which was further homogenised with an Ultra-Turrax® for 30s. Total RNA was extracted following instructions given in the TRIzol® manual. The concentration and purity of the obtained aqueous RNA samples were determined by UV spectroscopy using a NanoDrop® ND-1000 photo spectrometer (Thermo Scientific, Delaware, USA). Integrity of RNA samples was assessed by agarose gel (1%) electrophoresis under denaturing conditions followed by staining with ethidium bromide. Samples were stored at -80°C until further use.

3.2.8 Reverse transcription quantitative real time PCR (RT-qPCR)

To assess the mRNA expression of *L. salmonis* ABCC transporters identified by the bioinformatic approach described above, RT-qPCR primers were designed with Primer3 (<http://frodo.wi.mit.edu/>, accessed 28th May 2013) to yield 70-85 bp amplicons. Primer sequences are given in Table 3, which also compiles the GenBank accession numbers of ESTs corresponding to the transporters.

Table 3: Overview of four novel ABCC transporters investigated in *L. salmonis*. ESTs assembled to contigs, their size, primer sequences for RT-qPCR and respective amplicon sizes are given.

Name	Accession numbers of ESTs assembled	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
SL-MRP1	EX481436.1 EX481437.1 FK908937.1 FK908938.1	TTCGATGTGT AGTGATGCT GGTCTC	AAGTGCGAGTC CTTCAATCCTT CC	97
SL-MRP2	FK904156.1	CGCTTTTCCG TTTATCGAGT TTTTC	ACAGCAAAGT ACGACGGAAT CTA	100
SL-MRP3	FK917841.1 FK917842.1	TGGGACAAA GACAGTTGA TTTGCTT	CAGCAGCAGTA GCTTCGTCCATT AT	84
SL-MRP4	FK916336.1 FK916337.1 FK930382.1 FK930383.1	AATAGATCC CTCTGGCCA AACAAA	ATGGACCTCCTT GGTCATCGTAC TT	80

For each salmon louse sample, 500 ng total RNA were reverse transcribed using the High Capacity cDNA RT Kit (Life Technologies™) following the manufacturer's instructions. The

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obtained first-strand cDNA was stored at -20°C. RT-qPCR was carried out using the SYBR® Green method using a commercial kit (ABsolute™ QPCR SYBR® Green Mix, Thermo Scientific, Loughborough, UK) according to the instructions provided. RPS20 (ribosomal protein S 20) and eEF1a (eukaryotic elongation factor 1 alpha) were used as reference genes (Heumann et al., 2012). PCR reactions were performed in a Quantica® Real Time Thermal Cycler (Techne, Stone, UK). The thermocycling programme comprised an initial 15 min activation step at 95°C, followed by 40 cycles of 15 s denaturation at 95°C, 30 s annealing at 60°C and 30 s elongation at 72°C. Fluorescence was measured during the 72°C elongation step. Following completion of the PCR programme, a melting curve analysis was performed to confirm that only single products had been amplified. Crossing points were analysed using the PCR cycler's software Quantsoft®. Internal controls were included in each run, comprising samples where reverse transcriptase or the cDNA template had been omitted. Standard curves were derived from serial dilutions made from a reference cDNA pool. Each measurement was performed in triplicate and raw data quality checked. Relative expression ratios (RER) were calculated using the following formula (Pfaffl, 2001):

$$\text{RER} = \frac{(E_{\text{target}})^{\Delta\text{CP}_{\text{target}}(\text{control} - \text{sample})}}{(E_{\text{reference}})^{\Delta\text{CP}_{\text{reference}}(\text{control} - \text{sample})}}$$

3.2.9 Statistical analysis

Median effective concentration (EC₅₀) of EMB was calculated by probit analysis using Minitab 15 (Minitab Inc.). Relative expression ratios of MRPs were compared between different groups of salmon lice by one-way ANOVA followed by *post-hoc* comparisons with the Tukey-Kramer test using InStat 3.05 (GraphPad Inc., La Jolla, USA). Data were log-transformed prior to ANOVA testing and the homogeneity of variances confirmed by Bartlett's test. In all tests the significance level was set at $p < 0.05$.

3.3 Results

3.3.1 Identification of ABC transporter sequences in salmon louse

Nucleotide binding domains (NBDs) were extracted from *Daphnia pulex* ABC transporters (Sturm et al., 2009) using ScanProsite (profile PS50893) and used as query sequences in parallel tblastn searches of *L. salmonis* ESTs available at GenBank. The 127 EST hits found by this strategy were further analysed with SeqMan™ (LaserGene®) to obtain a minimal set of 18 contiguous sequences (contigs). Blastx searches were carried out to further characterise the 18 sequences obtained. Five contigs were eliminated as they were not coding for eukaryotic ABC proteins. The remaining 13 contigs represented salmon louse ABC transporters (Table 4). The best hits from blastx comparison with well-characterised protein sequences (UniProtKB/SwissProt database) indicated the likely ABC subfamily affiliation of the isolated

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contigs (Table 4). To confirm the subfamily assignment, NBDs were extracted from the isolated contigs as described above and were subjected to phylogenetic analysis together with NBDs of human ABC transporters (Figure 8).

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Table 4: Partial salmon louse ABC transporter cDNA sequences obtained by homology searches (TBLASTn) of ESTs in the GenBank database.

Subfamily	Contig number	Preliminary name	Length (bp)	Number of ESTs combined	Best BLASTX hit	Domains	E value
ABCB (half)	Contig 6	N/A	1080	5	ATP-binding cassette sub-family B, member 7 [<i>Rattus norvegicus</i>]	NBD	3.00E-89
ABCB (half)	Contig 9	N/A	1245	3	ATP-binding cassette sub-family B, member 8 [<i>Danio rerio</i>]	TM(partial)-NBD	4.00E-169
ABCB (full)	Contig 11	SL-PGY1	921	2	Multidrug resistance protein 3 [<i>Rattus norvegicus</i>]	NBD	1.00E-89
ABCC	Contig 5	SL-MRP1	937	5	Multidrug resistance-associated protein 1 [<i>Mus musculus</i>]	NBD2	7.00E-73
ABCC	Contig 7	SL-MRP2	1558	4	Multidrug resistance-associated protein 1 [<i>Macaca fascicularis</i>]	NBD2	2.00E-110
ABCC	Contig 13	SL-MRP3	1232	2	Multidrug resistance-associated protein 1 [<i>Homo sapiens</i>]	NBD2	1.00E-121
ABCC	Contig 14	SL-MRP4	830	1	Multidrug resistance-associated protein 4 [<i>Homo sapiens</i>]	NBD2	3.00E-77
ABCD	Contig 18	N/A	850	1	ATP-binding cassette sub-family D member 4 [<i>Homo sapiens</i>]	NBD	0.22
ABCE	Contig 3	N/A	1299	10	ATP-binding cassette sub-family E member 1 [<i>Homo sapiens</i>]	NBD(partial)-NBD	0.0

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ABCF	Contig 1	N/A	2048	27	ATP-binding cassette sub-family F member 2 [<i>Mus musculus</i>]	NBD-NBD	0.00
ABCF	Contig 4	N/A	1549	9	ATP-binding cassette, sub-family F member 3 [<i>Rattus norvegicus</i>]	NBD(partial)-NBD	1.00E-170
ABCF	Contig 12	N/A	1077	2	ATP-binding cassette, sub-family F, member 1 [<i>Rattus norvegicus</i>]	NBD	2.00E-97
ABCF	Contig 17	N/A	827	1	ATP-binding cassette, sub-family F member 3 [<i>Homo sapiens</i>]	NBD	8.00E-20

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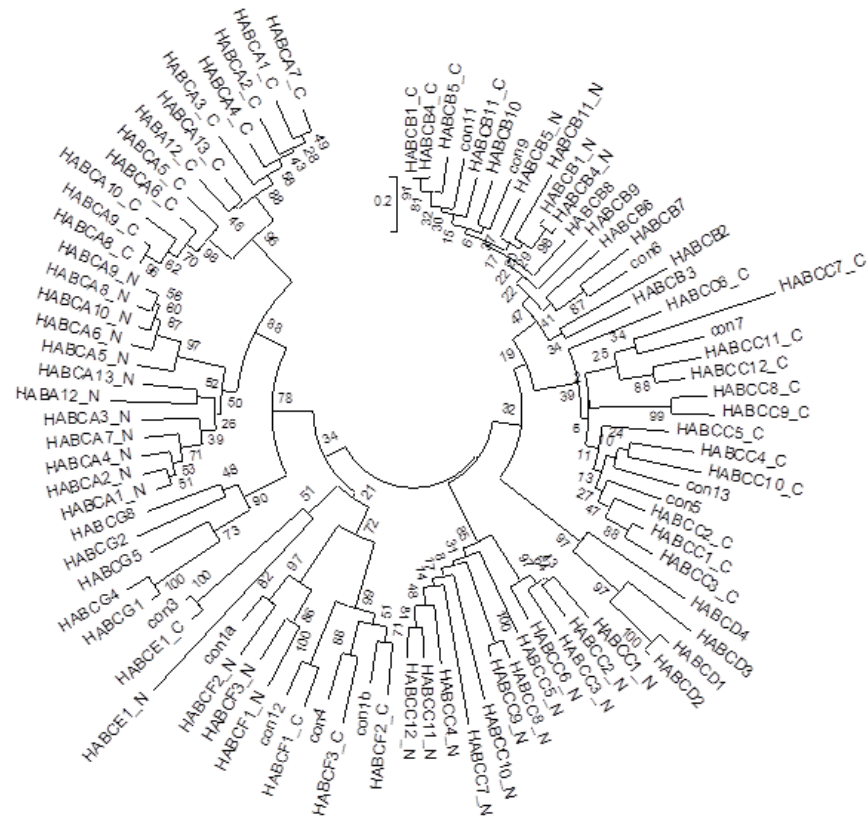


Figure 8: Phylogenetic analysis of human *H. sapiens* and salmon louse *L. salmonis* ABC transporter NBDs. Nucleotide binding domain sequences of ABC proteins were aligned with ClustalX and phylogeny established by generating a tree using the neighbour-joining method (Tamura *et al.* 2007) with bootstrapping set to 5,000 replicates. Relative support for the individual branches is given in percent. “HABC” indicates human ABC transporters, the following letter gives the subfamily and number of the member within the subfamily. For full transporters, the “_C” and “_N” suffix are added to indicate the C-terminal or N-terminal NBD, respectively. Partial cDNA sequences from *L. salmonis* ABC transporter NBDs are named “con”.

Sequences of *L. salmonis* clustered together with *H. sapiens* sequences of the same subfamily, supporting the previous assignments to ABC subfamilies (Table 4). ABC drug transporters are known to be found among the full transporters of subfamily B, as well as among members of subfamilies C and G. Accordingly, five out of the 13 isolated contigs, belonging to subfamilies B and C, were proposed as candidates for potential drug transporters in salmon lice.

The contig representing an *L. salmonis* ABCB full transporter has been fully cloned and characterised previously (Heumann et al., 2012). In this study, we investigated the mRNA expression of *L. salmonis* transporters of the ABCC subfamily showing similarities to MRPs in other organisms. For the purpose of identification within this study, the partial salmon louse MRP sequences were termed SL-MRP1 to SL-MRP4 (Table 4). The definitive naming of the transporters will require further research, including isolation of the full sequences.

3.3.2 Expression analysis

RT-qPCR was used to quantify mRNA levels of the isolated MRPs in salmon louse strains differing in their susceptibility to EMB. In addition to determining transcript levels in salmon lice prior to drug exposure, different exposure scenarios were used to study whether exposure to EMB increased expression of the MRPs over time. RPS20 or eEF1 α were used as reference genes for relative quantification. Because essentially identical results were obtained with both reference genes, data are shown only after normalisation with eEF1 α .

Following exposure of salmon lice to 200 $\mu\text{g L}^{-1}$ EMB for 3 hours, no significant effects of the treatment was found on transporter mRNA expression levels regardless of strain (Figure 9).

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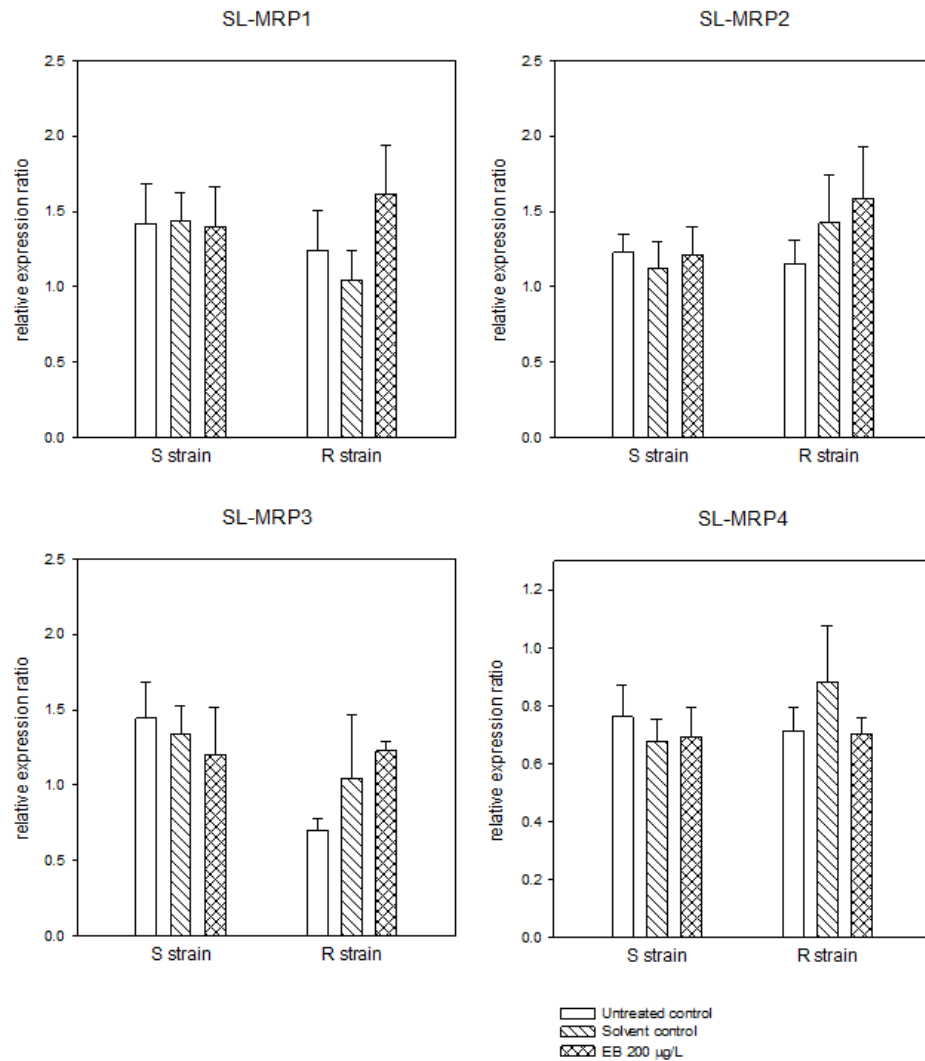


Figure 9: Messenger RNA expression levels of four MRPs in salmon lice with different genetic backgrounds and previous EMB exposure history. Adult male salmon lice of the drug-susceptible S strain or the moderately EMB-resistant PT strain were exposed to 200 µg L⁻¹ of EMB for 3 h, seawater (controls) or 0.01% DMSO (solvent controls). Relative expression ratios of the four MRPs transporters were measured with RT-qPCR using untreated, solvent-control and EMB-treated salmon lice. The reference gene was eukaryotic elongation factor 1 alpha (eEF1α). Mean relative expression ratios and standard deviations are shown, based on triplicate total RNA samples obtained from independent pools of four salmon lice. Variation of mRNA among treatments was not significant ($p > 0.05$).

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Moreover, transporter mRNA levels were similar between salmon lice of the two strains (Figure 9). In a second experiment using PT strain lice, exposure to 240 $\mu\text{g L}^{-1}$ or 410 $\mu\text{g L}^{-1}$ of EMB for 24 h had no apparent effects on the mRNA expression of MRPs (Figure 10).

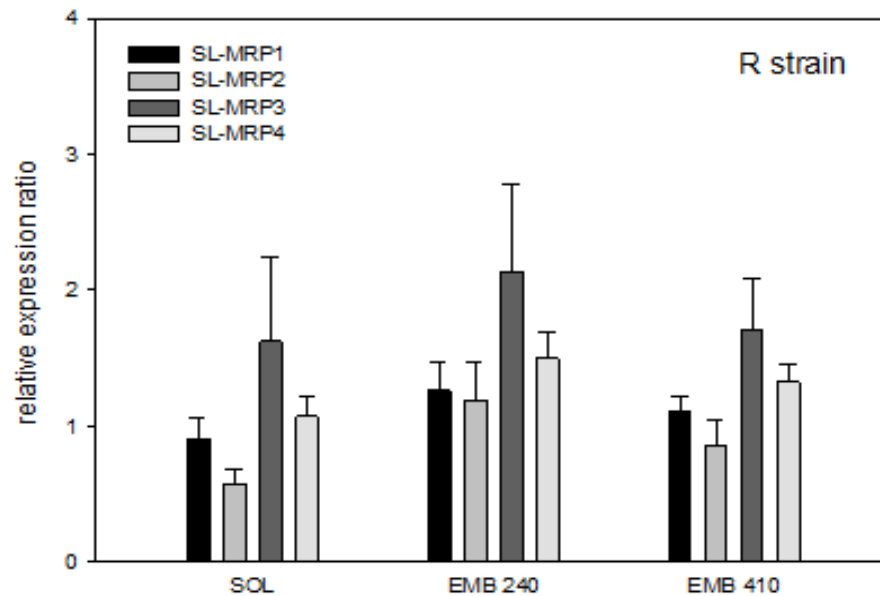


Figure 10: Effect of 24 h-exposure to EMB on the mRNA expression levels of four MRPs in salmon lice of the R strain. Adult male lice were exposed to 240 or 410 $\mu\text{g L}^{-1}$ EMB (S and R strain), incubated in seawater (controls) or seawater containing 0.01% DMSO (solvent controls). Solvent-control and treated lice were collected after 24 h for gene expression analysis. Data were normalised with eukaryotic elongation factor 1 alpha (eEF1 α). Means with standard deviation are shown for each group (n = 4-6 lice).

An additional experiment investigated the effects of long-term oral uptake of EMB by salmon lice on MRP expression, using an exposure scenario resembling a typical treatment. Salmon bearing *L. salmonis* of the SF strain were treated with medicated feed containing SLICE® (50

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$\mu\text{g EMB kg}^{-1} \text{ fish day}^{-1}$) for 7 days in the treated group (Stone et al., 2000), while louse-bearing fish of the control group continued to receive a control diet. Host fish were then maintained for a further three weeks before salmon lice were collected for transcript expression analysis. No significant differences in MRP relative expression ratios were observed between lice from treated and untreated salmon (Figure 11).

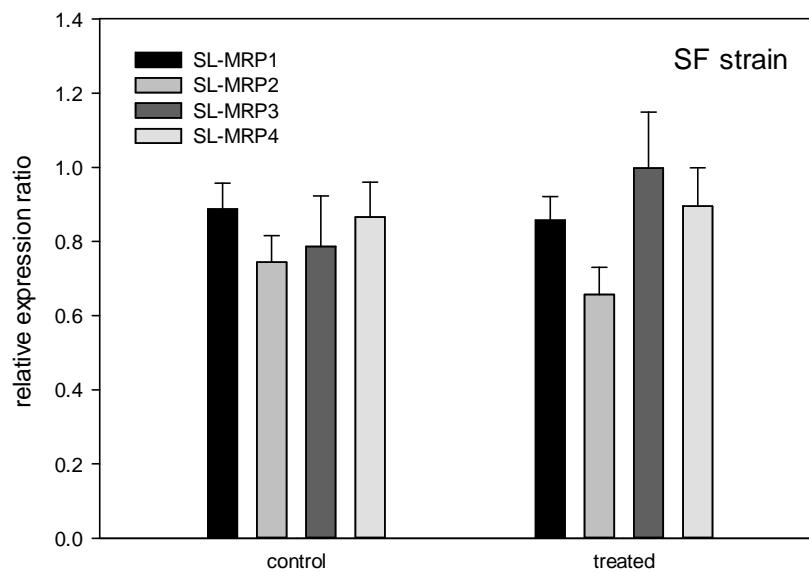


Figure 11: Effect of long-term exposure to SLICE® on relative expression ratios of four MRPs in salmon lice. Fish bearing salmon lice of the SF strain were fed SLICE®-fortified food ($50 \mu\text{g EMB kg}^{-1} \text{ fish day}^{-1}$) or a control diet for seven days and were then maintained under control conditions for another three weeks before salmon lice were collected for RT-qPCR analysis. Data were normalised with eukaryotic elongation factor 1 alpha (eEF1 α). Means with standard deviations are shown for each group (n = 12 lice).

3.3.3 Co-administration of EMB and ABC transporter inhibitors

The mRNA expression studies above provided no evidence for an increased expression of the four salmon louse MRPs following exposure to SLICE® or for their enhanced expression in moderately EMB-resistant strains of salmon lice. Apart from a slight up-regulation in response to $410 \mu\text{g L}^{-1}$ of EMB, similar negative results have been previously reported for expression changes of SL-PGY1 (Heumann et al., 2012). However, further ABC drug transporters may exist in salmon lice and could have remained undetected by the bioinformatic approach adopted here.

To investigate the potential involvement of ABC drug transporters in the toxicology of EMB in salmon lice, a pharmacological approach based on inhibitors of ABC transporter activity was adopted. If ABC drug transporters mediate the efflux of EMB and thus reduce the intracellular concentrations of the drug in exposed animals, the inhibition of ABC drug transporter activity could be expected to lead to higher intracellular levels of EMB and subsequently enhanced toxic effects. The calcium channel blocker, verapamil (VER), and the immunosuppressant drug, cyclosporin A (CSA), are known as inhibitors of P-gp (MDR1/ABCB1), but also inhibit MRPs (Barrand et al., 1993; Twentyman, 1992). In standard aqueous exposure bioassays, the median effective concentration (EC_{50}) of EMB in the S strain was $125 \mu\text{g L}^{-1}$ (95% confidence limits: 83-194) in the absence of inhibitors, $35.7 \mu\text{g L}^{-1}$ (23.4-53.8) in the presence of $10 \mu\text{M}$ CSA and $82.3 \mu\text{g L}^{-1}$ (46.1-129.7) in the presence of $20 \mu\text{M}$ VER (Figure 12 and 13). For the PT strain, the EMB EC_{50} was $289 \mu\text{g L}^{-1}$ (189-439) in the absence of inhibitors, $108 \mu\text{g L}^{-1}$ (71-167) in the presence of $10 \mu\text{M}$ CSA and $49.3 \mu\text{g L}^{-1}$ (13.2-92.9) in the presence of $20 \mu\text{M}$ VER. CSA, therefore, caused a significant shift ($p=0.006$) of the EMB dose-response curve towards lower

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concentrations (left shift) in both the S and the PT strain (Figure 12). In contrast, verapamil significantly ($p=0.001$) increased the toxic effect of EMB in the PT strain but not the S strain (Figure 13).

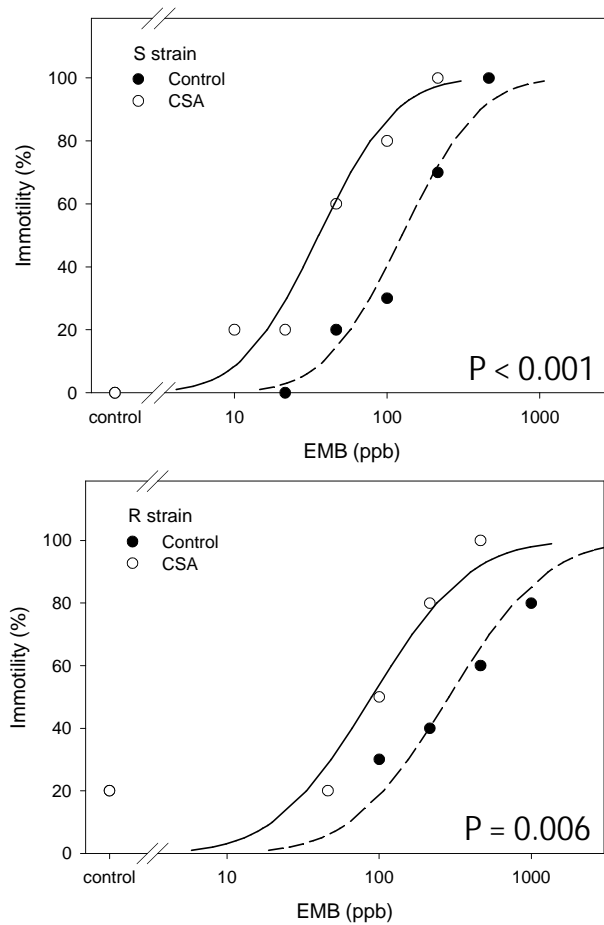


Figure 12: Effect of cyclosporin A (CSA) on acute EMB toxicity in salmon lice. Adult male salmon lice were exposed for 24 h to different EMB concentrations, provided alone or in combination with the ABC transporter inhibitor CSA (10 μM). Motility behaviour was then assessed and median effective concentrations (EC₅₀s) obtained by probit analysis. In the absence of inhibitors, the EMB EC₅₀ was 125 $\mu\text{g L}^{-1}$ (95% confidence limits: 83-194) in the S strain and 289 $\mu\text{g L}^{-1}$ EMB (189-439) in the PT strain. In the presence of CSA the EMB EC₅₀ was 35.7 $\mu\text{g L}^{-1}$ (23.4-53.8) in the S strain and 108 $\mu\text{g L}^{-1}$ (71-167) in the PT strain. Inhibitory effects were considered significant when the probability value P was below 0.05. One representative of three experiments is shown. Each data point summarises observations made on 10-20 lice.

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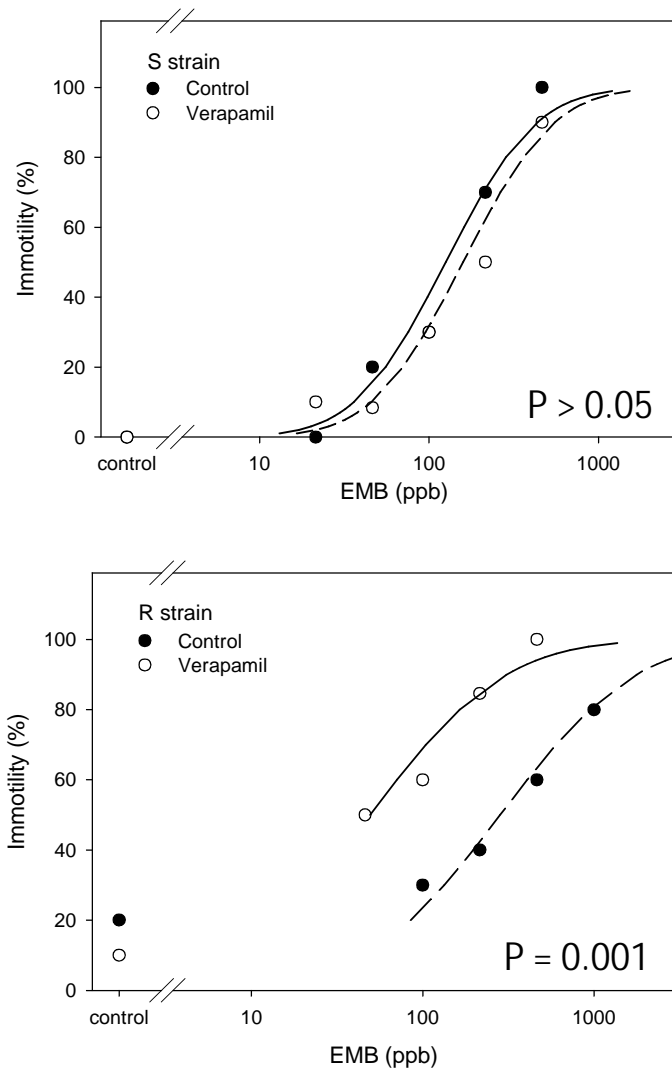


Figure 13: Effect of verapamil (VER) on acute EMB toxicity in salmon lice. Adult male salmon lice were exposed for 24 h to different EMB concentrations, provided alone or in combination with the ABC transporter inhibitor VER (20 μM). In the presence of VER, the EMB EC₅₀ was 82.3 $\mu\text{g L}^{-1}$ (95% confidence limits: 46.1-129.7) in the S strain and 49.3 $\mu\text{g L}^{-1}$ (13.2-92.9) in the PT strain. EC₅₀ values obtained in the absence of inhibitors and further details are given in the legend of figure 12.

3.4 Discussion

In this study, four *L. salmonis* MRPs of the ABCC subfamily of ABC transporters were identified. MRPs are potential drug transporters and could have relevance as factors modifying the efficacy of EMB. However, in the absence of EMB exposure, mRNA expression levels of the four MRPs were similar between salmon louse strains differing in EMB susceptibility, and MRP mRNA levels did not change following exposure to EMB. Accordingly, this study does not provide evidence for potential roles of the studied MRPs in EMB toxicity or drug resistance in salmon lice. To complement mRNA expression studies, an inhibitor-based approach was used to reveal potential roles of ABC transporters in EMB toxicity. CSA and VER are inhibitors of both P-gp and MRPs. CSA enhanced EMB toxicity in both the drug susceptible S strain and the EMB hyposensitive PT strain, whereas VER increased EMB toxicity only in the PT strain. The underlying causes for the apparent selectivity of the inhibitors is currently unknown and should be addressed in a separate study.

ABC drug transporters have been implicated to be involved in the resistance of nematodic parasites against avermectins (Wolstenholme et al., 2004). Most metazoan species possess multiple ABC drug transporters with overlapping substrate specificities. For instance, 15 P-gp homologues and 8 MRP homologues are present in the nematode model species *C. elegans* (Sheps et al., 2004). Due to the presence of multiple proteins with similar function, the specific roles of individual ABC drug transporters can be challenging to ascertain, especially in species lacking an available genome assembly. While the full number of ABC proteins in salmon lice is unknown, different studies have aimed to isolate ABC drug transporter cDNA sequences from this species. Two putative salmon louse homologues of the full transporter P-gp (MDR1/ABCB1) have been reported in an earlier study (Tribble et al., 2007). However, one of

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the sequences seems to belong to the ABCF subfamily, which is involved in ribosome assembly and does not contain drug transporters, while the second sequence appears to be an ABCB subfamily half transporter, which localises to intracellular membranes and has, to our knowledge, not been associated with drug transport (Heumann et al., 2012; Dean et al., 2001). A subsequent study isolated a P-gp homologue called SL-PGY1 from salmon lice (Heumann et al., 2012). However, exposure of salmon lice to EMB provoked, at best, only a moderate up-regulation of the pump, and no significant differences in SL-PGY1 mRNA levels were found between salmon louse strains of differing EMB susceptibilities (Heumann et al., 2012). In contrast, a Canadian study observed, among historical salmon louse samples from farms in New Brunswick, an increase of SL-PGY1 mRNA levels between 2002 and 2011, coinciding with a decrease in EMB susceptibility and suggesting possible links between SL-PGY1 expression and EMB susceptibility (Igboeli et al., 2012). The present study adds four more sequences encoding MRPs to the list of potential ABC drug transporters in salmon lice. The measurement of mRNA expression of the MRPs in salmon lice of different genetic background and EMB exposure history, however, did not provide evidence for a role of salmon louse MRPs in EMB toxicity or resistance.

ABC drug transporters are found in cell membranes, where they function as drug efflux pumps decreasing the intracellular levels of transport substrates. ABC drug transporters are usually predominantly expressed in organs involved in excretion, such as kidneys and liver, and in tissues forming external or internal boundaries, such as the gut epithelium or the capillary endothelia of the blood-brain barrier (Leslie et al., 2005). This expression pattern is in accordance with roles of ABC drug transporters in limiting the uptake and enhancing the excretion of toxic compounds. Indeed, animals lacking P-gp as the result of natural or targeted

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null mutations show a greatly enhanced susceptibility to toxic P-gp substrates such as ivermectin (Roulet et al., 2003; Schinkel et al., 1994). Similar changes in susceptibility to P-gp substrates can be provoked by the pharmacological blockage of P-gp with inhibitors (Mayer et al., 1997). In the present study, the P-gp inhibitor CSA enhanced the toxicity of EMB in both salmon louse strains studied. We speculate that ABC transporters inhibited by CSA in sea lice may play important roles in the biochemical defence against toxicants, for example limiting the effects of EMB in salmon lice, but may not show significant expression differences between the studied strains. In contrast, VER caused an increase in EMB toxicity in the studied hyposensitive strain PT, but had no effects in the drug-susceptible strain S. This observation is in accordance with the hypothesis that VER inhibits a biochemical mechanism contributing to the detoxification of EMB, possibly involving an ABC transporter. Paralleling the results from this study, VER has been reported to increase the efficacy of ivermectin and moxidectin in an avermectin-resistant strain of the sheep gastrointestinal nematode, *H. contortus*, showing elevated P-gp expression *in vivo* (Xu et al., 1998). We speculate that several ABC transporters showing distinct, but overlapping, patterns of inhibitor specificity could be involved in EMB detoxification in the salmon louse, and show differential expression between the studied strains. In addition to P-gp, different MRPs have been shown to transport ivermectin in mammalian cell culture systems. Moreover, two P-gps and four MRPs showed increased mRNA expression levels in two ivermectin-selected *C. elegans* strains. However, the putative role of genes ABCB and ABCC transporters in *L. salmonis* with decreased EMB susceptibility is currently not sufficiently understood.

The results of this study support the need for further research, involving both gene expression studies and toxicity tests involving ABC inhibitors, in order to ascertain the identity of ABC transporters affecting EMB toxicity in salmon lice.

3.5 Acknowledgements

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4 IDENTIFICATION OF ATP-BINDING CASSETTE (ABC) PROTEINS IN A SALMON LOUSE (*LEPEPHTHEIRUS SALMONIS*) TRANSCRIPTOME

The potential role of ABC transporters as factors influencing drug susceptibility in the salmon louse, *Lepeophtheirus salmonis* (Krøyer, 1837)
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“Identification of ATP-binding cassette (ABC) proteins in a salmon louse (*Lepeophtheirus salmonis*) transcriptome”

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(SC contributed to the design and performed the sample preparation, helped with the analysis and preparation the manuscript. JH took part in the identification and annotation of ABC transporter candidates, analysis and contributed to the manuscript. JBT advised on experimental design and data analysis. JEB supervised the work and gave input for the preparation of the manuscript. MB constructed the *L. salmonis* transcriptome, performed sequence annotation and assisted with writing the manuscript. AS conceived and supervised the study, contributed to data analysis and contributed to writing the manuscript.)

Abstract

The salmon louse causes significant economic damage in the mariculture of Atlantic salmon. In addition to farm management measures and biological control by cleaner fish, chemicals are used for the treatment of *L. salmonis* infections at fish farms. However, with only a restricted number of salmon delousing agents being available, the continued reliance on a limited range of parasitocidal drugs may favour the development of drug resistance in *L. salmonis*. Indeed, decreased treatment efficacies have been reported, at least locally or temporarily, for a number of substance classes including organophosphates, pyrethroids and avermectins. The ATP-binding cassette (ABC) gene superfamily is found in all biota and contains membrane proteins functioning as transporters, which include drug efflux pumps that can constitute drug resistance factors by reducing the internal exposure to biocidal drugs. In this study, we used next generation RNA sequencing (RNAseq) to construct a reference transcriptome representing key stages of the *L. salmonis* life cycle, and to support a genome-wide survey of the ABC gene superfamily of this economically important parasite. High-throughput sequencing of a pooled mRNA library (RNA-Seq) generated over 300 million sequence reads that were assembled into 37,681 transcripts, consisting of 33,833 unique genes.

4.1 Introduction

The large gene family of ATP-binding cassette (ABC) proteins has members in all biota (Higgins, 1992). Typical ABC proteins are composed of transmembrane and conserved nucleotide binding domains and function as primary transporters in trafficking processes across biological membranes (Higgins, 1992; Igarashi, 2004). The wide range of substrates transported by ABC proteins includes inorganic ions, metals, sugars, amino acids, peptides, lipids and organic chemicals (Dean, 2001; Higgins, 1992). ABC proteins functioning as drug transporters can contribute to chemical resistance phenotypes in cancers, pathogens and pests. Multidrug resistance (MDR) is defined as the reduced susceptibility of tumours to structurally and functionally unrelated cytostatic drugs and can be based on the expression of ABC efflux transporters reducing cellular drug accumulation (Gottesman et al., 2002). ABC transporters have further been linked to drug resistance in parasitic protozoans (Sanchez et al., 2010; Sauvage et al., 2009) and nematodes (Dicker et al., 2011; Prichard & Roulet, 2007), and to pesticide resistance in insects (Bariami et al., 2012; Heckel 2012; Lanning et al., 1996).

In metazoans, the ABC family is divided into eight subfamilies named ABCA to ABCH (Annilo et al., 2006; Dean, 2001; Sheps et al., 2004). Drug transporters are found in subfamilies ABCB, ABCC and ABCG and include the MDR-conferring proteins ABCB1 (also called MDR1 or P-glycoprotein), ABCC1 (also known as Multidrug resistance associated protein, MRP1) and ABCG2 (also known as the breast cancer resistance protein, BCRP) (Cole et al., 1992; Doyle et al., 1998; Gros et al., 1986; Roninson et al., 1984). In non-cancerous tissues, ABC drug transporters have physiological roles within the biochemical defence against toxicants (Leslie et al., 2005; Schinkel & Jonker, 2003). ABC drug transporters are predominantly expressed in

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tissues involved in excretion or constituting internal or external body boundaries. At these sites, ABC drug transporters often localise to the apical membranes of polarised epithelia and endothelia, resulting in directional transport of substrates into excreta (e.g., bile fluid or urine) and out of sanctuary sites (e.g., at blood-tissue barriers).

Caligid copepods, also called sea lice, are ectoparasites of wild and farmed marine fish (Boxaspen, 2006). In the Northern hemisphere, sea louse infections of farmed Atlantic salmon (*Salmo salar* Linnaeus, 1758) are mostly by the salmon louse, *Lepeophtheirus salmonis* (Krøyer, 1837) (Torrissen et al., 2013). In addition to farm management measures and the use of cleaner fish (Sayer et al., 1996; Skiftesvik et al., 2014), sea louse control relies heavily on chemical treatments applied as baths or through feed (Haya et al., 2005), causing estimated treatment costs of €305 million per annum globally (Costello, 2009). However, the continuous use of the same medicinal agents, or different agents with the same mode of action, can favour the development of drug resistance (Denholm et al., 2002). In *L. salmonis*, losses of efficacy have been reported for different treatments including hydrogen peroxide (Treasurer et al., 2000), organophosphates (Jones et al., 1992; Roth et al., 1996), pyrethroids (Sevatdal & Horsberg, 2003) and avermectins (Espedal et al., 2013; Lees et al., 2008).

At present, little is known about the molecular mechanisms of drug resistance in *L. salmonis*. Changes in the expression and/or sequence of molecular target sites have been suggested to contribute to decreased susceptibility of *L. salmonis* to organophosphates, pyrethroids and emamectin benzoate (Carmichael et al., 2013; Fallang et al., 2004; Fallang et al., 2005). Furthermore, it has been suggested that altered drug metabolism is by the activity of multiple cytochromes P450 in *H. contortus* (Laing et al., 2015) and has been proposed to affect pyrethroid toxicity in *L. salmonis* (Sevatdal et al., 2005). Moreover, two partial *L. salmonis* cDNA sequences have been suggested to represent homologues to ABCB1 potentially involved

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in EMB resistance (Tribble et al., 2007). However, subsequent studies revealed that these sequences were unrelated to ABCB1 and represented homologues of ABCF1 (Tribble et al., 2008) and ABCB8 (Heumann et al., 2012), two ABC proteins not functioning as drug transporters. Later, one ABCB1 homologue called SL-PGY1 and four MRPs have been isolated in *L. salmonis* (Heumann et al., 2014, Heumann et al., 2012). Sublethal EMB exposures provoked an up-regulation of the SL-PGY1 transcript (Heumann et al., 2012; Igboeli et al., 2012) but had no effect on mRNA levels of *L. salmonis* MRPs (Heumann et al., 2014). In summary, knowledge on *L. salmonis* ABC transporters is still fragmentary, and obtaining a more comprehensive picture of the ABC family in *L. salmonis* will be invaluable for research efforts aiming at unravelling the molecular determinants of drug resistance in this parasite.

Currently, a genome assembly exists only for one crustacean species, the waterflea, *Daphnia pulex* (Colbourne et al., 2012). Genomic resources available in other crustaceans mainly consists of publicly available expressed sequence tags (ESTs), generated from sequencing tissue- and stage-specific normalised (Qiao et al., 2012; Yasuike et al., 2012), non-normalised (O'Leary et al., 2006) or subtracted cDNA libraries (Hansen et al., 2007; Zhao et al., 2007). Available studies of transcriptomic responses in copepod to pollutants and environmental stressors include the analysis of *Tigriopus californicus* (Baker, 1912) responses to trace metal exposure by microarray techniques (Ki et al., 2009) and a study of *Calanus finmarchicus* (Gunner, 1765) responses to thermal stress by suppression subtractive hybridisation (Voznesensky et al., 2004).

In the present study, a *de novo* transcriptome was created in *L. salmonis* using Illumina RNA-Seq technology in order to carry out a comprehensive survey of the ABC gene family in this economically important fish parasite. Sequencing was based on a total RNA pool representing key stages of the *L. salmonis* life cycle, ensuring representation of major transcriptional events

in this custom sequence resource. This *L. salmonis* transcriptome was functionally annotated and characterised to allow the identification of novel candidate genes in *L. salmonis* ABC transporters, for further characterisation to establish any potential involvement in reduced drug susceptibility.

4.2 Materials and Methods

4.2.1 Salmon lice

L. salmonis samples from the different points of the life cycle were obtained from a laboratory-maintained strain (S) of the parasite that is susceptible to all major current salmon delousing agents. Details of *L. salmonis* husbandry conditions are provided elsewhere (Heumann et al., 2012). In brief, parasites were maintained on Atlantic salmon in circular tanks supplied with fresh seawater at ambient temperature, using a photoperiod corresponding to natural day length. To propagate cultures, egg strings were obtained from gravid females and allowed to develop to copepodids, which were used to infect fresh batches of host fish. Infection rates were maintained at levels that were unlikely to compromise fish welfare. Prior to the collection of *L. salmonis* from hosts, fish were anaesthetised with 100 mg L⁻¹ 2-phenoxyethanol for 3 min. All laboratory infections were carried out under UK Home Office licence and appropriate veterinary supervision.

Twenty-one *L. salmonis* samples of the S strain were collected, representing different points in the life cycle of the parasite (Johnson & Albright, 1991b). The collected material comprised of

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egg strings, nauplius and copepodid larvae, chalimus stages I to IV, as well as preadult I and II and adult stages, with some of these stages being further differentiated according to sex or age (Appendix S1). Samples representing earlier stages of the life cycle (nauplius – chalimus IV) consisted of pools of parasites. All samples were preserved in RNAlater, a high-salt RNA stabilisation solution (4.54 mol L⁻¹ ammonium sulphate, 25 mmol L⁻¹ trisodium citrate, 20 mmol L⁻¹ EDTA, pH 5.4), immediately after collection and then stored at -70°C. The two naupliar stages of *L. salmonis* develop relatively quickly (30.5 hours from nauplius 1 to 2 at 10°C) and are difficult to differentiate by size (0.54 – 0.56 mm) (S. C. Johnson & Albright, 1991a, 1991b). In order to obtain a set of samples collectively covering different points in the early larval development, egg strings obtained from gravid females were incubated in aerated seawater at two temperatures (8 and 10.5°C), and in each condition the emerging parasite larvae were collected at two time points (24 and 48 hours) by filtration. Free-living copepodids were obtained from cultures after 5 days of incubation (10.5°C). The host-attached stages were collected at the appropriate time points post-infection from anaesthetised salmon using forceps under low magnification microscopy, with stages being differentiated according to the criteria given by Johnson and Albright (Johnson & Albright, 1991b).

4.2.2 RNA Extraction and purification

Frozen samples were ground in liquid nitrogen using a pestle and mortar, and total RNA was immediately extracted from the homogenised sample using TRI Reagent[®] (Sigma-Aldrich, UK), following the manufacturer's protocol. After phase separation, RNA was precipitated from the aqueous phase by addition of 0.25 volumes isopropanol and 0.25 volumes of a high salt buffer (0.8 mol L⁻¹ trisodium citrate; 1.2 mol L⁻¹ sodium chloride), as recommended for samples with

high polysaccharide content (Chomczynski & Mackey, 1995) and resuspended in nuclease-free water. Total RNA was extracted from 21 different *L. salmonis* samples taken from key stages of the life cycle where each sample consisted of pools of individuals (Appendix S1). UV spectroscopy (NanoDrop ND-1000, Thermo Scientific, USA) was used to confirm purity of the RNA samples and establish concentrations, whereas RNA integrity was assessed by agarose gel electrophoresis and ethidium bromide staining.

4.2.3 Library construction and sequencing

A total RNA pool was created that included samples from the egg string, nauplii, copepodid, preadult (I and II) and adult stages for male and female *L. salmonis*, and incorporated 2.5 µg total RNA from each of 21 salmon louse samples (Appendix S1). This total RNA pool was further purified using RNeasy columns (Qiagen, UK). Transcriptome sequencing of the pool (EBI Sequence Read Archive (SRA) study ERP002482) was performed using Illumina RNA-Seq, with the sequencing library being prepared using the TruSeq™ RNA Sample preparation kit. Library preparation and sequencing was performed by The GenePool Genomics Facility, University of Edinburgh.

4.2.4 Data filtering and assemblies

Transcriptome sequencing requires high quality sequence reads for optimal assembly as sequencing errors can often create difficulties for short-read assembly algorithms. Therefore, a

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stringent filtering was performed to remove low-quality reads containing ambiguous nucleotides (“N”) or with a Phred score under 20. The sequences were also screened to remove any duplicates or low complexity sequences using PRINSEQ v0.20 (Schmieder & Edwards, 2011). The genome sequence for Pacific and Atlantic *L. salmonis* strains (NCBI assembly ASM18125v2) were retrieved from the salmon louse genome project website [<http://sealouse.imr.no/>] (Accessed: July 2012) and used for all subsequent transcriptome assembly processes. Two complementary sequence read assembly methods were chosen for this study. Firstly, TopHat v2.0.4 (Kim et al., 2013) was used to establish a reference-based assembly. Then, any unaligned reads were used by Trinity (release 2012-06-08) (Grabherr et al., 2011) in order to build a *de novo* assembly of the remaining reads or to extend reference-based transcripts. Finally, to lower the redundancy resulting from *de novo* assemblies, all transcripts shorter than 300 bp or exhibiting repeats, as detected by TRF 4.07b (Benson, 1999), were removed.

4.2.5 Gene annotation and analysis

To annotate the sequences obtained, we performed sequence similarity searches using the BLAST algorithm (Altschul 1990). The longest coding DNA sequences were determined for each transcript using getorf from the EMBOSS v6.5.7 package (Rice et al., 2000). ESTScan v2 (Iseli et al., 1999; Lottaz et al., 2003) was then used to confirm transcript coding regions and to determine sequence orientation. The coding sequences of the predicted transcripts were annotated using BLASTp (Basic Local Alignment Search Tool) searches against the GenBank Reference Proteins database (refseq_protein; 03/03/2013 release) from the National Centre for Biotechnology Information (NCBI), with an expectation value (e-value) cut-off of 10^{-4} and

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minimum alignment length of 33 amino acids being considered significant. Additionally, the transcripts were annotated using BLASTn searches against the UniGene/EST (*L. salmonis* datasets, 2013-12-18 release) databases.

The inferred annotations were used to retrieve Gene Ontology (GO) annotation for molecular function, biological process and cellular component (Ashburner et al., 2000). To avoid redundant functional assignments, the best-rated similarity hit with at least one GO annotation was chosen. A custom pipeline converted GO terms to GO Slim terms, using the Protein Information resource and Generic GO Slim files (Gene Ontology Consortium, 2014).

4.2.6 ABC transporter identification

The coding sequences of the predicted transcripts and downloaded EST dataset were searched using HMMER v3.1b1 (Eddy, 2011) for ABC transporter hidden Markov models (Appendix S2) from Pfam v27.0 (Finn et al., 2014), PRINTS v39.0 (Attwood et al., 2003), SMART v7.0 (Letunic, Doerks, & Bork, 2012), SUPERFAMILY v1.75 (Wilson et al., 2009) and PANTHER (Mi, Muruganujan, & Thomas, 2012). For each locus identified, a subsequent manual annotation was also carried out using BLASTp searches against the GenBank Reference Proteins database (Refseq_Proteins; 2014-04-07 release) from the NCBI.

4.2.7 Phylogenetic tree

Amino acid sequences of the NBDs were aligned using GramAlign v3.0 (Russell 2014); the multiple sequence alignment of proteins was converted into corresponding codon alignment

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using PAL2NAL v14 (Suyama 2006). Phylogenetic tree topology was estimated by a maximum likelihood search implemented in RAxML version 7.8.6 (Stamatakis 2006) using both the WAG and CAT models. One thousand bootstrap replicates were used to evaluate the support for different aspects of the optimal topology.

4.2.8 Data access

The raw sequence data from the study have been submitted to the EBI Sequence Read Archive (SRA) study ERP002482. The Annotated transcriptome has been deposited at the EBI European Nucleotide Archive (ENA) reference ERZ019407.

4.3 Results and Discussion

4.3.1 Paired-end sequencing and transcriptome assembly

To create a *L. salmonis* transcriptome sequence resource representing all key stages of the life cycle, including major metamorphosis steps, a total RNA pool was generated that included key stages of the salmon louse life cycle. This total RNA pool was subjected to Illumina paired-end sequencing using the Illumina HiSeq 2000 platform. In total, 389,927,940 raw sequence reads were obtained (194,963,970 paired-end reads; length 101 nt) (EBI SRA study ERP002482). Of those, 338,243,772 reads passed quality control filtering and were then used to generate a reference-based transcriptome assembly of 33,537 transcripts using the Atlantic *L. salmonis*

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genome as a reference ([<http://sealouse.imr.no/>]). The sequence reads that could not be aligned to the reference genome were subsequently used for *de novo* sequence assembly using Trinity software, which generated an extra 4,144 transcripts and extended 698 reference-based transcripts. Based on the high quality reads, 37,681 transcripts (Figure 14) were assembled (EBI ENA reference ERZ019407), consisting of 30,159 unique transcripts and 3,774 alternatively spliced transcripts (Table 5). Of these, 48.9% transcripts had a length of more than 1000 bp. The N50 length was 2,060 bp, whereby N50 represents the minimum length of contigs in the set containing the largest contigs. The bases of those contigs do, when combined, cover at least 50% of the total assembly (Miller et al., 2010).

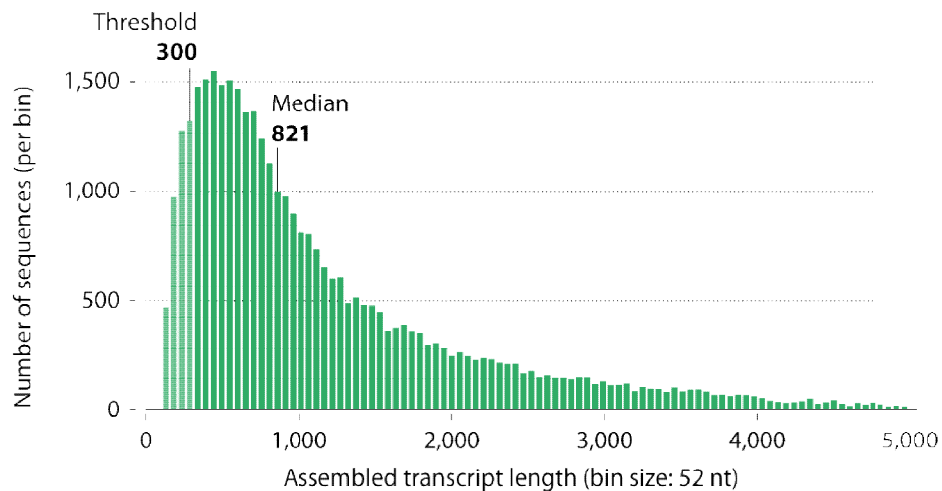


Figure 14: Overview of the *L. salmonis* transcriptome assembly. Size distribution of assembled transcripts (median 821 nucleotides). Cut-off at 300 nucleotides.

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Table 5: Statistics of the assembled sequences.

Raw data	
Number of reads	389,927,940
Read length	101
Total size of reads	39,382,721,940
After assembly	
Number of transcripts	37,681
N50	2,060
Total size of transcripts	50,718,754
After filtering (> 300 bps)	
Number of transcripts	33,933
Number of unique genes	30,159
Unique transcripts	27,086
Multiple transcripts	3,073
N50	2,100
Total size of transcripts	49,850,662
Shortest transcript	300
Longest transcript	24,684
Mean size	1,469
Median size	9,79
Mean GC%	36.9%
N%	0.03%

Finally, the 33,933 unique transcripts have an N50 length of 2,100 bp and a total length of 49,850,662 bp. Each gene had, on average, one splicing form per gene, with some genes having up to five (Table 5). To evaluate the quality of the assembled transcripts, all the usable sequencing reads were realigned to the transcripts. The sequencing depth ranged from one to 519,299 reads, with an average of 795 reads across whole transcripts. About 77% of the transcripts were supported by more than 10 reads, while 35% were supported by more than 100 reads.

4.3.2 Functional inference by searching against public databases

The assembled transcripts were annotated using BLASTp and BLASTn searches against the refseq_protein and UniGene databases, respectively. The results indicated that out of 30,159

genes, 28,547 (95%) and 8,640 (29%) showed significant similarity to known proteins or gene transcripts in refseq_protein and UniGene databases, respectively. To evaluate the coverage of the assembled transcriptome, 128,783 ESTs (> 100 bp) publicly available for *L. salmonis* (Yazawa et al., 2008) were aligned to the 33,933 transcript sequences generated in this study and vice versa. In total, 97,785 (76%) of all *L. salmonis* ESTs aligned to at least one transcript, while 15,507 (46%) of the transcripts had at least one corresponding *L. salmonis* EST.

4.3.3 GO functional classification analysis

GO annotations were assigned to the assembled *L. salmonis* transcripts/genes on the basis of refseq_protein annotations. In total, 28,547 genes had similarity to known gene products; however, only 4,954 (17%) were assigned GO annotations. A total of 3,009 different functional terms were assigned, with protein binding (25%) and nucleic acid binding (24%) identified as highly represented biological functions (Figure 15).

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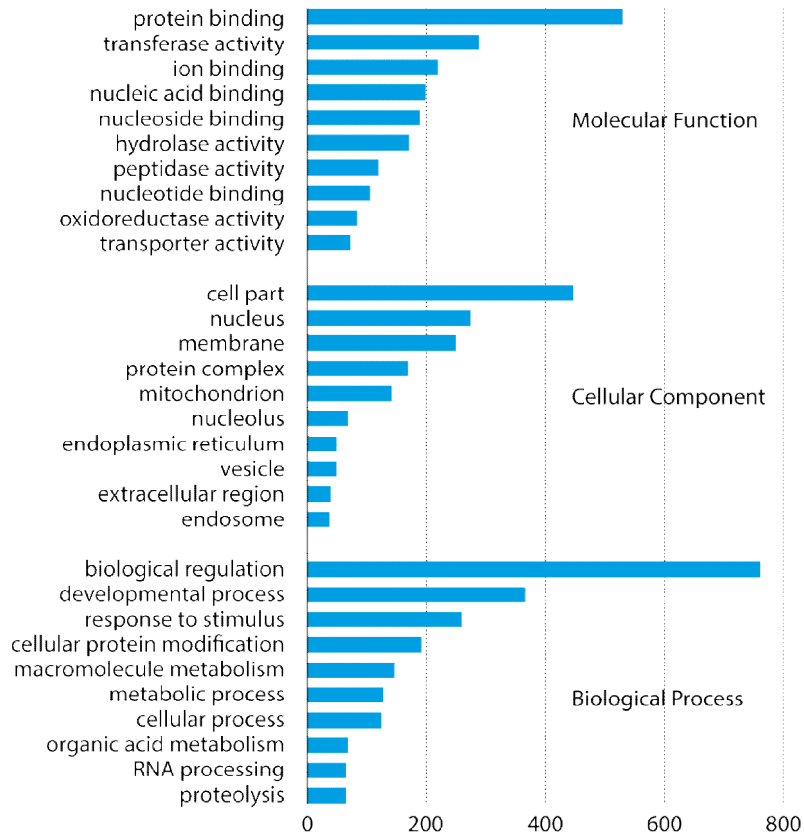


Figure 15: Multilevel Gene Ontology (GO) categorisation of the annotated transcripts. GO terms were assigned to 4,954 (17%) of the 28,547 annotated transcripts based on refseq_protein annotation. GO Annotations were first converted to GO-Slim annotations and the multilevel chart shows the top ten of each category to reduce the complexity of the chart.

4.3.4 Identification of ABC superfamily members

Based on well-established hidden Markov models for ABC transporters (Appendix S2), 33 transcripts out of the 30,159 unique genes were predicted to be ABC superfamily members. Similarly, out of 129,250 available ESTs, 186 were identified as ABC members and assembled into 28 contigs. Overall, 39 ABC genes were identified across families A to G (Table 6), of

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which 20 were found both in the transcriptome and the existing EST, while 13 were found only in the transcriptome and 6 only in the set of ESTs (Yazawa et al., 2008).

Table 6: Identification of drug resistance candidate genes in the *L. salmonis* transcriptome

ABC sub-group	EST supported transcriptome	Transcriptome only	EST contigs only	Total gene
A	1	2	0	3
B	2	1	0	3
C	7	7	1	15
D	1	1	2	4
E	1	0	0	1
F	3	0	3	7
G	5	2	0	7
Total	20	13	6	39

For a number of genes, alternative splicing forms were supported by alternative versions of ESTs (see full details, Appendix S3). The isolated ABC superfamily sequences are reported in Table 7 with preliminary names, accession numbers and detected domain architectures, based on the sequence range available. A maximum likelihood phylogenetic analysis grouped the *L. salmonis* ABC proteins into known ABC subfamilies with high bootstrap support (Figure 16). The subfamily assignment was re-evaluated and in all cases confirmed based on the presence of conserved protein motifs, domain architecture and manual annotation through BLASTp searches (Table 7).

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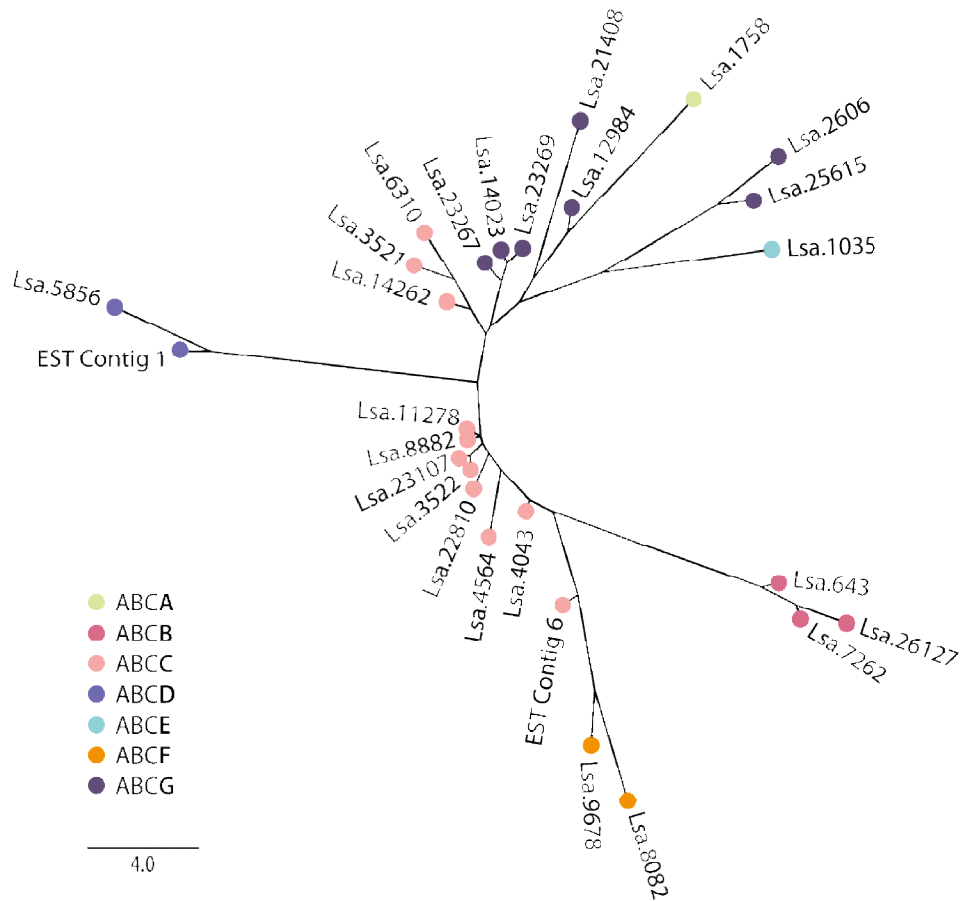


Figure 16: Unrooted phylogenetic tree of nucleotide binding domains of *L. salmonis* ABC proteins. Likelihood score of the constructed phylogenetic tree is indicated for each node. Filled circles represent nodes with bootstrap values over 90. Shaded clours indicate the different ABC protein subfamilies. ABC encoded gene references can be found in Table 7.

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Table 7: Characterised 39 *L. salmonis* ABC proteins.

Subfamily	ID	Predicted Classification	aa length	Identified topology
A	Lsa.1758	ABCA1	1583	TM - NBD - TM - NBD
A	Lsa.14583	ABCA4	127	TM
A	Lsa.1680	ABCA4	615	NBD - TM
B	Lsa.26127	ABCB10 (mitochondrial HT)	566	TM - NBD
B	Lsa.7262	ABCB7 (mitochondrial HT)	669	TM - NBD
B	Lsa.643	ABCB8 (mitochondrial HT)	687	TM - NBD
B	Lsa.4043	ABCB1	1448	TM - NBD - TM - NBD
C	EST_Contig_6	MRP14 / MRP1	339	NBD
C	Lsa.11278	ABCC1 / MRP1	1531	TM - NBD - TM - NBD
C	Lsa.22810	ABCC1 / MRP1	979	TM - NBD - TM
C	Lsa.29272*	ABCC1 / MRP1	185	TM
C	Lsa.4043	ABCC1 / MRP1	1448	TM - NBD - TM - NBD
C	Lsa.6310	ABCC1 / MRP1	1175	TM - NBD - TM
C	Lsa.8882*	ABCC1 / MRP1	1563	TM - NBD - TM - NBD
C	Lsa.23107*	MRP4	1381	TM - NBD - TM - NBD
C	Lsa.3521*	MRP4	926	TM - NBD - TM
C	Lsa.3522	MRP4	1293	TM - NBD - TM - NBD
C	Lsa.14264	MRP5-like	169	NBD
C	Lsa.4564	ABCC7	1466	TM - NBD - TM - NBD
C	Lsa.14261	ABCC8/9 (SUR)	198	TM
C	Lsa.14262	ABCC8/9 (SUR)	220	NBD
C	Lsa.14263	ABCC8/9 (SUR)	374	TM
D	EST_Contig_1	ABCD4 (peroxisomal membrane)	265	NBD
D	EST_Contig_2	ABCD4 (peroxisomal membrane)	355	TM - NBD
D	Lsa.10176	ABCD4 (peroxisomal membrane)	455	TM
D	Lsa.5856	ABCD4 (peroxisomal membrane)	656	TM - NBD
E	Lsa.1035*	ABCE1	670	NBD - NBD
F	Lsa.20458	ABCF1	109	NBD
F	Lsa.9678	ABCF2	660	NBD - NBD
F	EST_Contig_3	ABCF3	162	NBD
F	EST_Contig_4	ABCF3	125	NBD
F	EST_Contig_5	ABCF3	120	NBD
F	Lsa.8082	ABCF3	758	NBD - NBD
G	Lsa.12984	ABCG20	825	NBD - TM
G	Lsa.14023	ABCG20	737	NBD - TM
G	Lsa.21408	ABCG20	683	NBD - TM
G	Lsa.23267	ABCG20	716	NBD - TM
G	Lsa.23269*	ABCG20	773	NBD - TM
G	Lsa.2606*	ABCG5	702	NBD - TM
G	Lsa.25615	ABCG8	1001	TM - NBD - TM

EST_Contig_X: sequence provided in Appendix S3; peroxisomal membrane; TM: transmembrane domain; NBD: nucleotide binding domain. * Alternative splice forms exists

4.3.5 Representation of ABC subfamilies

ABCA proteins are full transporters involved in lipid transport. In humans, ABCA1 is involved in the formation of high density lipoprotein, while ABCA3, ABCA4 and ABCA12, respectively, have roles in pulmonary surfactant secretion, retinal integrity and keratinisation processes in the skin (Wenzel et al., 2007). Three members of the ABCA subfamily were identified in *L. salmonis* (Table 9).

The ABCB subfamily comprises both half and full transporters. ABCB half transporters often localise to intracellular membranes, particularly mitochondrial, and have cellular roles unrelated to drug transport, whereas ABCB full transporters, also called P-glycoproteins, are found in the cell membrane and include drug transporters (Dean, 2001). Three ABCB half transporters and one ABCB full transporter were found in *L. salmonis* in this study (Table 9). The half transporter Lsa.643 has previously been cloned and designated SL-Pgp1 (Tribble et al., 2007). However, Lsa.643 shows greater homology to ABCB8 than to the drug transporter MDR P-glycoprotein (ABCB1). Further *L. salmonis* half transporters represent homologues of the human mitochondrial transporters ABCB7 and ABCB10 (Table 9). ABCB7, ABCB8 and ABCB10 have roles in iron homeostasis and transport of Fe/S protein precursors (Dean, 2001). The full transporter Lsa.4043 (Table 9) is identical to SL-PGY1, an ABCB1 homologue cloned and characterised in a previous study (Heumann et al., 2012). SL-PGY1 is up-regulated in response to emamectin benzoate (EMB) exposure and has been reported to show greater mRNA levels in some *L. salmonis* isolates showing EMB resistance (Heumann et al., 2012; Igboeli et al., 2012).

The ABCC subfamily contains full transporters called multidrug resistance associated proteins (MRPs), the transport substrates of which include organic xenobiotics, xenobiotic conjugates

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and different metals (Deeley et al., 2006). Further ABCC subfamily members include the cystic fibrosis transmembrane conductance regulator (CFTR, ABCC7) and the sulfonylurea receptors (SUR1/2, ABCC8/9) (Deeley et al., 2006). In *L. salmonis*, 14 ABCC sequences were identified, which comprise 11 MRPs and three SUR homologues (Table 9). MRPs have roles in biochemical defence against toxicants in mammals Deeley et al., 2006; Vlaming et al., 2009) and invertebrates (Luedeking et al., 2005). In the free-living nematode *C. elegans*, *mrp-1* contributes to heavy metal resistance (Broeks et al., 1996), while in *D. melanogaster* (fruit fly) dMRP transports typical MRP1 (hABCC1) substrates (Szeri et al., 2009). The up-regulation of MRPs has been reported, together with that of P-glycoproteins and/or cytochromes P450, in drug-resistant nematodes (James & Davey, 2009; Yan et al., 2012) and pesticide-resistant mosquitoes (Bariami et al., 2012). Four of the 11 *L. salmonis* MRPs reported here (EST_Contig_6, Lsa.4564, Lsa.8882, Lsa.23107) have been identified during a previous study (Heumann et al., 2014), which failed to detect differences in transcript levels of these transporters between EMB-resistant and a drug-susceptible *L. salmonis*. Both mammalian and arthropod SURs have roles in regulating potassium channels (Bryan et al., 2007; Nasonkin et al., 1999). The apparent failure of this study to identify *L. salmonis* CFTR homologues was not unexpected. CFTR is lacking in arthropod and nematode genomes analysed so far (Dermauw et al., 2013; Sheps et al., 2004; Sturm et al., 2009), and is believed to have emerged following a gene duplication in the vertebrate lineage (Dassa & Bouige, 2001; Sebastian et al., 2013).

Four sequences of the ABCD subfamily were identified in *L. salmonis* in this study (Table 9). ABCD proteins are half transporters involved in the import of fatty acids and their precursors into the peroxisome (Theodoulou et al., 2006). Illustrating the vital function of ABCD proteins, silencing of ABCD expression by simultaneous knock-down using RNA interference (RNAi) almost completely suppressed production of progeny in *C. elegans* (Oi et al., 2002).

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Subfamilies ABCE and ABCF contain highly conserved atypical ABC proteins composed of a pair of linked nucleotide binding domains, but lacking transmembrane domains (Kerr, 2004). The present study found one ABCE and seven ABCF sequences in *L. salmonis* (Table 9). ABCE proteins have central roles in translation initiation (Chen et al., 2006), while ABCF proteins adopt functions in ribosome assembly and/or protein translation (Tyzack et al., 2000).

The ABCG subfamily contains half transporters showing a “reverse” domain order, with the NBD being located N-terminally to the TMD. Transport substrates of ABCG proteins include sterols and drugs (Kusuhara & Sugiyama, 2007). In human, ABCG1 and ABCG4 mediate the cellular transport of cholesterol to high-density lipoprotein, while ABCG5 and ABCG8 facilitate the intestinal and biliary efflux of cholesterol, plant, and shellfish sterols. In contrast, human ABCG2 (also called BCRP) is a drug efflux transporter (Krishnamurthy & Schuetz, 2006). In *D. melanogaster*, the ABCG proteins white, brown and scarlet form subunits of transporters of eye pigment precursors (Mackenzie et al., 1999), while ET23 is believed to regulate cellular ecdysone levels (Hock et al., 2000). In *L. salmonis*, seven ABCG sequences were identified (Table 9); however, phylogenetic analyses could not detect one-to-one orthologous relationships to vertebrate and *D. melanogaster* transporters (data not shown).

Members of the ABCH subfamily resemble ABCG proteins in domain organisation and are found in *Daphnia magna*, insects, nematodes and teleost fish (Annilo et al., 2006; Dean, 2001; Sheps et al., 2004; Sturm et al., 2009). No functions have so far been attributed to ABCH subfamily transporters. No members of the ABCH subfamily were found in *L. salmonis* ABC genes in this study (Table 9).

4.3.6 Conclusion

The annotation of expressed ABC proteins in *L. salmonis* represents a significant step towards an improved understanding of potential drug resistance factors in this species and related parasites. In particular, the high number of potential drug transporters identified will be invaluable to research efforts towards unravelling resistance mechanisms and improving control strategies.

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5 TIME COURSE TOXICITY
ANALYSIS AS A METHOD
FOR DRUG SUSCEPTIBILITY
ASSESSMENT IN THE
SALMON LOUSE
LEPEOPHTHEIRUS SALMONIS

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This chapter is a draft for publication, but further experiments will be conducted and an enhanced version will be submitted.

“Time course toxicity analysis as a method for drug susceptibility assessment in salmon lice”

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(J. Heumann developed the design and carried out the experiments, analysed the results and wrote the chapter, S. Carmichael critically assessed and improved the design, H. Christie carried out preliminary trials, D. Green provided analytical input, while A. Sturm and J. Bron supervised the work and helped with the analysis and writing.)

Abstract

Sea lice are marine fish ectoparasites belonging to the family Caligidae (Copepoda, Crustacea). A number of sea louse species are known to compromise the health and welfare of wild and farmed Atlantic salmon (*Salmon salar* L.). Currently, sea louse control on fish farms relies mostly on veterinary medicines. With only a limited range of drugs available for control, the continued use of compounds belonging to a restricted number of drug classes can promote the development of drug resistance. Successful resistance management requires the deployment of well-established methods to assess the drug susceptibility status of sea louse populations. Current standardised sea louse bioassays provide such a methodology, but require relatively large numbers of suitable stages of parasites, which can be difficult to obtain. The present study aims to investigate the feasibility of an alternative bioassay design based on time course toxicity analyses, which require significantly fewer test animals than standard 24-h dose-response bioassays. This could be advantageous under conditions where availability of suitable sea louse stages is restricted. Two well-characterised laboratory strains of the salmon louse, *Lepeophtheirus salmonis*, S (susceptible) and R (resistant), were employed for the study. These differ in their susceptibility to the drug emamectin benzoate (EMB) according to median effective concentrations (effective concentrations affecting half of the test population) obtained in standard dose-response bioassays. A similarly clear distinction between the two strains was observed when comparing their median exposure time (Elapsed time until half of the test population is affected at a certain concentration). Furthermore, drug susceptibility for individual salmon lice can be estimated, a feature that may be useful in studies aiming to examine heterogeneity of response or to identify molecular determinants of resistance.

5.1 Introduction

Sea lice (Caligidae: Copepoda) are marine fish ectoparasites feeding on the mucus and skin tissues of their hosts (Boxaspen, 2006). Sea louse infections of farmed Atlantic salmon (*Salmo salar*) mostly involve *Lepeophtheirus salmonis* (salmon louse) and *Caligus elongatus* in the Northern hemisphere and *Caligus rogercresseyi* in Chile (Costello, 2006). Currently, sea louse control on salmon farms mostly relies on the use of veterinary drugs, supplemented by farm management measures (Costello, 2009) and the use of cleaner fish (Sayer et al., 1996). Further alternative control methodologies have been suggested and include efforts to reduce host fish susceptibility through vaccination or selective breeding (Gharbi et al., 2009; Raynard et al., 2002), but, as of 2015, no commercial application is available. Other attempts to disrupt parasite host detection and/or attachment (Ingvarsdóttir et al., 2002) or to mechanically remove parasites using water jets (prototype stage) (Grydeland et al., 2013). However, these supplementary approaches to sea louse control are currently not sufficiently well developed to allow full replacement of the use of chemical delousing agents; therefore, the use of antiparasitics presently remains essential for sea louse control on fish farms (Burrige et al., 2010).

Only a limited number of anti-sea louse drugs is currently available (Burrige et al., 2010), minimising the options for rotation between drug classes with different modes of action (Denholm et al., 2002). The continued use of the same or a few related types of control agent can increase the likelihood for resistance to those drugs may develop in the parasite. The development of drug resistance is well documented in *L. salmonis*, and compounds for which loss of efficacy has been reported include organophosphates (Jones et al., 1992; Roth et al., 1996), pyrethroids (Sevatdal & Horsberg, 2003), hydrogen peroxide (Treasurer et al., 2000) and emamectin benzoate (Lees et al., 2008). Emamectin benzoate (EMB) resistance has further been

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reported for *C. rogercresseyi* (Bravo et al., 2008; Bravo et al., 2010). Accordingly, there is an urgent need to develop drug resistance monitoring and management strategies in salmon lice. This will assist in observing and constraining extant resistance as well as prevention of future resistance development.

Successful resistance management requires fast and reliable methods to assess the drug susceptibility status of parasite populations. Bioassays are used to monitor the reduction of drug susceptibility of a parasite population (French-Constant & Roush, 1990). Usually, a test population is exposed to different drug concentrations, and results are usually summarised as a median exposure level, *i.e.* the degree of exposure causing toxic effects in 50% of the test population. Assuming an aquatic organism takes up a toxicant from water at a stable rate and excretes it at a rate lower than the uptake rate, the resulting internal exposure is proportional to both the toxicant's concentration and exposure time. Standard salmon louse bioassays achieve gradual exposure levels by testing different toxicant concentrations while keeping exposure time constant (Sevatdal & Horsberg, 2003; Westcott et al., 2008), to derive median lethal or median effective concentrations (LC_{50} , EC_{50}) of the toxicant, *i.e.* the concentrations causing the relevant toxic effect in 50% of the parasites. Alternatively, different exposure levels can be obtained by combining a fixed toxicant concentration with variable exposure times, in which case the susceptibility of the population of test organisms to the toxicant can be expressed as the median lethal or median effective time (LT_{50} , ET_{50}).

While the requirement for multiple observations can account for increased workload required per test in time course analyses, fewer test organisms than in standard bioassays are needed per assay as the same batch of animals can be observed repeatedly. The availability of the appropriate developmental stages of salmon lice suitable for bioassays can be restricted at production sites when parasite levels approach the legal threshold for mandatory control

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interventions. Alternative bioassays involving only one drug concentration applied for a fixed exposure period have been proposed to allow for drug susceptibility assessment under such circumstances (Helgesen & Horsberg, 2013b; Whyte et al., 2013). While single dose/single exposure-time bioassays may be appropriate in many situations, their ability to resolve susceptibility differences between salmon louse populations is limited. In situations where more detailed information is required on drug susceptibility status, alternative salmon louse bioassays based on time course toxicity analyses could be extremely useful when parasite numbers do not permit the performance of standard salmon louse bioassays. However, to our knowledge, assay protocols based on time course toxicity analysis have not been established for this group of parasites.

The aim of the present study is to investigate the potential of a time course toxicity analysis as an alternative approach to conducting salmon louse drug sensitivity assessments. Two well-characterised laboratory strains of *L. salmonis* differing in susceptibility to the salmon delousing agent emamectin benzoate (Carmichael et al., 2013; Heumann et al., 2012) were used as biological models to establish a suitable protocol. Time course toxicity results were compared to standard dose-response bioassays with respect to their ability to detect differences in EMB susceptibility among salmon louse strains.

5.2 Materials and Methods

5.2.1 Salmon louse (*Lepeophtheirus salmonis*) strains and husbandry

Two *L. salmonis* laboratory-maintained strains differing in susceptibility to EMB (Heumann et al., 2012) were used in this study. The susceptible S strain was established in 2003 using salmon lice from a Scottish farm site where no chemical control agents other than hydrogen peroxide had been used. The EMB-resistant strain R was established in December 2008 using salmon lice from a different Scottish production site reporting variable EMB treatment efficacies. The strains have since been cultured under identical laboratory conditions using Atlantic salmon (*Salmo salar*) as host fish, as described in detail elsewhere (Heumann et al., 2012). To propagate cultures, *L. salmonis* egg strings were collected from gravid females and allowed to hatch and develop to copepodids, which were used to inoculate tanks containing fresh host fish. To collect *L. salmonis*, host fish were anaesthetised with 100 mg L⁻¹ 2-phenoxyethanol (99%; Sigma-Aldrich, Dorset, UK) in seawater for 3 minutes and parasites removed into clean aerated seawater using fine forceps. Infection rates were maintained at levels compatible with good fish welfare. All laboratory infections were carried out under UK Home Office licence and appropriate veterinary supervision.

5.2.2 Standard dose-response bioassay

Prior to use in bioassays, adult male *L. salmonis*, freshly collected from host fish, were allowed to recover in the dark for 2-4 h in filtered aerated seawater (SW) equilibrated to 12 °C. In previous experiments, when comparing results between parasites collected as described above and parasites collected from fish euthanized by a UK home office approved schedule 1 method (stunning followed by cervical dislocation), no effects of host fish anaesthesia on salmon louse motility or the ability of parasites to adhere to host skin or other surfaces were observed (data not shown). Exposure was performed in a temperature-controlled incubator set to 12 °C using polystyrene Petri dishes containing 70 mL of exposure solutions. Each vessel contained ten adult male salmon lice. EMB (technical grade, a gift from MSD Animal Health) was solubilised using polyethylene glycol 300 (PEG 300; Ph. Eur., Merck Chemicals, UK) before being diluted in filtered, aerated SW. Trials showed that PEG 300 had no effect on test animals. A stock solution was prepared and all other concentrations were made by serial dilution. Exposure solutions contained a final concentration of 0.01% (v/v) PEG 300 in all cases (except seawater controls). Each assay comprised of a series of five EMB concentrations (100, 130, 180, 230 and 300 µg L⁻¹ for S strain; 100, 200, 400, 800 and 1,600 µg L⁻¹ for R strain), SW controls as well as solvent controls containing 0.01% (v/v) PEG 300. Salmon lice were assigned to treatments at random. Three replicate vessels with ten animals each were used per combination of strain and experimental or control treatment (180 animals per strain). After 24 h of exposure, salmon lice were visually examined, carefully touched with a brush and their behaviour assessed matching one of three categories. Parasites rated as “live” were considered not affected, while those animals rated as “moribund” or “dead” were affected by EMB (see Table 8). Encryption of

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treatment labelling was used to ensure unbiased rating. Selected dishes were rated by two independent examiners to ensure consistency and independence of ratings.

Table 8: Effect criteria used by different authors to define sublethal responses in salmon louse bioassays.

Sublethal effect definition I¹		Sublethal effect definition II²	
Live - normal swimming behaviour (swim in a straight line), securely attaches to Petri dish, normal movement of extremities	Unaffected	Live – normal swimming behaviour (swim in a straight line), securely attaches to Petri dish, normal movement of extremities	Unaffected
Moribund – disabled swimming, but capable of weak, uncoordinated movement (loop swimming), inability to firmly adhere to Petri dish (short time, then drops off), minimal movement of extremities	Affected	Weak – poor or irregular swimming, inability to attach to Petri dish	Affected
		Immotile – immotile, minimal movement of extremities (twitching)	
Dead – inability to swim, floating in Petri dish, no movement of extremities		Dead - Inability to swim, floating in Petri dish, no movement of extremities	

¹ (Sevatdal & Horsberg, 2003; Westcott et al., 2008)

² (Igboeli et al., 2012; Saksida et al., 2013)

5.2.3 Time course bioassays

Salmon lice were exposed in a similar fashion to different EMB concentrations as for the standard bioassay. Experimental treatments and controls were run as triplicates (experiment 1) or duplicates (experiment 2 and 3) of ten adult males in disposable polystyrene Petri dishes

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containing 70 mL of the relevant test or control solution, using a total of 60 animals per strain. Susceptible salmon lice (S) were exposed to 400 and 800 $\mu\text{g L}^{-1}$ EMB, while resistant lice (R) were tested at 800 and 1,600 $\mu\text{g L}^{-1}$ EMB. At set time intervals throughout the exposure period, parasites were examined visually and allocated to one of four categories describing their swimming behaviour: “Live”, “weak”, “immotile” or “dead”. Lice affected by treatment belonged to categories “immotile” or “dead” (sublethal effect II, see Table 8). All animals were distributed randomly to each Petri dish containing seawater. After allocating all animals, seawater was carefully decanted and test solutions were added immediately. Furthermore, two sets of solvent and seawater controls were prepared. One set was examined at the same time as dishes containing EMB solutions, serving as internal control. These animals were repeatedly assessed in parallel to the drug-treated parasites. The other set of controls was wrapped with aluminium foil for protection against visual stimuli and left untouched over the course of the experiment; to be assessed only once when the final rating of the other treatments took place. A comparison between internal and external control would give an indication of whether repeated examination of salmon lice behaviour had an influence on behavioural responses.

In time course bioassays, the main factor controlling toxic response is the length of exposure, which cannot be concealed to the evaluator by encryption. Still, encryption of all labels was used to ensure unbiased assessment. All other experimental conditions were identical to those described above for the standard bioassay.

5.2.4 Statistics

In standard bioassays, median effective concentrations (EC50) of EMB, i.e. concentration of the drug causing immobility in 50% of the tested parasite strains, were derived from experimental

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data by probit analysis using the statistical programme Minitab 15.1 (MiniTab Inc., UK). In time course bioassays, median effective times (ET_{50}) and 95% confidence intervals were calculated using the reliability/survival time module of the commercial statistical programme Minitab (version 16.1), assuming a log-normal distribution. Fisher's exact test was used to compare bioassay outcomes between internal and external controls in a 2x2 contingency table design, in which categories "weak", "immotile" and "dead" were pooled and compared to the category "live". Fisher's exact test was carried out using the programme InStat3 (Graph Pad Inc., La Jolla, USA).

5.3 Results

Two laboratory strains of *L. salmonis* differing in EMB sensitivity served as biological models in this study. In 24 h standard bioassays with adult male parasites, EMB half-maximal effective concentrations (EC_{50}) were $160 \mu\text{g L}^{-1}$ (95% confidence intervals: 148 - $173 \mu\text{g L}^{-1}$) in the drug-susceptible strain S and $712 \mu\text{g L}^{-1}$ EMB (592 - $852 \mu\text{g L}^{-1}$) in the EMB-resistant strain R (Figure 17). In these bioassays, parasites are rated as "live", "moribund" or "dead" at the end of the test and moribund and dead animals are considered affected by the toxicant (sublethal effect definition I, Table 8).

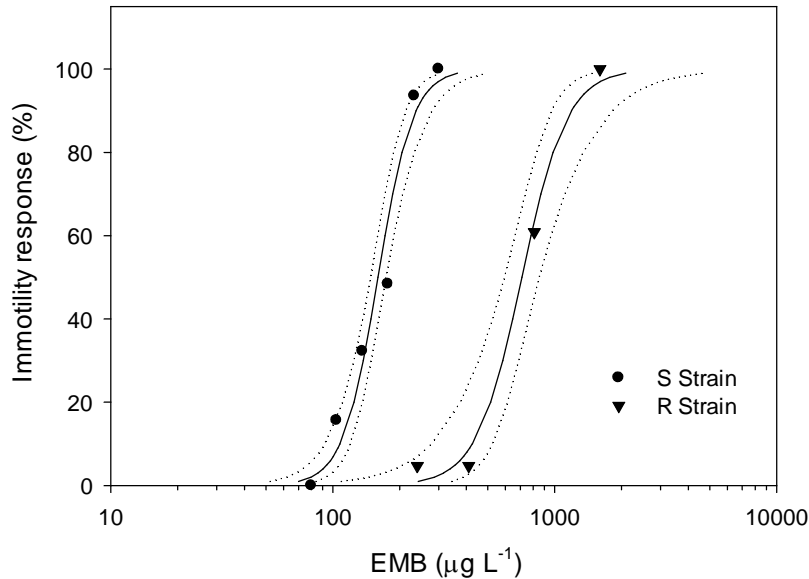


Figure 17: Dose-response bioassay testing for acute toxicity of emamectin benzoate (EMB) in two strains of *L. salmonis* (S = susceptible, R = resistant). Symbols represent the average immotility response among three replicate batches of ten adult male parasites following 24 h of aqueous exposure to the drug. Dashed curves represent 95% confidence intervals.

Based on the above estimates of strain sensitivities, EMB concentrations of 400 and 800 $\mu\text{g L}^{-1}$ (S strain) and 800 and 1,600 $\mu\text{g L}^{-1}$ (R strain) were selected for time course experiments addressing the temporal dependence of the toxicity response. In these tests, the behaviour of animals exposed to the toxicant was examined repeatedly at set time points, using similar criteria to those applied in standard bioassays. However, in order to allow for a more gradual assessment of parasite responses, the criterion “moribund” was subdivided into two categories called “weak” and “immotile” (Table 8). When results are summarised using the sublethal effect definition I, which is employed in standard bioassays, response levels in seawater controls were high (~45-90%) and showed no clear relationship to exposure time, while responses in EMB-

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exposed animals increased from initial values of ~30-60% to final values of 100% during the course of the experiment (Figure 18A-B). Solvent controls showed similar response levels as observed in seawater controls (data not shown).

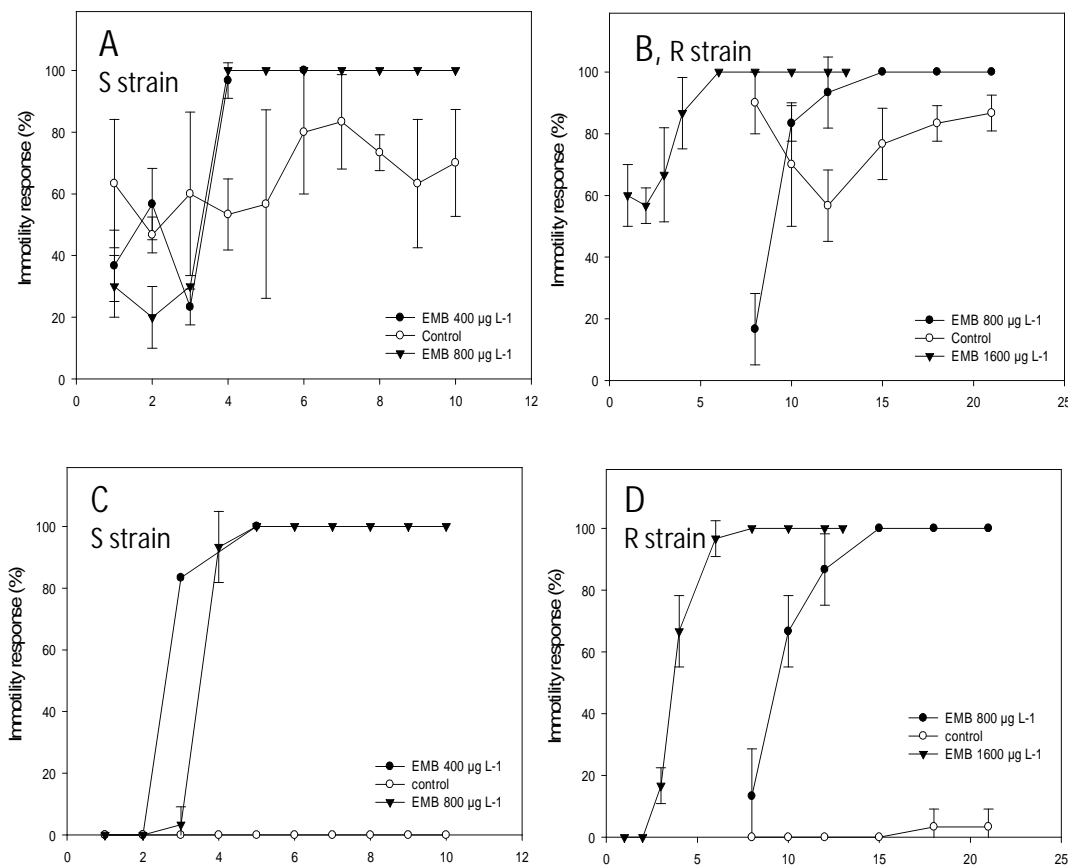


Figure 18: Time course of the acute toxicity of emamectin benzoate (EMB) in two strains of *L. salmonis*. Adult male parasites were exposed to 800 µg L⁻¹ of EMB or seawater as control and their behaviour assessed regularly. Graphic representation of response using sublethal effect criteria I (A, B) or sublethal effect criteria II (C, D) for S strain and R strain parasites, respectively. Symbols represent average immotility response among three replicates of ten parasites with standard deviation.

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Next, data were re-analysed using a recently suggested revised definition of the sublethal response in sea louse bioassays (Igboeli et al., 2012; Saksida et al., 2013), in which immotility or death of parasites is counted as toxic effect whereas live or weak animals are considered unaffected (sublethal effect definition II; Table 8). Using these effect criteria, response levels in the controls were close to zero (< 10%), while in parasites exposed to EMB an increase of response levels with time was observed (Figure 18C-D), allowing to estimate time required for 50% of the parasites to become affected during exposure to a given drug concentration. With 800 µg L⁻¹ EMB, the median effective time (ET₅₀) for sublethal effect II was 3.5 h (95% confidence intervals: 3.3 – 3.6 h) in the S strain and 9.6 h (9.0 – 10.3 h) in the R strain. In the S strain, 400 µg L⁻¹ EMB elicited a similar apparent time course of toxicity as 800 µg L⁻¹ EMB, but data obtained with the former concentration of the drug did not allow calculation of an ET₅₀. Compared to 800 µg L⁻¹ EMB, 1,600 µg L⁻¹ EMB had a greater apparent effect in the R strain, as reflected by an ET₅₀ of 3.4 h (3.0 – 3.9 h) (Figure 18C-D) (Table 9).

Table 9: Overview of three independent time course bioassays conducted between December 2011 and May 2014. Susceptible (S) and resistant (R) lice were exposed to 800 µg L⁻¹ EMB and the half maximal response time (ET₅₀) and 95% confidence intervals calculated.

Experiment (Date)	ET ₅₀ (95% confidence interval)	
	S strain	R strain
1 (December 2011)	12.9 h (11.2-14.9)	25.3 h (22.6-30.5)
2 (July 2012)	6.9 h (6.3-7.6)	23.5 h (20.6-26.7)
3 (May 2014)	3.5 h (3.3-3.6)	9.6 h (9.0-10.3)

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The time course bioassay involved multiple examinations of the same parasites, which could have affected behavioural responses. To control for potential effects of repeated assessments, external untreated controls and EMB treatments were included for both strains, which were examined only once at the end of the experiment. Compared to experimental controls subjected to repeated rating, more sea lice categorised as “live” and fewer animals rated as “weak” were observed in external controls (Figure 19). The difference in response between the two types of controls was significant in the assay with the R strain, but not for the S strain. In both types of controls, the number of sea lice showing a response according to sublethal effect definition I clearly exceeded 10%, the maximum response level allowed in the original protocols for standard sea louse bioassays. However, when using sublethal effect definition II, the response in both types of controls was below the 10% threshold (Figure 19).

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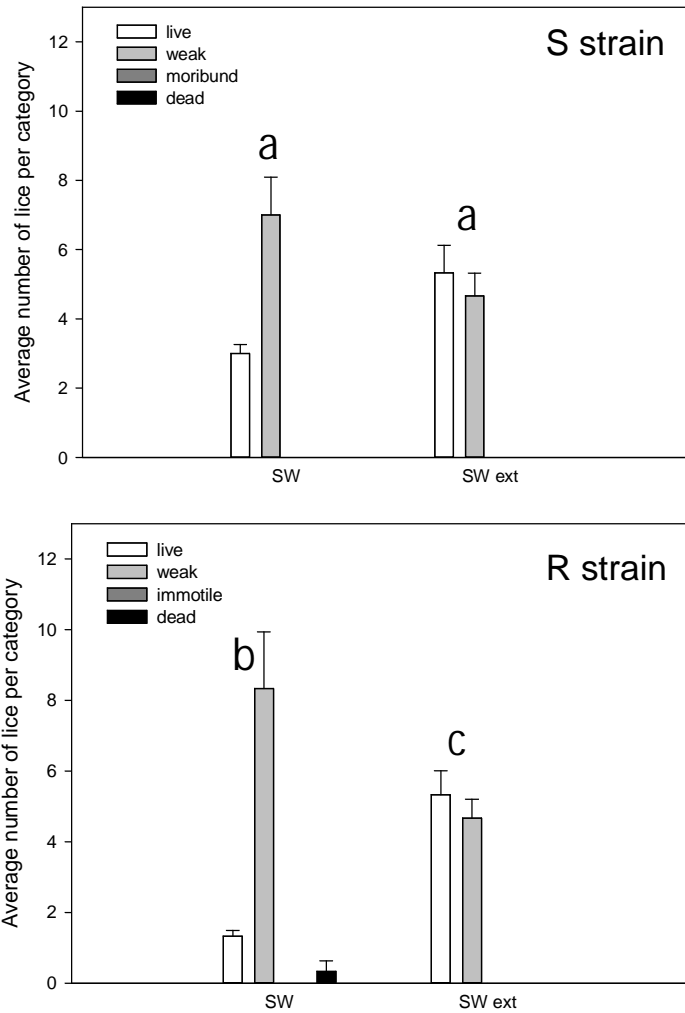


Figure 19: Average number and standard deviation of parasites per behavioural category for experimental seawater (SW) and external seawater (SW ext) controls. External controls were assessed once at the end of the time course bioassay, while experimental controls were rated multiple times before the final assessment. Triplicates of 10 adult male lice (S strain, R strain) were used. Different letters denote significant ($p < 0.05$) difference between two means.

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In order to test the consistency of the time course bioassay, both strains were investigated multiple times between 2011 and 2014. While ET_{50} values showed significant variability within each strain, a significant susceptibility difference between the two strains was detectable in all trials (Table 9).

5.4 Discussion

Bioassays are used to assess the toxicity of chemicals in aquatic organisms. They are usually based on analysing the relationship between different concentrations of a substance and its toxic effect over a fixed time period, and results are usually expressed as median lethal or effective concentrations (Pascoe & Edwards, 1989; Sprague, 1969). An alternative approach involves time course toxicity analyses, in which the manifestation of a toxic effect is established over a certain time period by repeated observations of a batch of organisms exposed to a fixed toxicant concentration, allowing the calculation of a median effective or lethal time (Rand, 2008; Sprague, 1969). Results obtained in the present study suggest, in principle, the feasibility of salmon louse bioassays based on time course toxicity analysis, which could complement current dose-response salmon louse bioassays in drug susceptibility assessments. The results of this study further highlight the importance of consistent and robust effect criteria in bioassays, an issue that recently has been the subject of debate (Igboeli et al., 2012; Saksida et al., 2013).

While mortality is a clear and specific bioassay endpoint, it shows limitations in terms of sensitivity (Sevatdal & Horsberg, 2003). Tests based on sublethal behavioural effects potentially offer improved sensitivity; however, clear definitions of such effects are required. The original definition of sublethal effects in toxicity tests with sea lice (sublethal effect I, Table 8) comprise

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a wide range of behaviours indicating sublethal effects, ranging from subtle alterations in swimming and/or attachment behaviour to complete impairment of locomotion. Standardised tests based on these criteria (SEARCH Consortium, 2006; Sevatdal & Horsberg, 2003; Westcott et al., 2008) have been found to perform well under laboratory conditions. However, in certain instances (e.g. field bioassays on salmon farms), elevated response levels of up to 60% in control treatments have been observed (Westcott et al., 2008). While the use of probit regression models incorporating “natural responsiveness” has been suggested to allow for sensitivity assessment in such cases (Westcott et al., 2008), effect levels of greater than 10% invalidate test results according to current international guidelines (SEARCH Consortium, 2006). High response levels in control treatments (>10%) were also observed during time course assays in the present study, and are reported to occur occasionally in the summer months during standard bioassays both at MERL (Dr William Roy, personal communication) and elsewhere (Gregg McArthur, North Atlantic Fisheries College, Shetland, UK). To improve the reliability of bioassays, revisions of sublethal effect criteria have recently been suggested (Igboeli et al., 2012; Saksida et al., 2013). The revised categories differentiate between subtle changes in parasite behaviour (“weak”) and more pronounced alterations (“immotile”), and only regard the latter as indicative of toxicant effects. Applying the revised criteria in time course bioassays in the present study allowed clear differentiation between drug treatments and controls, with control responses now being less than 10%, demonstrating the suitability of the revised criteria to detect sublethal effects.

Dose-response bioassays based on behavioural criteria usually involve only one assessment of animal behaviour, which takes place at the end of a defined exposure period. In contrast, time course bioassays require repeated examination of parasites. The repeated gentle probing of test animals could cause changes in their motivation to react to stimulation. Moreover, impaired

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swimming in time course bioassays could be due to fatigue caused by the examination procedure. To address this point, experimental (continuous assessment) and external controls (assessment only once) were compared. More weak animals were counted in experimental as compared to external controls, and these differences were significant for the test with strain R, but not strain S. Accordingly, repeated assessment may have reduced the responses of test animals to a certain degree. However, under sublethal effect criteria I, the number of weak animals by far exceeded the threshold of 10% in both internal and external controls. In contrast, the observed response according to the revised criteria was practically zero in both types of controls, suggesting that repeated examination of sea lice did not affect test results based on sublethal effect criteria II.

Apart from handling-induced stress, other potentially interfering factors in bioassays include the possibility of a decline of toxicant concentration over time. A recent study reported significant losses of EMB from solutions prepared in seawater over 24 h, with the degree of EMB depletion being dependent on the material of the container and the incubation time (Helgesen & Horsberg, 2013a). In glass containers, 41.9% of an initial concentration of $150 \mu\text{g L}^{-1}$ EMB was still present in seawater after 24 h. Slightly higher recoveries (48.9%) were obtained with high-density polyethylene dishes, whereas less EMB was detected at the end of the test when teflon or silanised glass containers were used (27.4% and 24.0%, respectively) (Helgesen & Horsberg, 2013a). It was not possible to cover this aspect in the present study. However, in an earlier study of our group using similar conditions as those employed in this report, 49.7% of a nominal EMB concentration of $200 \mu\text{g L}^{-1}$ was found in seawater after 3 h (Carmichael et al., 2013). These data demonstrate that, during salmon louse bioassays, actual concentrations of EMB in seawater can deviate significantly from nominal concentrations, presumably due to adsorption to the walls.

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In situations where only a limited number of test organisms is available, time course toxicity analyses allow the assessment of the susceptibility of a population to a chemical based on a limited number of test animals. Such a scenario is common for salmon farms, where continuous monitoring of salmon lice susceptibility to employed antiparasitics is required; yet, due to the low threshold of parasite abundance triggering therapeutic interventions, information about the drug susceptibility status of salmon lice is sometimes required when parasite stages suitable for bioassays, *i.e.* pre-adult II and adult salmon lice, are scarce. Single-dose bioassays employing a fixed exposure time have been proposed as a method of susceptibility assessment requiring fewer test organisms (Helgesen & Horsberg, 2013b; Whyte et al., 2013). While straightforward, such approaches are limited, however, with respect to the resolution achievable in detecting differences in drug susceptibility. Time course toxicity bioassays are currently labour-intensive. It might be feasible to employ automated image analysis systems for the assessment of parasite movement. Such a system (termed WormAssay) has been successfully used to monitor filarial nematode parasites like *Brugia malayi* (Marcellino et al., 2012). It is inexpensive, has increased throughput and allows the screening of macroparasites (Marcellino et al., 2012); and if found suitable, this technology might reduce the labour required for time course toxicity analysis.

With improvements regarding standardisation and labour requirements, the time course toxicity bioassay could provide a complementary methodology for situations where detailed information is required concerning the susceptibility phenotype. In addition, time course toxicity analyses offer the advantage that the response times of the different exposed organisms provide estimates of their individual drug susceptibilities, which may be useful in characterising heterogeneity of response and identifying molecular markers related to drug resistance.

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Considering earlier data obtained with the standard dose-response bioassay, one would expect to observe a 50-60% response of R strain lice after 24 h when exposed to 800 $\mu\text{g L}^{-1}$ EMB (EC_{50} is 712 $\mu\text{g L}^{-1}$, which is comparable to 779 $\mu\text{g L}^{-1}$ found for the R strain in other standard bioassays (Carmichael et al., 2013)). However, a faster response was observed, where half of R strain lice showed responses after 10 h when exposed to 800 $\mu\text{g L}^{-1}$ EMB. We suspect that seasonally changing environmental conditions during the parasite's development could play a role in the apparent variability of the salmon lice population investigated. A repeat of this experiment, possibly with a dose-response bioassay at the same time, could shed light on this phenomenon. Variability between experimental repeats has been observed not only for time course bioassays (Table 9), but also in dose-response bioassays (Carmichael et al., 2013; Espedal et al., 2013; Heumann et al., 2012; Igboeli et al., 2012). Potential factors leading to observed variability could be a) examiner's subjectivity when assessing salmon lice, b) variation in test chemicals used, c) inherent biological variability or d) seasonal influences and overall viability of the test animals. While training of examiners and experimental design strives to minimise these influence, it might not be possible to exclude them. Therefore, the extent of their influence should be further assessed.

In conclusion, the present study investigated the overall feasibility of using an altered bioassay design to assess the susceptibility of two strains of sea lice to the delousing agent EMB. The employed time course toxicity analyses require significantly fewer test animals, when compared to current sea lice bioassays using dose-response analyses. We found that this altered bioassay design delivered a similarly clear distinction regarding the EMB susceptibility of the two model strains as with the standard dose-response bioassay. This alternative methodology could be applicable in situations where the available number of test animals is too restricted to carry out

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standard bioassays. A further advantage of this modified bioassay is the estimation of a “time-to-response” for each individual animal, thus providing data for each parasite’s drug responsiveness. However, the time course bioassay is more labour-intensive and the procedure is not as established and tested as the standard dose-response bioassay. Further experiments using the modified bioassay should be aimed at improving standardisation and reducing expenditure of human labour, which would improve repeatability and make this bioassay more competitive.

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6 GENERAL DISCUSSION

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Production of farmed salmon has increased over the years (FAO, 2012) and so has resistance of sea lice to delousing agents (Denholm et al., 2002). Prevalence of infection increases with host density – an important theory of epidemiological research (Anderson & May, 1991). Empirical studies in human, agricultural and wild systems support this theory (Anderson & May, 1991; Arneberg et al., 1998; Burdon & Hilvers, 1982). Higher stocking density allows parasites to be faster and more successful in locating new hosts and often procreate more successfully (Arneberg et al., 1998). Delousing using chemical compounds is an important tool for salmon farmers to keep salmon lice burden under control. However, prolonged use of a single compound, like EMB, will lead to drug resistance as the compound's efficacy decreases and delousing becomes less effective (Bravo et al., 2008). Using a single class of chemical continuously is favouring parasites that survive the treatment cycle (French-Constant & Roush, 1990). As there is less natural competition, they will propagate with greater success and can eventually become dominant within the population. EMB was introduced in 2000 as being highly effective against all stages of salmon louse and has become widely used since (Bravo et al., 2008; Westcott et al., 2004). It has been used for many years to control infections with salmon lice and is only the latest drug losing its efficacy. Other delousing agents, with different modes of action, like the organophosphates or pyrethroids, have also lost their efficacy for delousing in some salmon production areas (Denholm et al., 2002), when resistant salmon lice strains emerged. Resistance to EMB has emerged in salmon lice populations in Europe and Canada (Hjelmervik et al., 2010; Westcott et al., 2010). Therefore, it is vital to improve our knowledge of the mechanisms conferring drug resistance in salmon lice, particularly at a molecular level. Other delousing methods independent of drugs, are currently developed – e.g. co-culturing with wrasse -, but, while promising, have not replaced chemical delousing (Skiftesvik et al., 2013). Having a better understanding of the processes conferring resistance

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could lead to improved monitoring tools and earlier detection of developing resistance. Furthermore, it could provide crucial insight for the development of new drugs for delousing.

Several studies suggest that ABC transporters (such as P-gp) play a major role in nematodes resistant to IVM, a compound chemically very similar to EMB (Prichard & Roulet, 2007). One study suggested that increased expression of P-glycoproteins in the gut epithelium of *L. salmonis* would decrease the absorption of EMB from ingested mucus (Tribble et al., 2007). Additional knowledge about P-gp homologues in salmon lice would be beneficial for understanding the molecular mechanisms conferring EMB resistance.

During this project, we identified a novel salmon louse P-gp. Rapid amplification of cDNA ends (RACE) was used to obtain its full-length open-reading frame. The predicted gene product has a length of 1,438 amino acids, was named SL-PGY1 and has high homology with P-gps in other species. P-gps, belonging to the ABCB subfamily, act as efflux pumps with a wide range of substrates, including toxicants (Epel et al., 2008). Overexpression of P-gp has been found to be one of the causative factors for drug resistance in human cancer cells (Ueda et al., 1987). They are also suspected factors contributing towards avermectin resistance observed in parasites (Williamson & Wolstenholme, 2012; Wolstenholme & Kaplan, 2012). In fact, co-administration of avermectins and an ABC transporter inhibitor (verapamil) lead to an increase in treatment efficacy in avermectin-resistant parasites (Kerboeuf et al., 2003). Therefore, drug efflux transporters belonging to the ABCB subfamily have been implicated as factors contributing to avermectin resistance in nematodes such as *C. elegans* or *H. contortus* (James & Davey, 2009; Sangster et al., 1999; Xu et al., 1998).

SL-PGY1 shares a high structural similarity with P-gps of vertebrate and invertebrate origin and a role for SL-PGY1 in EMB detoxification was investigated. In the absence of EMB, SL-PGY1

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mRNA expression level in S and R strain lice did not significantly differ from each other. However, EMB exposure had a variable effect on the expression of this P-gp, depending on length and mode of exposure. Three hours of exposure to 200 µg/L EMB in bioassays had no effect on the mRNA expression ratio of both model strains. Salmon lice of the moderately resistant SF strain were exposed to EMB while attached to their hosts, which had been fed EMB-fortified food. After three weeks, no differences in SL-PGY1 expression ratios were found when comparing treated and untreated parasites. However, sublethal exposure to 410 µg/L EMB in a 24 h standard bioassay resulted in a 2.9-fold increase in SL-PGY1 mRNA expression in parasites of the R strain. An investigation of single-nucleotide polymorphisms did not find any changes possibly associated with EMB resistance. We conclude that SL-PGY1 is not the sole factor responsible for reduced susceptibility to EMB, although it could play a role, as expression levels were increased in the R strain upon acute exposure to EMB. It appears that there is no single ABC drug transporter functioning as the major determinant for avermectin drug resistance (Heumann et al., 2012; Le Jambre et al., 1999; Lespine et al., 2012; Yan et al., 2012), suggesting the involvement of multiple factors, not necessarily limited to P-gps. Interestingly, the expression of SL-PGY1 differed between male and female parasites of the same strain, with males expressing significantly more mRNA. This could indicate that this transporter has additional physiological functions, which might differ between the sexes. Further research regarding the role of SL-PGY1 and other ABC transporters in *L. salmonis* is required.

Therefore, the role of other ABC drug transporters was also investigated. Multidrug-resistance proteins, part of the ABCC subfamily, are known to act as drug transporters in humans (Cole et al., 1992) and mice (Johnson et al., 2001) and could potentially also play a role in EMB

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resistance. During this project, we identified four novel MRPs in *L. salmonis*. We examined the relative expression levels of all four transporters in the two model salmon louse strains differing in their susceptibility to EMB. MRP levels did not differ between strains in the absence of EMB and did not change after exposure to the drug. There was no molecular evidence for a potential role of those four MRPs in EMB resistance. Further experiments involved a pharmacological approach employing well-documented inhibitors of ABC transporters. CSA and VER are known to inhibit ABC transporters, including human P-gps and MRPs (Meier et al., 2006; Saeki et al., 1993). This approach was chosen as these integral membrane proteins facilitate the export of substrates from the cytoplasm and form the “first line of defence” against toxicants (Epel et al., 2008). Therefore, they are predominantly expressed in tissues involved with excretion or in boundary-forming tissues, such as placenta, gut epithelia or blood-brain barrier (Leslie et al., 2005). They often localise to the apical membrane domain of polarised cells, which underlines their function in restricting uptake and facilitating efflux of cytotoxic substances (Zaja et al., 2008). Furthermore, lack of functioning ABC drug efflux transporters can result in hypersensitivity to substrates of those transporters, as was observed for P-gp-deficient Collie dogs (Roulet et al., 2003) and mice (Schinkel et al., 1994) becoming highly susceptible to IVM.

We investigated whether co-administration of ABC inhibitors would lead to a change in EMB susceptibility in *L. salmonis*. CSA increased the toxic effect of EMB in both tested salmon lice strains. Apparently, the targets of CSA are part of the defence mechanisms against toxic substances, and CSA’s presence is decreasing their capacity to limit the effects of EMB on the parasite. Furthermore, we assume they are expressed at comparable levels, as a similar effect was observed in both strains. Interestingly, VER increased the toxic effect of EMB in the resistant strain, but had no significant effects on the EMB-susceptible strain. We suspect that VER inhibits the function of biochemical factors involved in EMB detoxification, possibly ABC

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transporters. A similar result was observed in a study with the ruminant parasitic nematode *H. contortus*. There, VER co-administration increased the toxic effect of IVM and moxidectin in an avermectin-resistant strain with increased P-gp expression (Xu et al., 1998). Furthermore, co-administration of VER increased EMB toxicity in a laboratory-reared Canadian strain of *L. salmonis* (Igboeli et al., 2012). We hypothesise that a number of ABC transporters with distinct, yet overlapping patterns of inhibitor specificity are affected by those inhibitors. This reduces their capacity to remove EMB from the parasite, leading to an increased EMB concentration in *L. salmonis*.

The observed differences in EMB susceptibility between the S and R strains could also be linked to varying degrees of expression of resistance-conferring proteins, which are targeted by ABC transporter inhibitors like cyclosporin A and verapamil. For example, increased expression of four MRP mRNAs was associated with IVM resistance in *C. elegans* (James & Davey, 2009). With EMB being chemically very similar to IVM, use of inhibitors reversing IVM resistance could represent a way to reverse EMB resistance in *L. salmonis*, because inhibitor-mediated reversal of drug resistance was successful in IVM-resistant *H. contortus* (Bartley et al., 2009; Molento & Prichard, 2001).

The capability of ABC transporters to remove a wide variety of substances is a major obstacle for treating salmon louse infections. Combination therapies, whereby two or more drugs are simultaneously administered, are successfully used to treat nematodes infecting ruminants and horses (Geary et al., 2012). Combining several delousing agents to treat *L. salmonis* infections have been tested since 2009. In Norway, pyrethroids (deltamethrin, cypermethrin) and organophosphates (azamethiphos) have been used for combination therapy in several areas and there are signs for increasing resistance, especially against azamethiphos (S Sevatdal, <http://www.veso.no/sea-lice-selection-study>). Resistance to a triple combination of

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anthelmintics was also observed in the parasitic nematode *Ostertagia circumcincta* (Wrifley et al., 2006) and it is possible that the activity of ABC transporters is, at least partially, responsible for the limited success of combination therapy. This highlights the need to improve our understanding of the intricate role ABC transporters play in protecting *L. salmonis* from toxicants such as EMB. Future experiments could employ different, probably second- and third-generation ABC transporter inhibitors to do so.

The candidate gene approach, which yielded one P-gp (Chapter 2) and four MRP homologues (Chapter 3) in *L. salmonis*, was unlikely to be exhaustive, due to limited genetic information available for this parasite. In fact, as of 2014, the total number of ABC transporters in *L. salmonis* remains unknown. Known are: The previously mentioned P-gp homologue SL-PGY1 (Heumann et al., 2012); a protein showing homology with mitochondrial ABC transporters (SL-Pgp1) (Heumann et al., 2012; Tribble et al., 2008); four MRPs (Heumann et al., 2014) and SL0525, belonging to the ABCF subfamily, which is not known for drug transporters (Tribble et al., 2007).

A systematic, genome-wide survey of ABC transporters in *L. salmonis* was used to find additional members of this important gene family (Chapter 4). For this purpose, next-generation high-throughput RNA sequencing (RNA-seq) was employed. The reference transcriptome was created from a total RNA pool derived from salmon lice at key developmental stages. It was assembled against the *L. salmonis* genome (version July 2012) and encompassed 33,933 transcripts, corresponding to 30,159 putative genes, with 27,086 single transcripts, while the remaining transcripts were splicing variants. The transcriptome was annotated using BLASTn searches against the UniGene and BLASTp searches ref_seq databases. For 29 and 95 per cent of the transcripts, matching data was found in public databases, respectively. Furthermore, 76

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per cent of the 128,783 ESTs for *L. salmonis* aligned with at least one transcript. In addition, gene ontology analysis revealed that 95 per cent of the putative genes of the *L. salmonis* transcriptome had similarities to known gene product; yet only 17 per cent were annotated. Screening of the transcriptome for ABC transporters yielded 33 transcripts assumed to be members of the ABC transporter superfamily, while 186 ESTs were assembled into 28 contiguous sequences, putatively representing ABC transporters. Thereby, we identified a total of 39 putative ABC transporters in *L. salmonis*. Phylogenetic analysis placed all sequences into groups of known ABC transporters with high boot-straping values and, when possible, domain structure was analysed. This, and manual annotation, was used to confirm membership to the respective ABC subfamily of each sequence. Of further interest were subfamily B, C and G, as these contain drug-transporting ABC proteins (Dean et al., 2001).

For the ABCB subfamily, we found one full and three half transporter transcripts. Only full transporters, like mammalian P-gp, are known to contribute to the biochemical defence against toxicants (Leslie et al., 2005). The full transporter is identical to SL-PGY1, which was identified in a previous study (Chapter 2). Expression of this P-gp homologue was shown to increase upon EMB exposure and differs between sexes (Heumann et al., 2012; Igboeli et al., 2012). The half transporters show homology to human mitochondrial proteins ABCB7 and ABCB10, which are involved in iron metabolism and transport of Fe/S protein precursors (Dean et al., 2001).

For the ABCC subfamily, we found 14 sequences – 11 MRPs and 3 homologues to sulfonylurea receptors. Of particular interest are the MRPs, as they contribute to detoxification in humans (Deeley et al., 2006) and invertebrates. In zebra mussels, *Dreissena polymorpha*, expression of an MRP1 homologue is increased in response to heavy metal- and pesticide-induced stress (Navarro et al., 2012), while in *C. elegans*, an MRP1 homologue plays a role in protecting the

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nematode from heavy metal toxicity (Broeks et al., 1996). Increased expression of MRPs, in conjunction with P-gps and cytochromes P450, was observed in IVM-resistant *C. elegans* (James & Davey, 2009; Yan et al., 2012). Four out of 11 MRPs had been identified in previous EST searches and their expression ratios did not differ between S and R strain parasites (Chapter 3).

Seven sequences belonging to ABCG subfamily were found. Human ABCG transporters facilitate the transport of steroid alcohols and drugs (Kusuhara & Sugiyama, 2007). For example, ABCG1 and ABCG4 are involved in loading of cholesterol to high-density lipoprotein, while ABCG2 (also known as BCRP) is a drug transporter (Krishnamurthy & Schuetz, 2006). However, none of the *L. salmonis* ABCG transcripts identified showed sufficient homology to known drug transporters in other species.

The transcriptome-wide search and annotation of ABC transporters has substantially added to the current knowledge of ABC transports in *L. salmonis*. The increased number of putative drug transporters could allow for a deeper and more comprehensive understanding of this important gene family. Subsequently, this might enable researchers to improve efforts aimed at elucidating the underlying molecular mechanisms of factors involved in drug resistance in salmon lice.

Bioassays are a valuable tool to assess the toxicity of chemicals on aquatic organisms. The toxic effect is observed in relationship to the compound's concentration over a pre-defined period. The results are normally given as the effective concentration, where 50% of the test animals are immobilised or as lethal concentration, at which 50% of the test population has are dead. This dose-response-based approach has become the standard for monitoring the sensitivity of salmon lice to EMB (SEARCH Consortium, 2006; Sevatdal & Horsberg, 2003). Tests are usually conducted with pre-adult II or adult parasites to ensure the same developmental stage. However,

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relatively large numbers of parasites (~150) are required to conduct a dose-response bioassay, which may not always be feasible. Especially in a farm context, where animal welfare is paramount, chemical intervention happens at relatively low levels of infection. Therefore, it can be difficult to secure sufficient numbers of test animals.

To address this issue, single-dose bioassays using a fixed time of exposure have been proposed as an alternative requiring fewer test animals (Helgesen & Horsberg, 2013; Whyte et al., 2013). However, such a design might be insufficient for detecting differences in EMB susceptibility. Therefore, we tested the feasibility of an alternative bioassay design requiring fewer test animals than the dose-response bioassay, while being sufficiently sensitive to discriminate between *L. salmonis* strains with differing EMB susceptibility. This alternative approach uses time course toxicity analysis, where the toxic effect of a fixed chemical concentration is monitored over time. This yields the median time that has to elapse before 50% of test animals are affected or dead.

Similar to the standard dose-response bioassay, for the time course bioassay, a researcher observes behavioural changes of salmon lice in response to EMB. In order to ensure consistency, it is important to clearly define what constitutes an effect. Mortality as an endpoint is specific, but lacks sensitivity (Sevatdal & Horsberg, 2003). Observing sublethal behavioural effects has the potential to improve sensitivity, but requires exact definitions of those effects. For the widely used dose-response standard bioassay, the sublethal effect criteria encompass a wide range of behavioural effects, ranging from minor changes of the parasite's swimming performance over changes in their capacity to adhere to surface to full impairment of swimming. Under laboratory conditions, bioassays using these criteria (Sevatdal & Horsberg, 2003; Westcott et al., 2008) performed well. However, under certain circumstances, for example

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when performing field bioassays on fish farms, elevated response levels were observed in untreated controls (Westcott et al., 2008), exceeding the recommended threshold of 10% (SEARCH Consortium, 2006). During the course of this project, high response levels in controls were occasionally observed, which is apparently seasonal and occurs elsewhere (Gregg McArthur, North Atlantic Fisheries College, Shetland, UK), too. The cause is currently unknown (Dr William Roy, University of Stirling, personal communication). A revision of the wide range of sublethal effect criteria was recently recommended, in order to increase the reliability of bioassays (Igboeli et al., 2012; Saksida et al., 2013). The revised criteria would exclude parasites with minor behavioural alterations such as loop-swimming from being counted as affected by EMB. When applying these modified sublethal effect criteria, it was possible to clearly differentiate between animals of the S and R strain.

The time course bioassay requires multiple assessments over the observation period, while for dose-response bioassays, there is only a single assessment at the end of the experiment. Repeated examination is likely to be stressful for the test animals and the potential influence of the disturbance was assessed using two sets of controls. While there was an increased number of animals with minor behavioural changes, none of them was considered affected (chapter 5). The alternative bioassay design allowed for a clear distinction between the EMB-susceptible and EMB-resistant strain as the standard dose-response bioassay. Therefore, time course toxicity bioassays could be used to assess EMB susceptibility under circumstances when the number of available test animals is limited. However, as it is even more labour-intensive than the standard bioassay, it also lacks the possibilities for high throughput or automation in its current form. Computer-assisted image analysis might be useful to simplify the time course toxicity bioassay, when it is employed for the assessment of swimming behaviour, as it was done for nematodes (Marcellino et al., 2012). Still, an additional benefit of the proposed design is the possibility to

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record the response time of each exposed animal, which allows for conclusions regarding their individual susceptibility to EMB. This could be useful to investigate the individual responses within the test population and might help to identify molecular markers that indicate drug resistance.

In conclusion, the work presented in this thesis has significantly expanded the number of ABC transporters in salmon lice and investigated their potential role in EMB resistance. The genes identified here could become the foundation for future research intended to identify molecular markers associated with reduced EMB susceptibility.

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7 APPENDICES

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APPENDIX S1 – SUMMARY OF THE INDIVIDUAL SAMPLES USED TO GENERATE THE *L. SALMONIS* TOTAL RNA POOL.

Sample ID	Salmon louse stage
1	Egg strings (light colouration)
2	Egg strings (dark colouration)
3	Nauplius (24 h growth at 8°C)
4	Nauplius (24 h growth at 10.5°C)
5	Nauplius (48 h growth at 8°C)
6	Nauplius (48 h growth at 10.5°C)
7	Free-living copepodid
8	Attached copepod (24 h dpi)
9	Attached copepod (48 h dpi)
10	Chalimus I (72 h dpi)
11	Chalimus I (96 h dpi)
12	Chalimus II
13	Chalimus III
14	Chalimus IV
15	Preadult I male
16	Preadult II male
17	Adult male
18	Preadult I female
19	Preadult II female
20	Adult virgin female
21	Adult gravid female

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APPENDIX S2 – SUMMARY OF PROTEIN MODELS USED TO IDENTIFY ABC TRANSPORTS.

Motif ID	Description
PF00005	ABC transporter
PF00664	ABC transporter transmembrane region
PF06472	ABC transporter transmembrane region 2
PPTHR11384	ATP-binding cassette, sub-family D member
PTHR19211	ATP-binding transport protein-related
PPTHR19229	ATP-binding cassette transporter subfamily A
PF12698	ABC-2 family transporter protein
PR01868	ABC transporter family E signature
PS50893	ATP-binding cassette, ABC transporter-type domain
PS51012	ABC transporter integral membrane type-2 domain
SM00382	ATPases associated with a variety of cellular activities
SSF90123	ABC transporter, transmembrane domain, type 1

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APPENDIX S3—DETAILS OF THE CHARACTERISED 39 *L. SALMONIS* ABC PROTEINS

Subfamily	Accession	Transcript_ID	Predicted_Member_Name	Predicted_gene_acronym	Predicted_Location	Domain_structure_detected	Notes
A	HACA01002058	Lsa.1758	ABCA1	ABCA1		TM - NBD - TM - NBD	partial
A	HACA01016690	Lsa.14583	ABCA4	ABCA4		TM	partial
A	HACA01001963	Lsa.1680	ABCA4	ABCA4		NBD - TM	partial
B	HACA01029620	Lsa.26127	ABCB10 (mitochondrial HT)	ABCB10	mitochondrial	TM - NBD	
B	HACA01008343	Lsa.7262	ABCB7 (mitochondrial HT)	ABCB7	mitochondrial	TM - NBD	
B	HACA01000740	Lsa.643	ABCB8 (mitochondrial HT)	ABCB8	mitochondrial	TM - NBD	partial
B	HACA01004649	Lsa.4043	ABCB1	ABCC1		TM - NBD - TM - NBD	
C		EST_Contig_6	MRP14 / MRP1	ABCC1		NBD	partial
C	HACA01012937	Lsa.11278	ABCC1 / MRP1	ABCC1		TM - NBD - TM - NBD	
C	HACA01025969	Lsa.22810	ABCC1 / MRP1	ABCC1		TM - NBD - TM	partial
C	HACA01033013 HACA01033014	Lsa.29272*	ABCC1 / MRP1	ABCC1		TM	partial
C	HACA01007250	Lsa.6310	ABCC1 / MRP1	ABCC1		TM - NBD - TM	partial
C	HACA01010189 HACA01010190	Lsa.8882*	ABCC1 / MRP1	ABCC1		TM - NBD - TM - NBD	
C	HACA01026296 HACA01026297	Lsa.23107*	MRP4	ABCC4		TM - NBD - TM - NBD	
C	HACA01004060 HACA01004061	Lsa.3521*	MRP4	ABCC4		TM - NBD - TM	partial
C	HACA01004062	Lsa.3522	MRP4	ABCC4		TM - NBD - TM - NBD	
C	HACA01016337	Lsa.14264	MRP5	ABCC5		NBD	partial
C	HACA01005249	Lsa.4564	MRP7	ABCC10		TM - NBD - TM - NBD	
C	HACA01016334	Lsa.14261	ABCC8/9 (SUR)	ABCC8		TM	partial

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C	HACA01016335	Lsa.14262	ABCC8/9 (SUR)	ABCC9	NBD	partial
C	HACA01016336	Lsa.14263	ABCC8/9 (SUR)	ABCC9	TM	partial
D		EST_Contig_1	ABCD4 (peroxisomal membrane)	ABCD4	peroxisomal membrane	NBD partial
D		EST_Contig_2	ABCD4 (peroxisomal membrane)	ABCD4	peroxisomal membrane	TM - NBD
D	HACA01011675	Lsa.10176	ABCD4 (peroxisomal membrane)	ABCD4	peroxisomal membrane	TM partial
D	HACA01006738	Lsa.5856	ABCD4 (peroxisomal membrane)	ABCD4	peroxisomal membrane	TM - NBD
E	HACA01001204 HACA01001205	Lsa.1035*	ABCE1	ABCE1	NBD - NBD	
F	HACA01023315	Lsa.20458	ABCF1	ABCF1	NBD	partial
F	HACA01011095	Lsa.9678	ABCF2	ABCF2	NBD - NBD	
F		EST_Contig_3	ABCF3	ABCF3	NBD	partial
F		EST_Contig_4	ABCF3	ABCF3	NBD	partial
F		EST_Contig_5	ABCF3	ABCF3	NBD	partial
F	HACA01009266	Lsa.8082	ABCF3	ABCF3	NBD - NBD	
G	HACA01014872	Lsa.12984	ABCG20	ABCG20	NBD - TM	
G	HACA01016058	Lsa.14023	ABCG20	ABCG20	NBD - TM	
G	HACA01024391	Lsa.21408	ABCG20	ABCG20	NBD - TM	
G	HACA01026477	Lsa.23267	ABCG20	ABCG20	NBD - TM	
G	HACA01026479 HACA01026480	Lsa.23269*	ABCG20	ABCG20	NBD - TM	
G	HACA01003008 HACA01003009	Lsa.2606*	ABCG5	ABCG5	NBD - TM	partial
G	HACA01029057	Lsa.25615	ABCG8	ABCG8	TM - NBD - TM	partial

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