An investigation into the molecular determinants of salmon louse (*Lepeophtheirus salmonis* (Krøyer, 1837)) susceptibility to the antiparasitic drug emamectin benzoate.

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DECLARATION

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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Abstract

Caligid copepods, also called sea lice, are ectoparasites of marine fish, with *Lepeophtheirus salmonis* (Krøyer, 1837) emerging as a problem for mariculture of Atlantic salmon (*Salmo salar* Linnaeus, 1758) in the northern hemisphere. Annual costs of sea lice to global salmon farming was estimated to be in excess of €300 million in 2006, with the majority of this accounted for through expenses accrued from chemical treatments. Only a limited range of anti-sea louse drugs are available and licensed for the treatment of fish, and the continued use of only a few compounds creates a situation potentially favouring the development of drug resistance. Emamectin benzoate (EMB) is currently used as a salmon delousing agent, being employed as a 0.2 % in-feed pre-mix (SLICE®). Atlantic salmon farmers have reported increased incidence of reduced *L. salmonis* sensitivity to SLICE®, which has highlighted the requirement for further research into the molecular mechanisms controlling salmon louse resistance to EMB.

Genomic and transcriptomic research concerning *L. salmonis* drug resistance mechanisms has not often been reported, with previous transcriptomic studies using candidate gene approaches and genetic studies focussing on population genetics. Drug resistance in ecdysozoan invertebrates is associated with a variety of molecular mechanisms including target site mutations and changes in the expression of components in drug detoxification pathways. The research reported in this thesis was aimed at the exploration of mechanisms employed by *L. salmonis* to reduce the toxicity of EMB exposure, following a transcriptomic approach that utilised custom oligonucleotide (oligo) microarrays and a genetic approach that utilised Restriction-site associated DNA sequencing (RAD-seq) to identify Single Nucleotide Polymorphism (SNP) markers. An EMB-resistant (PT) and drug-susceptible (S) *L. salmonis* laboratory-maintained strain were to be used as a model for this research, as these two

strains differ in EMB susceptibility (~ 7-fold) and show stable susceptibility profiles through multiple generations, suggesting that this drug resistance phenotype may be a heritable trait.

Sequence resources available for salmon lice are limited as an annotated *L. salmonis* genome is currently under construction. Therefore, a significant amount of this study involved creating new resources to facilitate the analysis of EMB susceptibility. Suppression subtractive hybridisation (SSH) was used to enrich for transcripts that were differentially expressed between strains PT and S, which provided sufficient target sequence for the development of 15K oligo microarrays when combined with sequences assembled from existing *L. salmonis* ESTs. Additionally, transcripts were generated through sequencing a pooled sample representing key developmental stages of the *L. salmonis* life cycle, which were later used in the construction of a 44K oligo microarray.

The toxicity of EMB and other avermectins (AVMs) against ecdysozoan invertebrates is reported to be based mainly on their interaction with ligand-gated ion channels (LGIC), specifically glutamate-gated chloride channels (GluCl). However, γ-aminobutyric acid (GABA)-gated chloride channels (GABA-Cls) are also believed to be targeted by AVMs and neuronal acetylcholine receptors (nAChRs) can be allosterically modulated by the AVM compound ivermectin. Transcriptional responses in PT and S salmon lice were investigated using custom 15K *L. salmonis* oligo microarrays. In the absence of EMB exposure, 359 targets differed in transcript abundance between the two strains. GABA-Cl and nAChR subunits showed significantly lower transcript levels in PT compared to S lice, which was estimated at ~1.4-fold for GABA-Cl and ~2.8-fold for nAChR using RT-qPCR, suggesting their involvement in AVM toxicity in caligids. Although, salmon lice from the PT strain

showed few transcriptional responses following acute exposure (1 or 3 h) to 200 µg L^{-1} of EMB, a drug concentration tolerated by PT lice, but toxic for S lice.

RAD-seq analysis of both genders from *L. salmonis* strains S and PT identified 15 RAD-markers that show complete association with salmon louse strain, although these preliminary results will need further analysis to confirm marker association with reduced EMB susceptibility. Additionally, RAD marker *Lsa*101901 showed complete association with sex for all individuals analysed, being heterozygous in females and homozygous in males. Using an allele-specific PCR assay, this SNP association pattern was further confirmed for three unrelated salmon louse strains. Marker *Lsa*101901 was located in the coding region of the prohibitin-2 gene, which showed a sex-dependent differential expression, with mRNA levels determined by RT-qPCR about 1.8-fold higher in adult female than adult male salmon lice.

In conclusion, the identification of decreased transcript abundances for LGIC subunits in EMB-resistant salmon lice, and polymorphic SNP markers showing complete association with *L. salmonis* strains S or PT, provides suitable candidates for further investigation into their association with reduced EMB susceptibility. Further analysis will also be required to confirm whether EMB-induced mechanisms are not associated with reduced EMB susceptibility in *L. salmonis*. Additionally, the identification of sex-linked SNP *Lsa*101901 suggests that sex determination in the salmon louse is genetic and follows a female heterozygous system, with marker *Lsa*101901 providing a tool to determine the genetic sex of salmon lice. Improved knowledge of *L. salmonis* biology and the mechanisms potentially involved in EMB resistance, obtained during this study, may provide molecular markers that contribute to successful monitoring and management of this commercially important parasite of Atlantic salmon.

Keywords: drug resistance, sea lice, avermectin, ligand-gated chloride channel, cys-loop receptor, sex determination, RAD-seq, single nucleotide polymorphism.

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Abbreviations

Ach	Acetylcholine
AChE	Acetylcholinesterase
ATP	Adenosine triphosphate
aaUTP	5-(3-aminoallyl)-UTP
AGD	Amoebic gill disease
aRNA	Amplified Ribonucleic acid
ANOVA	Analysis of Variance
et al.	and others
T_a	Annealing temperature
ABC	ATP Binding cassette
Auto-PMT	Auto photo-multiplier tube
AVM	Avermectin
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BC	British Columbia
CoGP	Code of Good Practice
cDNA	Complementary Deoxyribonucleic acid
Contig	Contiguous sequence
ΔCP	Crossing point difference
Cy 3	Cyanine 3
Cy 5	Cyanine 5
Ct	Cycle threshold
COI	Cytochrome oxidase subunit I
CYP	Cytochrome P450 monooxygenase
CpG ODN	Cytosine-phosphate-guanine oligodeoxynucleotide motifs
CTP	Cytosine triphosphate
dpi	Days post infection
°C	Degrees Celsius
DNase	Deoxyribonuclease
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
Oligo dT	Deoxythymidylic acid oligonucleotide

DDT	Dichloro-diphenyl-trichloroethane
ddNTP	Dideoxyribonucleotide triphosphate
DMSO	Dimethyl sulfoxide
DTT	Dichloro-diphenyl-trichloroethane
S	Drug Susceptible
PT	EMB Resistant
EMB	Emamectin Benzoate
ENA	European Nucleotide Archive
e-value	Expectation value
EST	Expressed Sequence Tag
FI	Farm Isolate
FMA	Farm Management Areas
FMAg	Farm Management Agreement
FE	Feature Extraction
ZW/ZZ	Female heterogametic
FC	Fold Change
e.g.	For example (exempli gratia)
FPKM	Fragments Per Kilobase of transcript per Million
GABA-Cl	GABA-gated chloride channel
GABA	Gamma-aminobutyric acid
dbEST	GenBank Expressed Sequence Tag database
GE	Gene Expression
GO	Gene Ontology
GS	Genome Sequencer
GluCl	Glutamate-gated chloride channel
GlyCl	Glycine-gated chloride channel
GIH	Gonad-Inhibiting hormone
GSH	Gonad-Stimulating hormone
g	Gram
>	Greater than
GTP	Guanine triphosphate
h	Hour
H_2O_2	Hydrogen peroxide
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase

ID	Identity
ISA	Infectious Salmon Anaemia
IPM	Integrated Pest Management
Indels	Insertions and deletions
IVM	Ivermectin
IVT	*In vitro* transcription
KAAS	KEGG Automatic Annotation Server
kb	Kilo base pairs (1000 bp)
kdr	Knockdown resistance
KASP^{TM}	Kompetitive Allele Specific PCR
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LGIC	Ligand Gated Ion Channel
LG	Linkage group
L	Litre
LOEL	Lowest Observed Effect Level
LMW	Low Molecular Weight
ML	Macrocyclic lactone
MHC	Major Histocompatibility Complex
XX/XY	Male heterogametic
MERL	Marine Environmental Research Laboratory
MAS	Marker assisted selection
MCL	Markov Clustering Algorithm
EC_{50}	Median Effective Concentration
Mb	Mega base pairs (1,000,000 bp)
T_M	Melting temperature
mRNA	Messenger Ribonucleic acid
µg	Microgram
µg L^{-1}	Microgram per Litre
µl	Microlitre
ml	Millilitre
mM	Millimolar
MIAME	Minimum Information About Microarray Experiment
M	Molar
MW	Molecular Weight

MIH	Moult inhibiting hormone
MRP	Multidrug resistance-associated protein
MCS	Multiple Condition Solver
ng	Nanogram
NCBI	National Centre for Biotechnology Information
nAChR	Neuronal Acetylcholine Receptor
NGS	Next Generation Sequencing
NOEL	No Observed Effect Level
No RT	No Reverse Transcription
NTC	No Template Control
nt	Nucleotide
ER	Oestrogen receptor
Oligo	Oligonucleotide
OP	Organophosphate
PE	Paired-End
r	Pearson Correlation Coefficient
%	Percent
P-gp	P-glycoprotein
PMT	photo-multiplier tube
pg	Picogram
pmol	Picomole
PBO	Piperonyl butoxide
Poly (A) + RNA	Polyadenylated RNA
PEG_{300}	Polyethylene glycol 300
PCR	Polymerase Chain Reaction
PEC	Predicted Environmental Concentration
PCA	Principle Component Analysis
p	Probability
PGE_2	Prostaglandin E_2
QC	Quality Control
qPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative Trait Locus
x g	Relative centrifugal force (rcf)
RER	Relative Expression Ratio

REST	Relative Expression Software Tool
REA	Repressor of oestrogen activity
RMD-5	Required for meiotic nuclear division-5
RAD	Restriction site-associated DNA
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
rpm	Revolutions per minute
RNase	Ribonuclease
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RNA-seq	Ribonucleic acid Sequencing
RNAi	RNA interference
RT	Room Temperature
SEPA	Scottish Environmental Protection Agency
SSPO	Scottish Salmon Producers Organisation
SW	Sea water
SEP	Secretory/Excretory Products
SRA	Sequence Read Archive
SBH	Single-directional Best Hit
SNP	Single Nucleotide Polymorphism
SOL	Solvent
SFF	Standard Flowgram Format
SSH	Suppression Subtractive Hybridisation
i.e.	That is (id est)
TGICL	The Gene Indices Clustering tools
TTP	Thymine triphosphate
UV	Ultraviolet
UTR	Untranslated region
UK	United Kingdom
US	United States
USA	United States of America
UTP	Uracil triphosphate
v/v	volume/volume
H_2O	Water

Chapter 1
General Introduction

This thesis investigates 'the molecular determinants of salmon louse (*Lepeophtheirus salmonis* (Krøyer, 1837)) susceptibility to the antiparasitic drug emamectin benzoate'. The following provides some background context for this parasitic copepod, providing an overview of its impact on Atlantic salmon aquaculture and the control methods employed by the industry to manage this group of parasites. In consideration of the molecular mechanisms that may be involved in reduced susceptibility of *L. salmonis* to emamectin benzoate (EMB), several mechanisms will be discussed in respect to their association with previous incidences of reduced ecdysozoan susceptibility to antiparasitic agents. The current situation regarding reduced susceptibility of *L. salmonis* to various medicinal agents and the present understanding of the molecular mechanisms suggested to be associated with this reduction in *L. salmonis* will then be presented to highlight the importance of both this research area and the current work, which employs a global transcriptomic and genomic analysis strategy in the identification of candidate genes or markers putatively associated with reduced EMB susceptibility in *L. salmonis*.

1.1 Sea lice and marine fish

Sea lice are parasitic copepods belonging to the family Caligidae. This family comprises approximately 63 genera and 549 species (Chad and Boxshall, 2013a), including 263 and 125 species from the genera *Caligus* (Boxshall, 2013) and *Lepeophtheirus* (Chad and Boxshall, 2013b) respectively. Caligids are predominantly ectoparasites of marine fish that parasitise a wide range of hosts and feed on host mucus, skin and underlying tissues (Pike and Wadsworth, 1999). The principal subject of this thesis, *Lepeophtheirus salmonis* (Krøyer, 1837) (see Table 1.1 for full classification), is thought to be restricted to oceans of the northern hemisphere and is often referred to as the salmon louse as it mainly parasitises salmonids, particularly

from the genera *Salmo*, *Oncorhynchus* and *Salvelinus* (Kabata, 1979). *Caligus* species, including *C. elongatus* von Nordmann 1832, have a much wider host range and are distributed throughout the northern and southern hemispheres (Kabata, 1979). Intensive Atlantic salmon (*Salmo salar* Linnaeus, 1758) mariculture operations have experienced considerable problems with sea lice infection, with *L. salmonis* being responsible for the majority of farmed Atlantic salmon infections in Norway, Scotland and Ireland, and on the Pacific and Atlantic coasts of Canada and USA (Pike and Wadsworth, 1999). *C. elongatus* also parasitises farmed and wild salmonids in the northern hemisphere but is not considered to be as problematic as *L. salmonis*, probably due to its wider host range (Costello, 2006) and smaller size. Although *C. rogercresseyi* has a similarly wide host range to *C. elongatus* (Costello, 2006), it has emerged as a serious problem for Atlantic salmon production in Chile where production was restricted to a small region of southern Chile (Bravo *et al.*, 2008). In 2010, following the crash of the Chilean salmon industry due to a combination of infectious salmon anaemia (ISA) and sea louse infection, 65 % of global Atlantic salmon biomass from aquaculture (1.4 M tonnes) was produced in Norway and 11 %, 9 % and 7 % was produced in Scotland, Chile and Canada respectively (FAO, 2012a). Of the family Caligidae, the salmon louse (*L. salmonis*) has received by far the most intensive study as it is the most problematic and thus economically important species for global Atlantic salmon production, with an estimated global cost in 2006 of €305 million per annum (Costello, 2009).

Table 1.1 Full taxonomic classification of *L. salmonis*.

Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Crustacea Brünnich, 1772
Class	Maxillopoda Dahl, 1956
Subclass	Copepoda Milne-Edwards, 1840
Infraclass	Neocopepoda Huys & Boxshall, 1991
Superorder	Podoplea Giesbrecht, 1882
Order	Siphonostomatoida Thorell, 1859
Family	Caligidae Burmeister, 1834
Genus	*Lepeophtheirus* von Nordmann, 1832
Species	*salmonis* (Krøyer, 1837)

ITIS Taxonomic serial number: 89113 (ITIS, 2013).

Apia ID: 135782 (Boxshall, 2012).

Salmon louse infection of wild salmonids is assured by dispersal of planktonic louse stages in high salinity water during periods of optimal host density (Torrissen *et al.*, 2013). The development of commercial Atlantic salmon aquaculture gave rise to production sites that can often be close to wild salmonid migration routes, and which maintain captive salmonids at high densities for 12 - 24 months and potentially provide a continual host source for maintenance of the salmon louse life cycle in addition to wild sea trout that are also found in these areas (Torrissen *et al.*, 2013). Atlantic salmon farming began in the 1970s with the establishment of production sites for growth and maturation of Atlantic salmon. Global Atlantic salmon production has increased largely continuously since then, and was calculated at 1.4 million tonnes in 2010 with a value of US $7.8 billion (FAO, 2012b). Atlantic salmon is therefore the world's leading farmed marine species, with this growth in production projected to continue due to increasing demand for this food fish. Soon after the establishment of Atlantic salmon production sites it was found that cultured smolts were being infected by salmon lice. Pest control measures were then implemented for sea louse control, mainly consisting of chemical intervention, with additional general farm management practises used to break the sea louse infection cycle.

1.2 *L. salmonis* biology and life cycle

The *L. salmonis* life cycle includes planktonic and host-associated phases (Figure 1.1). The planktonic phase begins with hatching of nauplii from egg strings carried by gravid adult female lice, and comprises two non-parasitic nauplius stages and an infective copepodid stage that requires host-attachment for further development. The host-associated phase begins with the settlement of infective copepodids on the surface of host fish and continues through two permanently attached chalimus stages, two mobile preadult stages and one final mature and reproductively active adult stage (Johnson and Albright, 1991a). Nauplii and free-living copepodids are primarily passively dispersed by water currents, although these planktonic stages often display positive phototactic responses and also a limited amount of vertical swimming that allows diel vertical migration, enabling positioning in upper layers of the ocean during the day and sinking to lower strata at night, which is thought to increase host-parasite interactions (Bron *et al.*, 1993a; Heuch *et al.*, 1995). Planktonic sea lice have also been shown to display positive rheotaxis behaviour and additional movement in the avoidance of lower (< 29 %) salinity waters (Bricknell *et al.*, 2006) or during host semiochemical attraction (Bailey *et al.*, 2006). Nauplii and free-living copepodids are non-feeding stages that are limited by maternally deposited endogenous lipid reserves, with duration of the latter stage being 3-7 days depending on water temperature (Tucker *et al.*, 2000). Once copepodids locate and settle on a suitable host they secure attachment to the host by means of a frontal filament and feed on host mucus and skin. It was originally considered that attached copepodids undergo a major metamorphosis into chalimus I (Pike and Wadsworth, 1999) then repeat frontal filament attachment and continue feeding through three successive chalimus stages, facilitating gradual changes in body shape until chalimus IV closely resemble preadult I morphology (Bron *et al.*,

1991). More recently however, it has been recognised that the chalimus phase comprises only two stages, with a chalimus I stage comprising the chalimus stages previously described as chalimus I and II, and a chalimus II stage that comprises the stages formerly described as chalimus III and IV (Hamre *et al.*, 2013). The chalimus stages are non-mobile and feed at the point of attachment, whereas preadult and mature adults actively move across the surface of host fish but continue to graze on host mucus, skin and underlying tissues (Brandal *et al.*, 1979). These latter mobile salmon lice stages ensure attachment to the host through suction of the cephalothorax and gripping with antennae and maxilliped appendages. In addition, early preadults attach to the host using a chalimus-like filament during moult steps (Wagner *et al.*, 2008). The development time of the salmon louse is highly temperature dependent (Johnson and Albright, 1991b), with males developing substantially faster than females. As a consequence of the differences in developmental speed between the sexes, the timing of moulting to adult male and second preadult female lice is similar, which provides time for males to locate an immature mate and guard her until full female maturation when mating can proceed (Bron *et al.*, 1993b). After the final female moult, adult males attach and cement spermatophores to the female genital complex in an effort to prevent successful mating with other males (Ritchie *et al.*, 1996). The adult male mate-searching, pre-copula mate-guarding and polygamous mating behaviour is believed to have evolved under conditions of restricted female availability where reduced energy investment in mate searching improves fitness and reproductive success (Ritchie *et al.*, 1996). The female salmon louse produces two egg strings that can each contain 100 – 1000 eggs, depending on a range of factors including water temperature, time of year and female abdomen size (Costello, 2006). Gravid female lice can produce between 6 and 11 pairs of egg strings during a reproductive lifespan (Heuch *et al.*, 2000). An egg-

bearing female salmon louse can therefore release high numbers of planktonic nauplius stages and cause subsequent parasite transmission to farmed and wild salmonid hosts. For this reason, ovigerous female louse numbers are used as the key indicator for the infection levels at many production sites, and are the prime target for disruption of the salmon louse reproductive lifecycle.

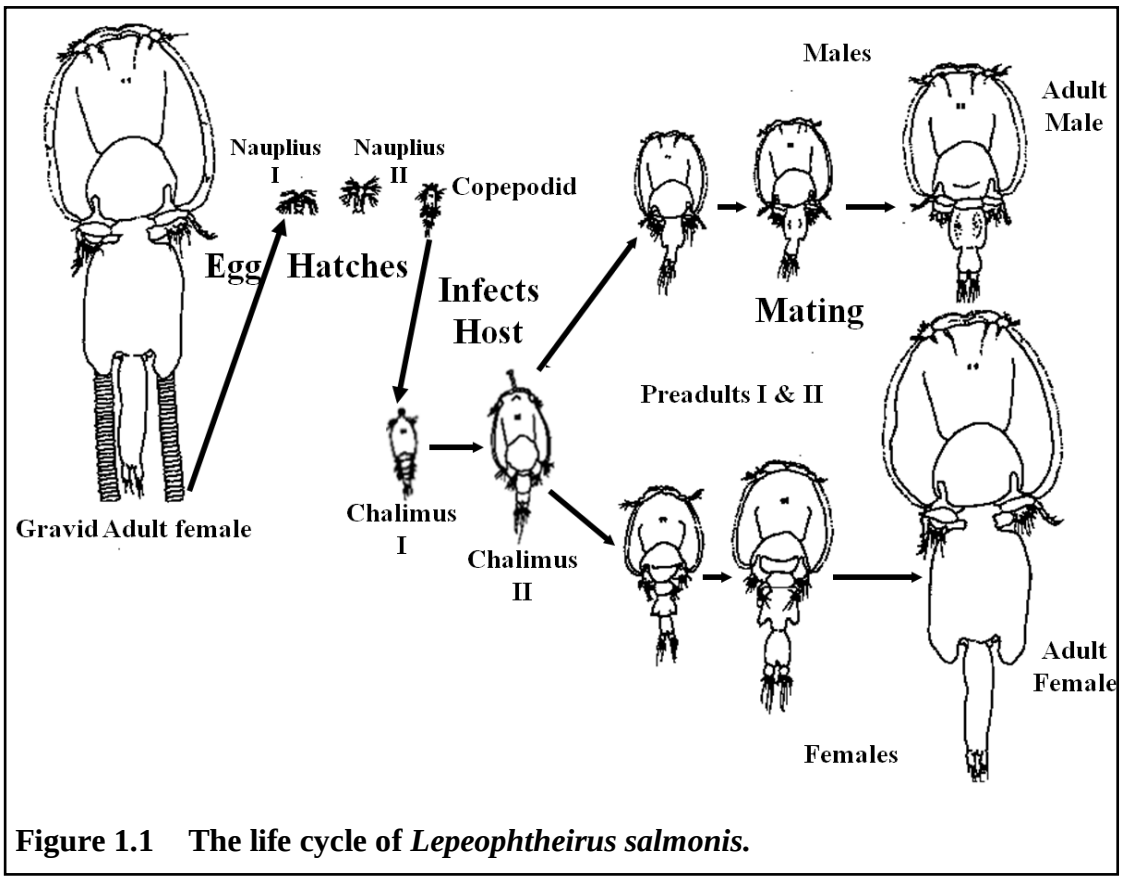


Figure 1.1 The life cycle of *Lepeophtheirus salmonis.*

1.3 Host-parasite interactions

In an era where there is a limited number of effective chemotherapeutants for control of *L. salmonis* on Atlantic salmon farms (see section 1.5.8 onwards), there is an urgent need for the development of non-chemical based salmon louse control methods. The development of such methods first requires detailed knowledge of the intimate relationship between parasite and host to identify aspects of these interactions that could be manipulated.

1.3.1 *L. salmonis* hosts

The host range of *L. salmonis* is mainly restricted to salmonids from the genera *Salmo*, *Oncorhynchus* and *Salvelinus* (Kabata, 1979). In the North Atlantic there are two main *L. salmonis* hosts; Atlantic salmon (*S. salar*) and sea trout (*Salmo trutta* Linnaeus, 1758), although the arctic charr (*Salvelinus alpinus* (Linnaeus, 1758)) is also known to host the salmon louse. However, in the Pacific ocean *L. salmonis* commonly parasitises *Oncorhynchus* species, particularly rainbow trout (*O. mykiss* (Walbaum, 1792)), pink salmon (*O. gorbuscha* (Walbaum, 1792)) and chum salmon (*O. keta* (Walbaum, 1792)). Additionally, *L. salmonis* have been reported on non-salmonid species including white sturgeon (*Acipenser transmontanus* Richardson, 1836), sand lance (*Ammodytes hexapterus* Pallas, 1814) (Kabata, 1979), saithe (*Pollachius virens* (Linnaeus, 1758)) (Bruno and Stone, 1990), sea bass (*Dicentrarchus labrax* (Linnaeus, 1758)) (Pert *et al.*, 2009) and three-spine stickleback (*Gasterosteus aculeatus* Linnaeus, 1758) (Jones *et al.*, 2006a). In the latter case, *L. salmonis* is considered to be able to reach pre-adult stage on *G. aculeatus*, with explanations for this reduced numbers of adults including mobilisation of mature *L. salmonis* from *G. aculeatus* to a salmonid host (Jones *et al.*, 2006b) or predation by other three-spine sticklebacks (Pert *et al.*, 2012).

Two *L. salmonis* sub-species are now considered to exist, located respectively in the Atlantic and Pacific oceans, with comparisons of ESTs, the mitochondrial genome, 16S rRNA and Cytochrome oxidase subunit I (COI) DNA sequences between the Pacific and Atlantic *L. salmonis* populations showing several differences between the lineages and a lower genetic diversity in Pacific populations (Yazawa *et al.*, 2008). This genetic divergence suggests the beginning of speciation between Pacific and Atlantic *L. salmonis* populations that may have co-evolved with their respective hosts (Yazawa *et al.*, 2008). Opening of the Bering Strait ~5 million years ago created the first continuous connection that allowed migration of fish and associated parasites between the Pacific and Atlantic oceans (Marincovich and Gladenkov, 1999), whereas *L. salmonis* and host co-evolution is thought to have occurred over the last 2.5 to 11 million years (Yazawa *et al.*, 2008). The COI gene was used in the identification of differences in *L. salmonis* population genetic structure in the Pacific ocean (Boulding *et al.*, 2009), however, previous studies were unable to identify such genetic structure and these authors concluded that *L. salmonis* populations must be homogeneous in the Pacific due to high levels of host salmonid migration (Messmer *et al.*, 2011). A weak but statistically significant population genetic structure was identified in *L. salmonis* from the Atlantic ocean (Glover *et al.*, 2011), although previous studies had failed to find significant difference between samples throughout the North Atlantic (Scotland, Norway and Canada), between different host species (Atlantic salmon and Brown trout) or between wild and farmed salmonids (Shinn *et al.*, 2000; Dixon *et al.*, 2004; Todd *et al.*, 2004) other than significant but low differentiation between small groups of *L. salmonis* in Ireland (Nolan and Powel, 2009). It is unclear from any of these studies whether there is significant population genetic structure in either the Atlantic or Pacific *L. salmonis* lineages but these populations do seem to be distinct from each other. This

thesis will mainly address the problems associated with Atlantic *L. salmonis* infection of salmonids in the North Atlantic with references being made to the situation with Pacific *L. salmonis* for comparative purposes.

1.3.2 Atlantic salmon

The Atlantic salmon is an andromous salmonid species that is indigenous to the North Atlantic (Verspoor *et al.*, 2007); particularly Western Europe and the east coast of North America. It has further been introduced as a farmed species to the west coast of North America, Chile and Tasmania (Seear *et al.*, 2010a). *S. salar* is a member of the teleost family Salmonidae within the Protacanthopterygii subgroup that diverged from a common ancestor and went through a tetraploidization event 25 to 100 million years ago; meaning that extant salmonids are now considered to be pseudo-tetraploid as they are in the later stages of reverting to a more stable diploid state (Koop *et al.*, 2008). The life cycle of Atlantic salmon begins with spawning in fresh water, usually between autumn and spring. Subsequently alevins (yolk-sac fry) hatch and become juvenile fry after 3 – 6 weeks then develop into parr during the autumn. The parr may remain in fresh water for a period of 1 to 6 years until they have grown sufficiently (Verspoor *et al.*, 2007), when a programmed physiological adaptation takes place in spring/early summer called 'smoltification' that allows migration to the open ocean for rapid growth and development of mature salmon in a saltwater environment (Hoar, 1988). Adult Atlantic salmon feed and develop in the ocean for 1 to 4 years before returning to spawn in the river they originated from, when physiological adaptations are again required to allow transition back from the saltwater to freshwater environment (Verspoor *et al.*, 2007). The Atlantic salmon life cycle is now routinely manipulated for aquaculture, through optimisation of spawning time and maturation using intensive hatcheries and sea cages to ensure continuous production of food fish with uniform size

and quality (Bromage *et al.*, 2001). Production cycles used for commercial Atlantic salmon farming involve intensive fresh water rearing of smolts and then transfer of these smolts to sea water after 8 - 9 months (0 + smolts) or 16 - 17 months (1 + smolts) post-hatching, for growth and maturation until mature fish reach market size (3 – 6 kg) (Duncan *et al.*, 1998; Marine Harvest, 2012). Out-of season smolt release strategies ensure that market size Atlantic salmon can be produced throughout the year (Duncan *et al.*, 1998). As salmon are stocked in relatively high densities during the marine stage of commercial aquaculture production, an ideal environment is created for salmon louse infection of susceptible fishes.

1.3.3 Salmon louse attachment and host response

1.3.3.1 Salmon louse host location and attachment

Free-living *L. salmonis* copepodids are thought to use mechanoreceptors to detect a number of physical and chemical cues in the process of host location (Mordue and Birkett, 2009), including positive reaction to reflective patterns of fish scales (Browman *et al.*, 2004) and currents produced by swimming fish (Heuch and Karlsen, 1997). It is also suggested that *L. salmonis* possess dedicated chemoreceptors (Hull *et al.*, 1998) that are used in the recognition of host specific odours (Fields *et al.*, 2007) or salmon louse pheromones, released for mate location and social aggregation (Mordue and Birkett, 2009). Electrophysiological studies of adult salmon louse responses to whole fish extract indicated greater levels of response to low molecular weight (LMW) soluble compounds from salmon flesh compared to controls (Fields *et al.*, 2007). Further investigations demonstrated that salmon lice were attracted to salmonid-conditioned water, suggesting the involvement of semiochemicals in the host-parasite interactions (Devine *et al.*, 2000; Pino-Marambio *et al.*, 2007). The recognition of a potential host by a copepodid initiates bursts of high speed swimming and more frequent turning

behaviour, termed ‘circle attacks’ (Heuch and Karlsen, 1997). This behaviour may have evolved to ensure that the copepodid remains in the vicinity of salmonid hosts which will increase the frequency of host-parasite encounters, and circle attack behaviour aids in the physical attachment to the host (Heuch and Karlsen, 1997).

1.3.3.2 Salmonid host responses to salmon louse infection

All host-attached salmon lice are considered to feed on host mucus, skin and underlying tissues with mature adults, especially adult females, also ingesting blood as a result of damage to epidermal capillaries during the feeding process (Brandal *et al*., 1979; Pike and Wadsworth, 1999). The first line of defence against parasites and infectious disease for Atlantic salmon is a layer of mucus that coats the epithelia of the gills, skin and gut (Easy and Ross, 2009). Infection of Atlantic salmon post-smolts (200 – 250 g) with *L. salmonis* under normal ocean conditions usually results in low parasite abundances (< 0.01 *L. salmonis* g^{-1} host fish) that may cause initial stress-related reductions in host feeding and feed conversion efficiency (Nolan *et al.*, 1999; Wagner *et al.* 2008). Host infection with higher parasite abundances (0.3 – 0.5 adults g^{-1} host fish) may result in tissue damage, bleeding, increased mucus discharge (Wagner *et al.* 2008) and altered mucus protein composition (Easy and Ross, 2009). Large skin lesions often form on salmonids with high levels of mobile salmon lice infection (> 0.5 – 0.75 adults g^{-1} host fish), which leads to complications such as anaemia, secondary infection, loss of osmotic and hydromineral balance and chronic stress responses, although tissue necrosis and mortality may occur in the most severe cases (Wagner *et al.*, 2008). Catecholamines and cortisol are major hormones involved in the teleost stress response, which is a conserved adaptive physiological mechanism to perceived or real threats to normal homeostasis that is generally characterised by directing metabolic energy away from growth and reproduction and towards physiological mechanisms contributing to

the maintenance of normal homeostasis (Barton and Iwama, 1991; Wendelaar Bonga, 1997). In addition to effects on growth and reproduction the endocrine stress responses in teleosts has also been implicated in a reduction of non-specific immunity and increased susceptibility to secondary infections (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Krasnov *et al.* studied the effects of chalimus infection (18 days post infection (dpi)) and cortisol administration on gene expression in Atlantic salmon skin (Krasnov *et al.*, 2012). The authors demonstrated that cortisol administration was implicated in the suppression of host immune and tissue repair gene expression responses and also potentially influences the host response to salmon louse damage. Salmonid host species have been found to possess differing levels of susceptibility to *L. salmonis*, with Atlantic salmon and sea trout (*S. trutta*) showing the greatest susceptibility and very little inflammatory response to salmon louse infection (Fast *et al.*, 2002); whereas the most resistant pink salmon (*O. gorbuscha*) rapidly rejects *L. salmonis* through development of systemic and attachment site specific inflammatory response (Jones *et al.*, 2007). Atlantic salmon are known to have a thinner epidermal layer, with fewer mucus cells that are more sparsely distributed and possess lower proteolytic activity than more resistant salmon species, which may help to explain these differences in susceptibility as Atlantic salmon do not possess as robust an immune system as pink salmon (Fast *et al.*, 2002). Genetic variability in the susceptibility of Atlantic salmon to *L. salmonis* has been shown to exist in spawning stocks and full-sib families (Glover *et al.*, 2004; Glover *et al.*, 2005; Kolstad *et al.*, 2005; Gjerde *et al.*, 2011; Torrissen *et al.*, 2013) with a heritability of lice counts of 0.07 - 0.33 (Torrissen *et al.*, 2013). Quantitative Trait Locus (QTL) regions were identified on Atlantic salmon linkage group (LG) 6, that were associated with increased salmon louse abundance. This linkage group was shown to contain MHC II genes, although it was

suggested by Gharbi *et al.* that the QTL region associated with *L. salmonis* susceptibility may include regulatory elements that control MHC II gene expression (Glover *et al.*, 2007; Gharbi *et al.*, 2009). When considering the striking difference in *L. salmonis* susceptibilities between Atlantic salmon and Pink salmon that also differ considerably with respect to immune responses to *L. salmonis* infection, it seems that the host inflammatory response is an important factor in these complex host parasite interactions that influences susceptibility level.

1.3.3.3 L. salmonis immunomodulation of Atlantic salmon

Pro-inflammatory and anti-inflammatory components, including trypsin-like enzymes and prostaglandin E_2 (PGE_2), have been identified in *L. salmonis* secretory/excretory products (SEP), with clear changes in SEP constituents throughout parasite development suggesting that each component has different immunomodulatory effects on the host (Fast *et al.*, 2003; Fast *et al.*, 2004; Fast *et al.*, 2007). Salmon louse host immunomodulation may therefore have evolved to provide favourable conditions for parasite feeding and survival, depending on specific developmental requirements of the parasite throughout host-associated stages (Fast *et al.*, 2007), with PGE_2 thought to stimulate vasodilation and reduce host cellular responses at feeding sites (Fast *et al.*, 2007). Transcriptomic responses were analysed in Atlantic salmon during *L. salmonis* infection, throughout the attached copepodid and chalimus parasite stages (Tadiso *et al.*, 2011). These authors found two distinct phases of host immune response, an initial innate immune response at 1 dpi and a second response at 5 – 10 dpi that coincided with the salmon louse transition from copepodid to chalimus stage which suggests changes in host-parasite interactions throughout the *L. salmonis* life cycle (Tadiso *et al.*, 2011). It has also been hypothesised that *L. salmonis* shows host preferences through the release of different enzymes on 'unsuitable hosts', such as Coho salmon

(*Oncorhynchus. kisutch*), with respect to more susceptible hosts such as Atlantic salmon (*S. salar*) (Fast *et al.*, 2003). The current evidence therefore suggests that *L. salmonis* have developed mechanisms to promote parasitism through the release of compounds with a limited effect that is localised to the site of attachment. These mechanisms do not seem to elicit organism-wide immunomodulation and some salmonid hosts have the ability to counter these effects to varying degrees. Further characterisation of *L. salmonis* host immunomodulatory mechanisms and host responses may uncover mechanisms that could potentially be manipulated in the control of *L. salmonis* populations on Atlantic salmon production sites.

1.4 Interactions of salmon louse populations originating from farmed and wild salmonid populations

Atlantic salmon and sea trout population abundances have been falling dramatically over many years and in the former case this has often been associated with reduced marine survival of migrating salmon (Todd, 2008). Wild Atlantic salmon nominal catches have declined from 7,237 tonnes in 1960 to 1,539 tonnes in 2010 (ICES, 2011), while Atlantic salmon aquaculture has increased productivity during the same period to 1.4 M tonnes in 2010 (FAO, 2012b). Although declines in wild Atlantic salmon and sea trout numbers began many years before the establishment of commercial Atlantic salmon farming, and occurs in regions with and without Atlantic salmon production sites, some groups nevertheless maintain that infection of outwardly migrating smolts with salmon lice originate from farm sites are responsible for this decline (Costello, 2009). It is, however, suspected that complex interactions of several other anthropogenic, biological and physical factors unrelated to Atlantic salmon production, influence variations in wild salmonid stock size (Torrissen *et al.*, 2013) such as climate change and pollution. Todd *et al.* noted that sea surface temperatures

have been warming in sub-Arctic North Atlantic oceans over recent years, which they hypothesise has contributed to the return of underweight fish to spawning grounds (Todd *et al.*, 2008). It has also been suggested that the fresh water environment can have a negative impact on outwardly migrating smolts (Russell *et al.*, 2012). Juvenile salmon have been found to grow faster in some rivers and migrate to sea earlier due to climate change, pollution and industrial exploitation of rivers, which may result in reduced sea survival of these smaller smolts (Russell *et al.*, 2012).

Salmon farms do present a significant source of hosts for *L. salmonis* and hydrodynamic modelling suggests that planktonic salmon lice could potentially be dispersed up to 30 km from initial source (Amundrud and Murray, 2009; Costello, 2009; Salama *et al.*, 2013). The infective copepodids will therefore have potential to infect wild salmonids that migrate past salmon farm sites. Transmission of infective louse stages from salmon production sites has been associated with infection of juvenile wild salmonids migrating past commercial sites, where Krkošek *et al.* estimated that 80% of wild pink salmon mortalities in the Broughton Archipelago (BC, Canada) was attributed to salmon louse infection and predicted 99 % population crash in 3.9 generations (Krkošek *et al.*, 2007). These conclusions were contested by several other groups, including Marty *et al.* who concluded that factors other than salmon lice were responsible for premature wild salmon mortalities in Broughton Archipelago (Marty *et al.*, 2010). These authors also suggested wild salmonid production was not negatively associated with salmon lice or Atlantic salmon numbers from salmon farms, although the number of pink salmon returning to spawn in autumn did predict *L. salmonis* numbers on farmed fish the next spring and also accounted for 98 % of the salmon lice prevalence on outgoing wild juvenile pink salmon (Marty *et al.*, 2010). The predicted pink salmon population decline in Broughton Archipelago has since not materialised

(Peacock *et al.*, 2013). In the North Atlantic substantial declines in wild sea trout numbers were suggested to be associated with increased infection risk in areas containing Atlantic salmon farming sites in Scotland (MacKenzie *et al.*, 1998; Butler, 2002; Middlemass *et al.*, 2010; Middlemass *et al.*, 2013), Ireland (Tully *et al.*, 1999; Gargan *et al.*, 2012) and Norway (Bjorn *et al.*, 2001; Bjorn *et al.*, 2007). It is evident that wild and farmed hosts have the potential to cross infect each other, although farmed hosts are thought to be the most likely source due to localised epizootic episodes occurring when ovigerous females were recorded on farm sites that have a high density of hosts. However, a study of wild sea trout from sites on the east and west coast of Scotland recorded more *L. salmonis* on *S. trutta* sampled on the east coast than west coast (Urquhart *et al.*, 2010). These results contradict suggestions of farm-associated mortalities as, while there are a high number of Atlantic salmon farms on the west coast, no farms exist on the east coast and this may therefore suggest influences of other factors on sea mortality of post-smolts, although the sea trout sampled on the east coast were larger and could therefore harbour a higher lice density. A study of Atlantic salmon post-smolts, sea trout and arctic charr in Norway found that, as sea trout and artic charr feed within nearshore waters, they have a higher infection risk than wild Atlantic salmon (Bjorn *et al.*, 2007) that migrate to open ocean to feed for 1-4 years (Verspoor, 2007). Bjorn *et al.* also found that arctic charr returned to fresh water earlier than the other two species studied (Bjorn *et al.*, 2007), although other studies have reported the premature return of sea trout to fresh water as a possible response to salmon louse infection (MacKenzie *et al.*, 1998; Wells *et al.*, 2007). Salmon louse infection has been shown to influence the mortality of post-smolt sea trout (Dawson, 1998) and Atlantic salmon (Hvidsten *et al.*, 2007) but it has also been shown that other factors such as acidification may have a significant influence on sea water mortality

(Finstad *et al.*, 2007). The effects of sea louse infections on the early sea water mortality of wild salmonids in the Atlantic has been studied in Norway (Hvidsten *et al.*, 2007; Skilbrei *et al.*, 2013) and Ireland (Jackson *et al.*, 2011a&b; Gargan *et al.*, 2012; Jackson *et al.*, 2013a&b) by an indirect approach involving the release and recapture of smolts and comparison of the effects of prophylactic administration of anti-salmon louse treatment with untreated controls. The release and recapture studies from Norway concluded that the odds ratio of recapturing treated salmon than untreated controls was 1.17:1, with a study between 1996 - 1998 finding that significantly more treated smolts survived in 1998 compared to controls (Hvidsten *et al.*, 2007). A 9 year release and recapture study of Burrishoole stock smolts concluded that salmon louse infection of outwardly migrating smolts was not consistently a predictor of mortality (Jackson *et al.*, 2011a), which was also found in a study of Atlantic salmon river stocks from the south and west coast of Ireland between 2002 and 2006 (Jackson *et al.*, 2011b). A separate study in Ireland involving the release and recapture of 74,324 smolts between 2004 and 2006 found that EMB treated smolts were 1.8 times more likely to return than untreated controls and the authors concluded that salmon louse mortality of wild salmonids could be significant in this area of Ireland (Gargan *et al.*, 2012). When considering both the Norwegian and Irish data on release and recapture of treated and untreated salmon smolts it was shown that the odds ratio of recapturing treated smolts was 1.11:1 to 1.2:1 (Torrissen *et al.*, 2013). The mortality rates were found to be highly variable between treatment groups with many smolts only moderately affected or unaffected by salmon lice and others being severely affected, with risk of infection being highly dependent on study location and release dates with large annual variability in ocean survival (Torrissen *et al.*, 2013). There may be several random effects that influence this variability in the effects of *L. salmonis* on Atlantic salmon smolts, associated with

reduced EMB treatment efficacies and the differences in health status and feeding of smolts in the open ocean prior to return. It is evident that as the numbers of commercially produced Atlantic salmon exceed those of wild salmon, louse infection is likely to be an ongoing problem, with re-infection originating from both farm sites and wild migratory salmon (Torrissen *et al.*, 2013). There is not currently any conclusive evidence that suggests that salmon lice are the main contributory factor to the declines in wild salmonid populations. It would seem that complex interactions of numerous different biological, physical and anthropogenic factors have contributed to this decline, although it is necessary to control salmon louse infection on Atlantic salmon farms as infection of wild salmonids will contribute to this problem.

1.5 Control and management of salmon lice in aquaculture

1.5.1 Integrated Pest Management (IPM)

Integrated Pest Management (IPM) was first developed for the prevention and suppression of insect pests in the protection of crop plants through co-ordinated integration of multiple control tactics (Radcliffe *et al.*, 2013). The Food and Agriculture Organisation of the United Nations (FAO) defines IPM as follows:

"Integrated Pest Management (IPM) means the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms." (FAO, 2013).

IPM strategies have been successfully employed in the control of pest species in many industries and are being adopted for the control of salmon lice in the Atlantic

salmon mariculture industry. In aquaculture, IPM strategies are being developed to consider all current treatment methods, in specific hydrographical regions, for co-ordinated control and prevention of *L. salmonis* and where appropriate other infectious diseases of aquaculture species. In recent years, attempts have been made to develop IPM in Scotland (Rae, 2002; SSPO, 2013), Norway (Heuch *et al.*, 2005; Torrissen *et al.*, 2013), Ireland (Jackson *et al.*, 2002) and more recently in Canada (Brooks, 2009), through collaboration of government agencies, aquaculture producers and wild fishery groups (SSPO, 2013). Geographical regions that contain commercial aquaculture are arranged into hydrographical areas and working groups are established for each area to include all commercial producers and wild fisheries that operate in the areas (Rae, 2002). Working groups can then coordinate salmon louse monitoring, chemotherapeutant treatment and other farm management practices for effective control of salmon lice (Heuch *et al.*, 2005). The most effective IPM strategies are those that utilise all available treatment methods, including antiparasitic compounds and non-chemical based methods, on a rotational basis to prevent the development of resistance in salmon louse populations to specific medicinal compounds.

1.5.2 Regulations for monitoring salmon lice numbers and drug susceptibility

Regulatory agencies in Scotland, Norway, Ireland and Canada recommend specific regimes for monitoring salmon lice numbers and chemotherapeutant sensitivities (Brooks, 2009). This data are recorded and monitored over short- and long-term periods for the identification of trends in reduced treatment success and to facilitate the adoption of alternative control measures and prevent development of serious salmon louse outbreaks. In Scotland, farm management areas (FMA) have been defined in the Code of Good Practice for Scottish Finfish Aquaculture (CoGP) (http://www.thecodeofgoodpractice.co.uk) and require co-ordination of salmon louse

control and fish health monitoring by companies within FMA, according to a Farm management agreement (FMAg) (SSPO, 2013). The CoGP defines best practices for all aspects of farm management including guideline treatment-trigger levels for *L. salmonis*. In Scotland, levels are defined as a mean abundance of ≥ 0.5 adult females between February - June and ≥ 1.0 adult females between July – January (Lees *et al.*, 2008a). In Norway, similar procedures are in place where the Salmon Lice Directive defines procedures for surveillance, prevention and treatment of salmon lice on Norwegian Atlantic salmon farms, including treatment trigger levels of 0.5 mature female or 3 mobile lice between January and August and 1 mature female or 5 mobile lice during the rest of the year (Torrissen *et al.*, 2013). Chemotherapeutant treatment efficacy is recorded through the documentation of salmon louse counts before and after treatment and the use of bioassay methods to determine the sensitivity of salmon lice populations to various antiparasitic treatments (Sevatdal and Horsberg, 2003; Sevatdal *et al.*, 2005a). These bioassay methods record salmon louse responses following aqueous exposure to chemotherapeutants, with results expressed as median effective concentrations (EC_{50}) of the veterinary drug in question (SEARCH, 2006; Westcott *et al.*, 2008). There are often large variations in bioassay methods between commercial companies and across geographical regions, including the use of solvents, salmon louse sex and life stage that are included and the selection of drug concentration, which makes it difficult to compare results between laboratories (Whyte *et al.*, 2013). Bioassays for testing sea louse sensitivity are also labour intensive and require large numbers of lice which has prompted the recent development of simplified single dose bioassays for field assessments, in Norway (Helgesen and Horsberg, 2013a) and Canada (Whyte *et al.*, 2013).

1.5.3 Farm management practices

Farm management practices are routinely employed to reduce infectious diseases and avoid unnecessary chemotherapeutant use (Brooks, 2009). Atlantic salmon production sites are often stocked with healthy single year class smolts and routinely fallowed between production cycles, which have proven effective in interrupting salmon louse breeding and ensuring that newly introduced fish do not become infected by lice cultured on the previous year class (Bron *et al.*, 1993c). These fallow periods can vary from < 4 weeks to 52 weeks throughout a year (MSS, 2011). Culture pens are thoroughly cleaned to remove any growth on the nets, which ensures there are high water currents through the pens which reduces the opportunity for salmon louse attachment to a suitable host. Removal of diseased fish, with compromised immune systems, is also important as they are more susceptible to louse infection and would provide a continual source of lice for infection of the healthy population and thereby reducing the overall health of the cage population (Brooks, 2009). As part of IPM, it is recommended that all farm sites within a management area co-ordinate management practices such as fallowing, to control sea lice populations throughout the hydrographic area.

1.5.4 Cleaner fish application

A number of wrasse species were shown to actively feed on host-attached mobile salmon lice on Atlantic salmon during trials in 1988 and 1989 (Bjordal, 1990). Wrasse were subsequently sourced from wild fisheries for control of salmon lice on Atlantic salmon farms at stocking densities of 1 wrasse to 25 - 150 Atlantic salmon (Treasurer, 2002). Successful wrasse control of salmon lice was demonstrated on commercial Atlantic salmon production sites in Norway (Bjordal, 1990), Scotland (Treasurer, 1994) and Ireland (Deady *et al.*, 1995). Several wrasse species have since been used,

including Goldsinny wrasse (*Ctenolabrus rupestris*), Ballan wrasse (*Labrus bergylta* Ascanius, 1767), Ascanius corkwing wrasse (*Symphodus melops* (Linnaeus, 1758)), rock cook (*Centrolabrus exoletus* (Linnaeus, 1758)), cuckoo wrasse (*Labrus ossifagus* Linnaeus, 1758) and scaled-rayed wrasse (*Acantholabrus palloni* (Risso, 1810)) (Torrissen *et al.*, 2013). Two additional fish species, Cunner fish (*Tautogolabrus adspersus* (Walbaum, 1792) and Lumpfish (*Cyclopterus lumpus* Linnaeus, 1758), have also been shown to be effective as cleaner fish (Groner *et al.*, 2013). To ensure efficient cleaner fish control of salmon lice it was found that cage conditions needed to be strictly managed (Deady *et al.*, 1995), as several factors could influence cleaner fish effectiveness, including the presence of refuges for wrasse, the size and shape of nets and biofouling of nets (Costello, 1996; Treasurer, 1996; Groner *et al.*, 2013). Wrasse are more inclined to feed on net biofouling than salmon lice (Deady *et al.*, 1995), with feeding behaviour also being influenced by water temperature, parasite dispersal patterns and anti-salmon louse treatment strategies (Groner *et al.*, 2013). There was a reduction in wrasse use in commercial Atlantic salmon aquaculture due to concerns that wild wrasse may act as a vector for disease (Treasurer, 2002), and low survival over winter periods unless shelter was provided from temperature fluctuations (Deady *et al.*, 1995). However, in recent years cleaner fish are increasingly being adopted as a non-chemical based salmon louse treatment due to increasing incidence of reduced chemotherapeutant effectiveness and evidence that these fish are not efficient disease vectors. As a result, methods for commercial wrasse production are being optimised and supplementary feeds are being developed in an attempt to optimise cleaner fish use in aquaculture (Groner *et al.*, 2013).

1.5.5 Salmon louse vaccine development

There have been continuing attempts to develop a vaccine against *L. salmonis*, as successful vaccination would offer numerous benefits over antiparasitic treatments including no requirements for discharge consents or withdrawal periods and less chance of resistance being developed by the parasite (Raynard *et al.*, 2002). Vaccines have successfully been developed against blood-feeding parasites including one targeted against the cattle tick (*Boophilus microplus* (Canestrini, 1888)) (Willadsena *et al.*, 1995). The antibodies are ingested by the parasite, as part of a blood meal, and target concealed antigens found on tick gut digestive cells which leads to increased cell membrane permeability, cell lysis and ultimately parasite death. It has been routinely demonstrated that *L. salmonis* also consumes blood as a result of tissue damage during feeding (Brandal *et al.*, 1976), which suggests that it should also be possible to deliver antibodies at therapeutic doses to the gut of *L. salmonis*, however a successful vaccine has yet to be developed. Parasite gut physiology and cellular/biochemical food processing may influence successful vaccination as *L. salmonis* haemolymph has similar osmolarity to sea water (Hahenkamp and Fyhn, 1985), indicating that the salmon louse gut may impair antibody function or degrade them prior to antiparasitic effects (Raynard *et al.*, 2002). Grayson *et al.* immunised Atlantic salmon with partially purified *L. salmonis* and *C. elongatus* extracts and found that treated hosts gained partial immunity against *L. salmonis*, antibodies targeting these salmon louse extracts were also found to bind *L. salmonis* gut epithelium (Grayson *et al.*, 1995). A second study expressed recombinant salmon louse proteins and subsequently selected three antigens that affected female egg production, through screening expression libraries using monoclonal and polyclonal antibodies (Andrade-Salas *et al.*, 1993). Carpio *et al.* performed vaccination trials using a recombinant my32 protein, designed to the *my32*

gene identified in *C. rogercresseyi*, and recorded reductions of *C. rogercresseyi* and delayed salmon louse development on the immunised fish (Carpio *et al.*, 2011). Further unpublished studies using an *L. salmonis* homolog to my32 did not show the same reduction in salmon lice numbers, although RNA interference (RNAi) experiments targeting this my32 homologue lead to a loss of egg strings from treated lice or the presence of egg strings that did not hatch (Nilsen, 2012 personal communication). An alternative vaccination strategy has been suggested that involves promoting the blockage of salmon louse immunosuppressive compounds or facilitating host antibody production at the site of parasite attachment and feeding (Raynard *et al.*, 2002).

1.5.6 Functional feed development

Covello *et al.* orally administered immunostimulatory compounds to Atlantic salmon and found that unmethylated DNA containing cytosine-phosphate-guanine oligodeoxynucleotide motifs (CpG ODN) and brewer's yeast extract (AllBru NuPro) decreased *L. salmonis* infection levels compared to controls, which was attributed to increased host inflammatory responses (Covello *et al.*, 2012). In a separate study, CpG ODN compound was orally administered at a lower therapeutic dose (2 mg kg^{-1}) in combination with a triple dose of the oral anti-sea louse treatment SLICE® (150 µg kg^{-1}) (Poley *et al.*, 2013). Reduction in salmon lice numbers on administration of CpG ODN or an alternative yeast extract (Aquate®) confirmed previous results, however, it was also found that host immunostimulation did not improve SLICE® efficacy but seemed to improve parasite survival (Poley *et al.*, 2013). It was suggested that host immunostimulation may have an additional effect of decreasing SLICE® efficacy through increasing P-glycoprotein expression in the parasite (Igboeli *et al.*, 2013). The study of repeated infection of Atlantic salmon with *L. salmonis* after host CpG ODN immunostimulation suggested that immunostimulatory effects may not only enhance

inflammatory and innate responses but may also provide prolonged protection through the acquired immune system (Purcell *et al.*, 2013).

1.5.7 Selective breeding of salmon louse resistant salmonids

Stock breeding programmes are currently being developed to select Atlantic salmon populations with enhanced resistance to *L. salmonis* by utilising marker assisted selection (MAS), which first requires the identification of candidate resistance genes/markers (Jones *et al.*, 2002). Recently, when studying the genetics of Atlantic salmon susceptibility to *L. salmonis* a QTL region was identified on Atlantic salmon linkage group (LG) 6, that is associated with increased salmon louse abundance and is thought to contain MHC II genes, which will provide valuable markers for these breeding programmes (Glover *et al.*, 2007; Gharbi *et al.*, 2009). Candidate gene expression has also been studied in Atlantic salmon (Fast *et al.*, 2006; Fast *et al.*, 2007) and pink salmon (Jones *et al.*, 2007; Jones *et al.*, 2008). More recently global transcriptomic studies have been undertaken with Atlantic salmon (Skugor *et al.*, 2008; Tadiso *et al.*, 2011; Krasnov *et al.*, 2012), pink salmon (Sutherland *et al.*, 2011) and comparisons of salmon louse -susceptible and -resistant salmon species (Braden *et al.*, 2012). These expression studies have identified numerous immune-related genes that exhibit differential gene expression in response to salmon louse infection and/or between susceptible and resistant species, that may also be useful for salmon louse resistant salmonid breeding programmes.

1.5.8 Chemotherapeutant treatment

1.5.8.1 Chemotherapeutant treatment methods

As Atlantic salmon are cultured at high density in open sea cages, attempts of chemical sea louse control have had to respond to the challenge to be able to deliver therapeutic doses of medicinal agents to high numbers of salmonid hosts in a manner

that is logistically possible, cost effective and results in successful control of the parasite. Many of the earliest anti-sea louse treatments were applied using immersion bath methods, although due to concerns regarding the wider environmental impact of these methods alternative techniques were developed that incorporated the medicinal agent in the feed.

1.5.8.1.1 Bath immersion treatments

Bath immersion methods require a reduction of the water volume in the fish cage, accomplished using a full tarpaulin or canvas skirt, and the addition of a known volume of active compound to the water (Rae, 2002; Guo and Woo, 2009). When employing these treatments, final therapeutic concentrations can not be confidently predicted as water volumes are not accurately controlled and there is a reliance on natural chemotherapeutant mixing (Guo and Woo, 2009). For effective treatment it is necessary to select a chemotherapeutant dose high enough to kill parasites but low enough to minimise toxicity to the host fish, although it is often the case that Atlantic salmon are exposed to variable concentrations, with chemical 'hotspots' potentially toxic to the host (Roth *et al.*, 1993) and other areas with low concentrations that expose salmon lice to sub-lethal levels of the agent (Guo and Woo, 2009). On completion of a bath treatment the tarpaulin or canvas skirt is removed to disperse the chemotherapeutant into surrounding waters which has been suggested to be potentially hazardous to marine organisms in close vicinity to the salmon farm (Willis and Ling, 2004). Bath treatments have often been administered using a cage-by-cage strategy that requires a succession of individual cage treatments until every cage on a farm has been treated (Sevatdal and Horsberg, 2003; Guo and Woo, 2009). This strategy not only has an excessive environmental impact due to prolonged release of a toxic substance but may also provide host refuges for salmon lice in untreated cages throughout the treatment period

(Campbell *et al.*, 2006). There has recently been increasing use of well boats for performing bath treatments, where Atlantic salmon are transferred from cages into large well boats using vacuum, and after a resting period the chemotherapeutant is added using a dosing system (ACFFA, 2011). The use of well boat treatments allows closer control over chemotherapeutant concentrations and mixing, with additional control of effluent release and filtering to prevent release of viable salmon lice into the surrounding waters (Bravo *et al.*, 2010; Stormoen *et al.*, 2013).

1.5.8.1.2 In-feed treatments

Oral administration of anti-salmon louse treatments has often proven more efficient than bath immersion methods, although the effectiveness of this strategy relies on medicated feed consumption by the host salmon to ensure active agents reach target tissues and are subsequently ingested by the parasites (Berg and Horsberg, 2009). There are several factors that can influence the feeding rate of fish, such as hierarchical behaviours within a cage population with larger more dominant fish consuming more than the smaller fish (Berg and Horsberg, 2009). Additionally, elevated stress levels can cause a loss of appetite, which may occur to fish that have a high lice load, are densely stocked in cages or have been exposed to toxic chemicals or extreme temperatures. Variable feed intake often leads to sub-optimal active ingredient concentrations within tissues of some fish, meaning that lice colonising these fish will not be exposed to therapeutic levels of the antiparasitic agent and will subsequently survive the treatment cycle. The survival of these salmon lice often means that genetic mutations causing partial drug resistance increase in frequency in the gene pool, and because of genetic recombination some individuals in the next generation may combine several of such mutations to show a greater level of drug resistance (Denholm *et al.*, 2002). Tissue concentrations of the anti-sea louse agent EMB will only reach therapeutic levels

several days after the initial treatment cycle upon feed metabolism by the host and distribution of EMB and metabolites to target tissues (Kim-Kang *et al.*, 2004). In comparison, salmon lice are exposed to toxic levels of compounds more or less instantly when administered using bath immersion methods. In-feed treatments, however, may also provide sustained periods of antiparasitic protection and are often safer to handle and less hazardous to the wider environment (Guo and Woo, 2009).

1.5.8.2 Chemotherapeutant compounds used for L. salmonis control

Organophosphates are a class of neuroactive agents that act by inhibiting the enzyme acetylcholinesterase (AChE), the role of which is to cleave and thereby inactivate the neurotransmitter acetylcholine at cholinergic synapses (ffrench-Constant *et al.*, 2004). Inhibition of AChE causes the accumulation of acetylcholine, leading to an excessive stimulation of acetylcholine receptors at the postsynaptic membrane and subsequent toxicity. The organophosphate (OP) trichlorphon was one of the earliest anti-salmon louse treatments (Brandal and Egidius, 1979) that was subsequently replaced by dichlorvos (2, 2-dichlorovinyl dimethyl phosphate) (Grave *et al.*, 1991). OPs are administered using immersion bath methods and are only effective against mobile salmon louse stages, which means that multiple treatments are often required to ensure removal of adult lice that matured from chalimus lice surviving the preceding treatment. OPs were continually used for treatment of salmon lice infections of Atlantic salmon in Scotland and Norway, when in 1989 dichlorvos use was severely restricted by the Scottish Environmental Protection Agency (SEPA) due to reduced effectiveness and concerns over its environmental impact (Rae, 2002). In the early 1990s dichlorvos was replaced by an alternative OP, azamethiphos as licensed product Salmosan, which was deemed safer for handling and ~10 times more effective than dichlorvos, although cross-resistance of salmon lice to these OPs was highly likely (Roth *et al.*, 1996).

Pyrethrum is a natural insecticide obtained as an extract of *Chrysanthemum cinerariifolium* and *Chrysanthemum coccineum* flower heads, which contain toxins termed pyrethrins that show a selective toxicity to arthropods (Casida, 1980). Pyrethrins express their toxic action on arthropods by disrupting normal ion permeability of nerve membranes through targeting neuronal voltage-gated sodium channels (Casida *et al.*, 1983). While pyrethrum was found to be effective for controlling salmon lice (Burka *et al.*, 1997), it was only briefly used for this purpose, reflecting the relatively high cost of this natural pesticide in addition to its sensitivity for environmental degradation. For salmon louse control, pyrethrum was replaced by structurally and pharmacologically similar synthetic pesticides called pyrethroids (Casida *et al.*, 1983; Burka *et al.*, 1997). In particular, a salmon delousing agent containing cypermethrin (Exis®) was introduced during the 1990's (Hart *et al.*, 1997) and alternative medicines based on deltamethrin (AlphaMax®) (Roth, 2000) and high-*cis*-cypermethrin (BetaMax®) subsequently became available. Pyrethroids are administered using immersion bath or well boat methods. Cypermethrin was authorised for use in Scotland during 1999 when > 90% of treatments in Norway also used this class of compound (Fallang *et al.*, 2005).

Hydrogen peroxide (H_2O_2) is a strong oxidising agent that has been used as an immersion bath treatment against salmon lice in Scotland and Norway since the early 1990s (Thomassen, 1993; Rae, 2002). Currently two H_2O_2-based products, SalarTect® and Paramove®, are available. H_2O_2 has a very narrow safety margin, becoming increasingly toxic to fish at temperatures above 14ºC and losing effectiveness at temperatures below 10ºC. Moreover, H_2O_2 can cause gill damage to salmonids after prolonged exposure (Johnson *et al.*, 1993; Bruno and Raynard, 1994) and is only effective for the removal of mobile salmon lice stages, with 85-100 % efficiency (Treasurer and Grant, 1997). H_2O_2 is not considered hazardous to marine life as it

degrades rapidly to water and oxygen, which has contributed to its continued use in regions where discharge consents are required for other chemotherapeutic agents (Burridge *et al.*, 2010). H_2O_2 is also employed in the control of amoebic gill disease (AGD), a well-recognised problem for salmon aquaculture in Tasmania and a more recent problem for Scottish salmon aquaculture. Treatment of AGD can therefore provide coincident reductions in salmon louse infection levels (Adams *et al.*, 2012).

The benzoylphenyl urea compounds teflubenzuron (Calcide®), [1-(3, 5-dichloro-2, 4-difluorophenyl)-3-(2, 6-difluorobenzoyl) urea] (Branson *et al.*, 2000) and diflubenzuron (Lepsidon®), [1-(4-chlorophenyl)-3-(2, 6-difluorobenzoyl) urea] (Horsberg and Høy, 1991) are chitin synthesis inhibitors administered orally as a medicated in-feed preparation. Accordingly, these compounds are effective against all moulting louse stages, which need to form a new cuticle, but have little if any effects on adult salmon lice. Benzoylphenyl ureas are also toxic to non-target crustacean species, which lead to restricted use in aquaculture due to concerns over the potential adverse effects of chitin synthesis inhibitors on such species (Burka *et al.*, 1997).

Avermectins (AVMs) are macrocyclic lactones that are used as antiparasitic agents (Davies and Rodger, 2000), with ivermectin (IVM) being an AVM that is widely applied in human and veterinary medicine. AVMs are believed to act through modulating glutamate- and γ-aminobutyric acid (GABA)-gated chloride channels of the invertebrate nervous system, resulting in an influx of chloride ions that leads to nervous impulse disruption and ultimately death of the parasite (Kass *et al.*, 1980; Arena *et al.*, 1995). While IVM is effective against salmon lice (Johnson and Margolis, 1993) it has not been licensed for routine use in aquaculture in the UK and Europe, although the compound has been used under the 'Cascade principle' for treatment of severe cases of Atlantic salmon lice infestations during the 1990s, based on case-to-case emergency

prescriptions (Davies and Rodger, 2000). The AVM compound emamectin benzoate (EMB) was licensed in 1999 as an oral treatment for Atlantic salmon. EMB is the active ingredient in the commercial premix SLICE®, and is a stable salt compound of the AVM derivative, emamectin 4”-deoxy-4”-epi-methylamino-avermectin B1, which is a mixture of two homologous compounds 4”-deoxy-4”-epi-methylamino-avermectin B_{1a} and 4”-deoxy-4”-epi-methylamino-avermectin B_{1b} (Kim-Kang *et al.*, 2004). The recommended SLICE® feeding regime is administration of 5 g medicated feed per kilogram of salmon biomass for seven consecutive days to provide a daily dosage of 50 µg SLICE® per kilogram biomass (Stone *et al.*, 2000a), which was initially shown to provide ~10 weeks parasite protection from the start of the treatment regime in field trials (Stone *et al.*, 2000b).

The environmental impact of EMB was studied through the analysis of EMB and metabolite concentrations around a commercial Atlantic salmon farm (Telfer *et al.*, 2006). EMB has low water solubility and so high concentrations were not expected in solution, although the authors recognised potential for negative impacts on sediment dwellers, filter feeders and surface dwelling crustacean inhabiting the surrounding environment, due to EMB adsorption to particulate material. The main source of EMB was identified as uneaten food material and Atlantic salmon biliary excretion and the largest environment impact of SLICE® use on production sites appeared to be enrichment of organic material in sediments below the cages and surrounding areas. The effects of EMB on the surrounding environmental was found to be short-term due to degradation of EMB and metabolites, with residues found in sediments and fauna at levels that would not have a detrimental effect on survival (Telfer *et al.*, 2006). Additionally, a separate study found that EMB use was unlikely to adversely affect planktonic copepods in the vicinity of Atlantic salmon farms, as concentrations that

caused toxic effects to these species were considerably higher than the Predicted Environmental Concentrations (PEC) for EMB (Willis and Ling, 2003) which was further confirmed in a. 31 month field study (Willis *et al.*, 2005). It has been shown that exposure of *Homarus americanus* (American lobster) to 0.6 – 0.8 µg g^{-1} EMB induced premature moulting in preovigerous and ovigerous females, which the authors hypothesised may be due to EMB interference in moult inhibiting hormone (MIH) modulation of the moulting process (Waddy *et al.*, 2002). Further studies reported a no observed effect level (NOEL) for EMB of 0.12 µg g^{-1} and lowest observed effect level (LOEL) of 0.22 µg g^{-1} in ovigerous female *H. americanus* (Waddy *et al.*, 2007a), and an EC_{50} of > 589 µg g^{-1} and 644 µg g^{-1} for juvenile and adult lobsters respectively (Burridge *et al.*, 2004). The latter study found that as *H. americanus* tissue EMB levels increased, consumption of EMB medication feed by the lobster decreased (Burridge *et al.*, 2004). Moreover, ovigerous female *H. americanus* have been shown to prefer their natural feed when offered the option of medicated feed, although they will eat SLICE® medicated feed (Waddy *et al.*, 2007b). It was concluded from these studies that under current SLICE® use, *H. americanus* feeding around Atlantic salmon production sites were unlikely to consume toxic levels of EMB, although a recent study found that repeated exposure of American lobster to low EMB (0.06 and 0.125 µg g^{-1} at 8 and 4 doses respectively) seems to induce premature moulting to a greater extent than single higher EMB doses (Waddy *et al.*, 2010). As the cumulative EMB dose after repeated exposure was higher than the dose lobsters would be expected to consume, further studies were required to investigate the effects of repeated exposure to lower doses (Waddy *et al.*, 2010).

1.6 Mechanisms of resistance to antiparasitic compounds

While chemical agents allow effective short- and medium-term control of parasites, there is a risk that drug resistance may develop in treated parasite populations over long-term periods of exposure. The formation of drug resistance in parasites is a classical example of an evolutionary process driven by natural selection as first proposed by Charles Darwin. The repeated treatment of a parasite population with drugs constitutes a selection pressure that can cause enrichment of genotypes in the gene pool that confer a fitness advantage under conditions of drug exposure (Wolstenholme *et al.*, 2004). Theoretical considerations predict that selection pressure increases with increasing treatment frequency and an increasing percentage of the parasite population being exposed to the drug during treatments. As most control agents have specific modes of action, a majority of these resistance mechanisms, which are discussed in detail below, are also substance-class specific. Accordingly, the intensity of selection pressure for resistance development can be reduced by the implementation of a rotation strategy that uses drugs with distinct modes of action (Denholm *et al.*, 2002).

While little is known about potential mechanisms of drug resistance in crustaceans including *L. salmonis*, the molecular mechanisms of resistance to chemical control agents in insects and nematodes are comparatively well studied. Recently proposed taxonomies based on molecular evolution place insects, crustaceans and nematodes in one large clade called Ecdysozoa (Telford *et al.*, 2008), within which insects and crustaceans form a sub-clade called Pancrustacea (Regier *et al.*, 2010), suggesting that crustaceans are phylogenetically closer to insects and nematodes than previously assumed. In the view of the fact that most salmon delousing agents contain active ingredients that are also used as insecticides and/or anthelmintics, it appears

likely that molecular mechanisms of drug resistance in salmon lice may overlap with those described in insects and nematodes.

Resistance of insects to control agents is a problem that emerged soon after the beginning of large-scale insecticide use for the control of undesired species (Heckel, 2012) such as phytophagous insects, insect parasites of domestic animals or humans and insects acting as disease vectors. The two main molecular mechanisms through which insecticide resistance can become established are firstly modifications of the insecticide's target site that disrupts binding of the control agent, and secondly changes in the pharmacokinetics of the insecticide that results in enhanced detoxification and reduces internal exposure to the toxicant. The latter mechanism can be based on enhanced expression of biotransformation enzymes and/or drug transporters (ffrench-Constant *et al.*, 2004; Wolstenholme *et al.*, 2004).

An association between insecticide resistance and the mutation of an insecticide target site was first found in fruit fly (*Drosophila melanogaster* Meigen, 1830) populations resistant to cyclodienes and the phenylpyrazole fipronil, which showed a single-amino acid substitution (Ala302) in a GABA receptor subunit termed Rdl (resistance to dieldrin) (ffrench-Constant *et al.*, 1993). Homologues to the Ala302 mutation were also identified in GABA receptors of insecticide-resistant isolates of other insect species (ffrench-Constant, 1994), demonstrating parallel evolution of this resistance mechanism. Another example of insecticide resistance linked to a target site mutation is provided by the *knockdown resistance* (kdr) phenotype (Busvine, 1951; Farnham, 1977), which is characterised by the decreased sensitivity of insects to DDT (dichloro-diphenyl-trichloroethane) and pyrethroids (Soderlund and Knipple, 2003). A single amino acid substitution (L1014F) in a voltage-gated ion channel, believed to be the target of DDT and the pyrethroids, was associated with kdr in a *Drosophila* mutant

(*para*^{ts}) (Loughney *et al.*, 1989). Interestingly, equivalent mutations of voltage-gated ion channels were subsequently identified in resistant isolates of the housefly (*Musca domestica*) (Williamson *et al.*, 1993) and numerous other pest species (Soderlund and Knipple, 2003), again demonstrating parallel evolution in response to a specific toxic stressor. To quote a final example of insecticide resistance based on a target site mutation, OP resistance in the fruit fly has been linked to single amino acid mutations of acetylcholinesterase (AChE) that reduces its sensitivity to OP inhibition (Fournier *et al.*, 1989). The analysis of OP resistant strains of *Anopheles gambiae* Giles, 1902 and *Culex pipiens* Linnaeus, 1758 revealed similar amino acid changes in AChE, providing another instance of parallel evolution (Weill *et al.*, 2003; ffrench-Constant *et al.*, 2004).

An example of insecticide resistance based on increased detoxification is given by OP resistant strains of the aphid *Myzus persicae* (Sulzer, 1776) (Field and Devonshire, 1998; Devonshire *et al.*, 1998; Field *et al.*, 1999) and the mosquito *C. pipiens* (Raymond *et al.*, 1998), which show markedly increased levels of esterase expression that is believed to inactivate OPs by sequestration. The dramatic increases in esterase levels required for OP sequestration can be achieved through gene amplification (*i.e.* the evolutionary acquisition of multiple copies of the gene) and/or altered esterase gene regulation (Field *et al.*, 1999; ffrench-Constant *et al.*, 2004). The overproduction of OP deactivating enzymes is a common but physiologically expensive mechanism of OP resistance. OP resistance in the Australian sheep blowfly (*Lucilia cuprina*) was found to be based on a unique alternative mechanism, where a single-amino acid mutation of a carboxylesterase enzyme conferred the ability to hydrolytically inactivate OP insecticides (Oppenoorth and van Asperen, 1960; ffrench-Constant *et al.*, 2004).

The large cytochrome P450 (CYPs) gene family encodes enzymes that function as monooxygenases (Bernhardt, 1995; Nelson, 1998). Certain CYP subfamilies contain

members with roles as biotransformation enzymes, with most organisms possessing multiple CYP genes that are involved in biotransformation and show distinct but overlapping substrate specificities. Multiple CYP genes have been reported to be involved in insecticide resistance phenotypes (Bergé *et al.*, 1998). For instance, increased expression of *Cyp6g1* was found in a DDT resistant *D. melanogaster* strain (Daborn *et al.*, 2002), whereas increased *Cyp12d1* and *Cyp6g1* (Brandt *et al.*, 2002) or *Cyp6a8* (Le Goff *et al.*, 2003) expression was evident in other DDT-selected fruit fly strains. Furthermore, neonicotinoid resistance of *M. persicae* was associated with enhanced *Cyp6Cy3* expression (Puinean *et al.*, 2010), whereas two pyrethroid resistant *Aedes aegypti* strains showed increased levels of seven different CYP genes, including five from the *Cyp9J* family (Bariami *et al.*, 2012).

The large ABC (ATP-binding cassette) transporter gene superfamily contains proteins that are located in the cell membrane where they function as broad specificity drug efflux pumps, in addition to members with other diverse roles (Higgins, 1992; Dean *et al.*, 2001; Jones *et al.*, 2009). While these ABC drug efflux transporters have initially been described as drug resistance factors in human cancers (Cole *et al.*, 1992; Doyle *et al.*, 1998; Gottesman *et al.*, 2002), recent data suggests that changes in ABC drug transporter expression levels can contribute to ecdysozoan invertebrate resistance against control agents. For instance, resistance of tobacco budworm (*Heliothis virescens*) populations against pyrethroids and carabamate insecticides coincided with an increased expression of the ABC transporter P-glycoprotein (Lanning *et al.*, 1996a), and pyrethroid resistant *A. aegypti* strains mentioned above showed enhanced levels of one subfamily B ABC transporter, in addition to changes in CYP gene expression (Bariami *et al.*, 2012). Further studies suggest roles for ABC transporters from subfamilies B and C in the resistance of nematodes to anthelmintic agents (Blackhall *et*

al., 1998; Xu *et al.*, 1998; Ardelli and Prichard, 2004; Ardelli *et al.*, 2006; Ardelli and Prichard, 2008; Bartley *et al.*, 2009; James and Davey, 2009; Bourguinat *et al.*, 2011; Dicker *et al.*, 2011).

The toxic action of AVMs was proposed to be based primarily on interaction with glutamate-gated chloride channels (GluCl) in *Caenorhabditis elegans* (Maupas, 1900) (Cully *et al.*, 1994) and *D. melanogaster* (Cully *et al.*, 1996). It has since been demonstrated that AVMs also interact with GABA-gated chloride channels (GABA-Cl) in insects and nematodes (Kane *et al.*, 2000; Feng *et al.*, 2002). Resistance to AVMs in some nematode strains has been attributed to mutations in GluCl and GABA-Cl subunits that decreases channel binding affinity for the drug (Njue and Prichard, 2004; McCavera *et al.*, 2009). Other molecular mechanisms associated with the development of AVM resistance in ecdysozoan invertebrates includes increased ABC transporter activity (Blackhall *et al.*, 1998; Xu *et al.*, 1998; Buss *et al.*, 2002; Ardelli *et al.*, 2006; Bartley *et al.*, 2009; Pohl *et al.*, 2011) and changes in the activity of mechanisms responsible for drug metabolism such as CYPs (Kwon *et al.*, 2010; Pu *et al.*, 2010; Chen *et al.*, 2011).

1.7 Reduced susceptibility of *L. salmonis* to antiparasitic treatments

Following the continual use of OPs for control of salmon lice reduced efficacy of dichlorvos (Jones *et al.*, 1992) and azamethiphos (Roth *et al.*, 1996) were reported in Norway and Scotland (Denholm *et al.*, 2002). Similarly, reduced H_2O_2 sensitivity was reported for *L. salmonis* from farm sites in Norway and Scotland that had regularly used this treatment since the early 1990s compared to salmon lice without previous H_2O_2 exposure, suggesting the development of H_2O_2 resistance (Treasurer *et al.*, 2000). The pyrethroids, deltamethrin and cypermethrin, were also heavily used in Scotland and

Norway from 1999 and within a few years reports of reduced pyrethroid efficacy against *L. salmonis* began to appear (Sevatdal and Horsberg, 2003; Sevatdal *et al.*, 2005a). Residual AChE activity was analysed in salmon lice sampled from several Atlantic salmon production sites in Canada and Norway between 1999 and 2002 (Fallang *et al.*, 2004). A study measuring total and organophosphate-resistant activities of AChE in *L. salmonis* sampled from Canadian and Norwegian field sites found higher levels of organophosphate-resistant AChE in Canadian salmon lice compared to those sourced from Norway (Fallang *et al.*, 2004). As OPs have been extensively used in Norway, but much less so in Canada, these results were interpreted as evidence for the existence of target-site resistance mechanisms responsible for OP resistance (Fallang *et al.*, 2004). However, OP susceptibility analysis of salmon louse populations was not performed in this study (Fallang *et al.*, 2004). The analysis of pyrethroid susceptibility in *L. salmonis* using the CYP inhibitor (piperonyl butoxide (PBO)) suggested that CYPs may be involved in pyrethroid detoxification, as PBO pre-treatment increased pyrethroid sensitivity in *L. salmonis* (Sevatdal *et al.*, 2005b). Additionally, the identification of a novel point mutation in target sodium channels from 11 *L. salmonis* samples taken from areas with previous reports of reduced pyrethroid susceptibility suggests that target site mutation may also be associated with reduced *L. salmonis* pyrethroid susceptibility (Fallang *et al.*, 2005).

The EMB based feed (SLICE®) was made available in 1999 for the treatment of salmon louse infection of Atlantic salmon (Stone *et al.*, 1999) and has since been used in Norway, Scotland and Canada. As EMB is administered orally, different factors can contribute to treatment failures, including inaccurate calculations of fish biomass, inadequate incorporation of EMB in medicated feed or poor consumption of the feed by the salmon (Bravo *et al.*, 2008; Berg and Horsberg, 2009; Jones *et al.*, 2012).

Nevertheless, a number of studies have reported reduced sensitivity of *L. salmonis* and *C. elongatus* to EMB in Norway, Scotland and Ireland (Lees *et al.*, 2008a, 2008b; Heumann *et al*., 2012; Horsberg, 2012). Similarly, EMB resistance of *C. rogercresseyi* in Chile has been attributed to exclusive use of EMB since 2000 and continual AVM use since the early 1990s (Bravo *et al.*, 2008; Bravo *et al.*, 2010).

Monitoring trends of reduced treatment efficacy has proven difficult as the emergence of resistant sea louse populations often occurs only once treatment failures are evident (Jones *et al.*, 2013), at which time resistance alleles will already be widespread throughout problematic populations (Wolstenholme *et al.*, 2004). In an attempt to identify potential trends in reduced EMB treatment efficacy, epidemiological studies have been conducted to analyse historical data on salmon louse counts and treatment episodes over successive years (Jones *et al.*, 2013). Epidemiological studies found that EMB was still effective in British Columbia (BC), Canada, which differs from other Atlantic salmon producing regions largely due to a wider distribution of farm sites and a high numbers of wild Pacific salmon, which provide untreated host populations that could represent refuges for salmon lice from drug selection pressure (Saksida *et al.*, 2010; Jones *et al.*, 2012). Genetic differences between Pacific and Atlantic *L. salmonis* lineages may also account for these observed differences in susceptibility (Yazawa *et al.*, 2008; Saksida *et al.*, 2010). Atlantic salmon farms in the Bay of Fundy region of New Brunswick, Canada are concentrated in a smaller geographical area, which may explain reductions in EMB efficacy and variable mean lice abundance between farm sites in this region (Jones *et al.*, 2012). Lee *et al.* investigated EMB treatment efficacy using historical data from 50 Scottish commercial fish farms (Lees *et al.*, 2008b), showing that EMB efficacy in Scotland varied between years, with lowest sensitivities found in the last year (2006) and winter treatments were

more likely to fail than spring ones (Lees *et al.*, 2008a). Comparisons of EMB sensitivity data between Scotland and New Brunswick identified a lower but steadier increase in EMB resistance in Scotland and a more rapid increase in New Brunswick, which was attributed to the fact that >95% of salmon louse treatments in New Brunswick during the study used EMB whereas approximately 50 % of treatments in Scotland were EMB based, thereby reducing selection pressure for resistance due to the use of alternative antiparasitic products (Jones *et al.*, 2013). There is now comprehensive evidence for reduced SLICE® efficacy throughout the Atlantic salmon farming industry; however, there is limited knowledge of the molecular mechanisms that reduce salmon louse susceptibility to EMB. It is therefore important that we build on current knowledge of the molecular mechanisms involved in the development of AVM resistance in insects and nematodes to establish the underlying mechanisms responsible for *L. salmonis* resistance to EMB.

1.8 Thesis aims and objectives

1.8.1 Overall aims

The main aim of this study was to improve our understanding of reduced EMB susceptibility in *L. salmonis* and to provide genomic tools that will facilitate the study of wider salmon louse biology. Evidence from the current literature would suggest that resistance to EMB could be due to target site modifications or changes in the mechanisms responsible for drug detoxification. Additionally, the molecular mechanisms underpinning EMB resistance may or may not involve changes in transcription. A global transcriptomic strategy using custom *L. salmonis* microarrays was selected to investigate the molecular mechanisms involving transcriptional changes that may be involved in reduced EMB susceptibility. This strategy was to be used in the investigation of constitutive differences in gene expression and EMB induced changes

in gene expression using drug susceptible and EMB-resistant *L. salmonis*. A global transcriptomic analysis strategy was selected as it avoids the limitations associated with candidate gene approaches that are often based on specific assumptions regarding drug resistance mechanisms and require detailed knowledge of the biological systems being studied. The University of Stirling's Marine Environmental Research Laboratory (MERL) maintains two *L. salmonis* strains that were employed as the biological model for these investigations of EMB susceptibility in *L. salmonis*, as they differ in EMB susceptibility and show stable susceptibility profiles when tested using aqueous EMB bioassays. An alternative strategy was employed to explore the existence of genetic polymorphisms that may be associated with reduced EMB susceptibility but are not detected using a transcriptomic approach. This strategy used Restriction-site associated DNA sequencing (RAD-seq) which facilitates the identification of genetic markers in the *L. salmonis* genome without access to genome sequence or detailed knowledge of the biological systems being studied. There have been no previous studies concerning the using of microarrays to study drug resistance in *L. salmonis* or the use of RAD-seq for the identification of genetic markers in this species.

1.8.2 Objectives

The key objectives of the research presented in this thesis may be summarised as follows:

1. **Development of transcriptomic resources for *L. salmonis*.**

In the absence of a suitably annotated *L. salmonis* genome and with limited available sequence resources, new sequence was to be generated during this study that would facilitate the design of oligo microarrays. Custom oligo probes could then be designed to discovered target sequences, in addition to existing *L. salmonis* ESTs, for the design of microarrays to be used for global transcriptomic analysis of *L. salmonis*.

2. **Comparison of constitutive gene expression between two *L. salmonis* strains with differing susceptibilities to EMB**.

A custom oligo microarray for *L. salmonis* was to be used for transcriptomic analysis of a drug susceptible (S) and an EMB-resistant (PT) *L. salmonis* strain, for the identification of genes that are constitutively differentially expressed between the strains that may indicate molecular mechanisms involved in reduced susceptibility to EMB.

3. **Analysis of transcriptomic responses to EMB exposure in two *L. salmonis* strains with differing susceptibilities to EMB.**

A custom oligo microarray for *L. salmonis* was to be used for analysing transcriptomic responses of *L. salmonis* strains S and PT to EMB exposure in order to identify genes indicative of responsive molecular mechanisms that reduce toxicity of EMB exposure and thereby reduce susceptibility.

4. **Identification of genetic markers associated with reduced EMB susceptibility and gender in *L. salmonis*.**

RAD-seq was to be employed in the identification of genetic markers in laboratory-maintained *L. salmonis* strains S and PT that may be associated with reduced EMB susceptibility. Additionally, the mechanisms responsible for sex determination and/or differentiation are poorly understood for the salmon louse and therefore these RAD-seq experiments were also used for the identification of sex-linked genetic markers in *L. salmonis*. With distinct differences in development time and EMB susceptibility observed between male and female salmon lice, it is hoped that the identification of sex-linked genetic markers might provide a tool to determine the genetic sex of *L. salmonis* that could be useful in the development of alternative control strategies.

Chapter 2

The development of transcriptomic resources for the salmon louse, *Lepeophtheirus salmonis*

2.1 Introduction

In reflection of the high economic and ecological relevance of caligids, particularly the salmon louse, *Lepeophtheirus salmonis* (Krøyer, 1837), the general biology of caligid copepods and their host interactions have increasingly attracted research attention. Recent topical areas include the biological mechanisms determining host susceptibility and the potential mechanisms by which the parasite develops resistance to chemical treatments.

Recent advances in genomic and transcriptomic methodologies offer considerable opportunities to significantly advance our understanding of the biology of caligid copepods, however, few genomic resources exist in this species-rich crustacean sub-phylum. The driving force behind the development of crustacean genomic resources has very often been generated either by the needs of commercial exploitation of a particular species (Cesar *et al.*, 2008) or the use of a species as a model to study environmental and evolutionary physiology (Tagmount *et al.*, 2010) or ecotoxicology (Watanabe *et al.*, 2008). A substantial proportion of the existing resources for crustaceans are associated with the model aquatic species *Daphnia pulex* Leydig, 1860, and commercially important Malacostracans such as *Litopenaeus vannamei* (Boone, 1931) (Pacific white shrimp, Decapoda) and *Homarus americanus* Milne Edwards, 1837 (American lobster, Decapoda) (Stillman *et al.*, 2008). Significant contributions to crustacean genomic resources have now been generated from research into the parasitic copepod *L. salmonis* (Yasuike *et al.*, 2012). Additionally, with general acceptance of the global warming phenomenon, there has been a considerable amount of research into thermal adaptation of *Petrolisthes cinctipes* (Randall, 1839) (flat porcelain crab, Decapoda) with the hope of improving our understanding of the impact of climate change on aquatic species (Tagmount *et al.*, 2010). Following the release of a *D. pulex* genome assembly

in 2011 (Colbourne *et al.*, 2011), it has been suggested that copepod species are also ideally suited to be used as model species, as copepod haploid genome size can range from 0.14 to 12.46 pg. Copepods have been used for the study of climate change, biodiversity or evolutionary responses of invasive species, such as *Eurytemora affinis* (Poppe, 1880) that have adapted from marine to freshwater habitats (Bron *et al.*, 2011). Among the copepoda, *Tigriopus californicus* (Baker, 1912) has been the subject of genomic studies of metal toxicology (Ki *et al.*, 2009) while *Calanus finmarchicus* has been investigated with respect to genomic responses to thermal stress (Voznesensky *et al.*, 2004) or surfactant exposure (Hansen *et al.*, 2010).

A large majority (92 %) of copepod expressed sequence tags (ESTs), publicly available in 2010, originated from parasitic copepoda (Yasuike *et al.*, 2012), with *L. salmonis* and *Caligus rogercresseyi* providing 62 % and 15 % of these resources respectively (Bron *et al.*, 2011). The *L. salmonis* genome project [http://sealouse.imr.no/] was launched in 2010 and an annotated genome is expected to be published in 2013. Publicly available sea lice ESTs originate from five parasitic copepod species, defined as Pacific *L. salmonis* (64,666 EST), Atlantic *L. salmonis* (57,349 EST), *C. rogercresseyi* (32,135), *C. clemensi* (14,821 EST) and *Lernaeocera branchialis* (16,441 EST) (Yasuike *et al.*, 2012). Mitochondrial genomes have been made available for three sea louse species; *L. salmonis* (16,148 bp; Yazawa *et al.*, 2008), *C. rogercresseyi* (13,468 bp) and *C. clemensi* (13,440 bp) (Yasuike *et al.*, 2012). The availability of mitochondrial and nuclear genome sequence has enabled genetic studies revealing distinct Atlantic and Pacific lineages (Yazawa *et al.*, 2008), a finding that was confirmed in a later study (Yasuike *et al.*, 2012). Salmon louse sequence resources have also facilitated transcriptomic analysis of complex traits, such as parasite development and egg production (Eichner *et al.*, 2008) and stress responses to extreme

temperature and salinity conditions (Sutherland *et al.*, 2012), through the development of cDNA or oligonucleotide-based microarrays (Bron *et al.*, 2011). These global transcriptomic studies often provide candidate genes that can be further characterised using transcriptional and translational analysis (Kvamme *et al.*, 2004; Skern-Mauritzen *et al.*, 2007; Dalvin *et al.*, 2011) or through use of direct gene knockout RNA interference studies (Campbell *et al.*, 2009; Dalvin *et al.*, 2009).

The aim of this element of the research was to generate a custom oligonucleotide (oligo) microarray for *L. salmonis*, in order to provide a new tool for use in studies of the molecular determinants of drug susceptibility in *L. salmonis* (reported in Chapters 3 and 4 of this thesis). The design of the microarray was first based on the use of existing *L. salmonis* EST resources available in GenBank, which were downloaded and assembled into a minimal set of contiguous sequences (contigs). In addition, subtracted cDNA libraries were constructed, with tester and driver cDNA derived from laboratory-maintained *L. salmonis* strains differing in emamectin benzoate (EMB) susceptibility, and sequenced to enrich for transcripts potentially involved in the development of EMB resistance in the salmon louse. Finally, *de novo* transcriptome sequence was assembled from sequence reads generated using the Illumina Hi-Seq platform and RNA-seq technology to sequence a sample pool from key stages of the *L. salmonis* life cycle, ensuring representation of major transcriptional events in the custom sequence resource.

2.2 Materials and methods

2.2.1 Salmon louse strains and husbandry

Two salmon louse (*L. salmonis*) laboratory-maintained strains differing in susceptibility to EMB (Heumann *et al.*, 2012), were used in this study. The susceptible strain S 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 moderately EMB-resistant salmon louse strain PT was established in December 2008 using salmon lice from another Scottish production site with reports of variable EMB treatment efficacies. The strains have since been cultured under identical laboratory conditions, as described in detail elsewhere (Heumann *et al.*, 2012). In brief, salmon lice were maintained on Atlantic salmon (*Salmo salar*) with an initial weight of 500 – 1000 g in circular tanks supplied with fresh seawater at ambient temperature, using a photoperiod corresponding to natural day length. To propagate salmon louse cultures, egg strings were allowed to hatch and develop to copepodids, which were used to inoculate a tank containing naïve host fish. Prior to the collection of salmon lice from hosts, fish were anaesthetised with 100 mg L^{-1} 2-phenoxyethanol. Infection rates were maintained at levels that were unlikely to compromise fish welfare. All laboratory infections were carried out under UK Home Office licence and appropriate veterinary supervision.

2.2.2 RNA extraction and purification

Frozen samples were ground in liquid nitrogen using a pestle and mortar, and further mechanically homogenised using a rotating probe homogeniser (Ultra-Turrax®). 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 M trisodium citrate; 1.2 M

sodium chloride; Appendix 1), as recommended for samples with high polysaccharide content (Chomczynski and Mackey, 1995). The total RNA was resuspended in nuclease-free water. For the construction of subtracted cDNA libraries, total RNA from 60 untreated adult males from either strain (S or PT) were further purified using RNeasy columns (Qiagen, UK), pooled and subjected to poly (A)+ RNA isolation using the Poly (A) Purist™ kit (Ambion®, UK). To generate *L. salmonis* transcriptome sequence total RNA was extracted from 21 different salmon louse samples taken from key stages of the life cycle where each sample consisted of pools of individuals (Appendix 2). The 21 total RNA samples were pooled and then further purified using RNeasy columns (Qiagen, UK). 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.

2.2.3 Assembly of contiguous sequence from *L. salmonis* ESTs

A total of 129,225 ESTs (> 100 bp) for *L. salmonis,* as described by Yasuike *et al.* (Yasuike *et al.*, 2012), were downloaded during December 2010 in FASTA format from the GenBank Expressed Sequence Tags database (dbEST) held by the National Centre for Biotechnology Information (NCBI) [http://www.ncbi.nlm.nih.gov/nuest]. This EST list was manually assembled into the groups 'Nilsen Norway', 'Atlantic Norway', 'Atlantic Canada' and 'Pacific' according to the originating sequencing project and then quality filtered to remove poly A tails and sequences with a length < 100 nucleotides. The Lasergene software package SeqMan II (version 6.1) for Windows (DNAStar Inc., USA) was used to remove contaminating cloning vector and bacterial sequence from the sequences.

The four EST groups were individually processed to create consensus sequence (contigs) using The Gene Indices Clustering Tools (TGICL) (Computational Biology and Functional Genomics Laboratory [http://compbio.dfci.harvard.edu/tgi/software] (The Gene Index Project, USA)), following the manufacturers' instructions. Briefly, a multi-fasta file containing all the trimmed EST sequences was loaded and the ESTs were clustered into groups of similar sequences using the megablast pairwise alignment software. These sequence clusters were then passed to the cap3 assembly software that created consensus sequences through multiple alignments of the sequences within each cluster. The resulting contig and singleton sequences were reported in separate FASTA files that were downloaded for subsequent annotation.

The assembled contig sequences were annotated using BLASTx (Basic Local Alignment Search Tool) searches against the non-redundant proteins (nr), UniprotKB/Swiss-Prot (Swissprot) and Reference Proteins (refseq_protein) GenBank databases at the National Centre for Biotechnology Information (NCBI), with annotation hits being considered significant when having a BLASTx expectation value (e-value) $< 1 \times 10^{-4}$. All sequences were further annotated with GO identifiers using Blast2Go software for Windows® using Java Webstart (Centro de Investigación Príncipe Felipe, Spain).

The 15K microarray design 1 (Table 2.1) was subsequently constructed using probes designed to 10,056 annotated and 5,052 unannotated sequences from this library of contig sequences. When constructing 15K microarray design 2 (Table 2.1) only 9,710 of these annotated sequences were used, to allow incorporation of oligo probes designed to target sequence that was generated from subtracted cDNA library sequencing as discussed in section 2.2.4 of this chapter. Finally, the 44K microarray design 1 (Table 2.1) incorporated oligo probes that were designed to 9,429 of these

annotated target sequences. The remaining probes included on the 44K microarray design 1 were designed to sequence from subtracted cDNA library sequencing (section 2.2.4) and salmon louse transcriptome sequence (section 2.2.5).

2.2.4 Subtracted cDNA library construction and sequencing

Suppression subtractive hybridisation (SSH) was used to prepare cDNA libraries enriched in transcripts differentially expressed between strains S and PT using commercial methods (PCR-Select™, Clontech, Takara Bio Inc., USA) (Adapter and primer sequences detailed in Appendix 3). Subtractions were performed in both directions, *i.e.* using cDNA derived from each strain (S or PT) either as the tester or the driver. A pool of cDNA from each subtraction, containing an equal amount of both subtracted cDNA libraries, was used for generating a 454 sequencing library using the GS FLX Titanium Rapid Library Preparation kit (Roche Applied Science, UK), following manufacturer's instructions. Adaptive Focus Acoustics™ (AFA™) using the S220 High Performance Ultrasonicator (Covaris® Inc., KBiosciences, UK) was employed to randomly shear the cDNA, blunt ends were repaired and MID adapters ligated to the DNA fragments prior to sequencing using the Genome Sequencer™ (GS) Titanium FLX instrument (Roche Applied Science, UK) (EBI Sequence Read Archive (SRA) study ERP002190). GS FLX Titanium library preparation and sequencing was performed by The GenePool Genomics Facility (University of Edinburgh, UK). Sequence reads were assembled using the GS De Novo Assembler (Newbler) v2.5.3 software (Roche Applied Science, UK) using default parameters after trimming of MID adapter and primer sequences.

These contig sequences were annotated as detailed in section 2.2.3 and used in the design of oligo probes that were incorporated in 15K microarray design 2 (Table 2.1). Two oligo probes were designed to each of the 1916 annotated and 783 unannotated

contig sequences generated from *L. salmonis* subtracted cDNA library sequencing, giving a total of 3832 annotated and 1566 unannotated sequences being used in 15K microarray design 2.

2.2.5 Salmon louse transcriptome sequencing

2.2.5.1 Salmon louse life stages included

The *L. salmonis* life cycle includes planktonic and host associated phases. The planktonic phase begins with hatching of nauplii from egg strings carried by gravid adult female lice, and comprises two non-parasitic nauplius stages and an infective copepodid stage that requires host-attachment for further development. The host-associated phase begins with the settlement of infective copepodids on the surface of host fish and continues through two permanently attached chalimus stages, two mobile preadult stages and one final mature and reproductively active adult stage (Johnson and Albright, 1991a). It was originally considered that attached copepodids underwent a major metamorphosis into chalimus I (Pike and Wadsworth, 1999), then repeated frontal filament attachment and continued feeding through the successive three chalimus stages, facilitating gradual changes in body shape until chalimus IV closely resembles preadult I morphology (Bron *et al.*, 1991). More recently however, it has been recognised that the chalimus phase comprises only two stages with the stages formerly described as chalimus I and II both now being classed as chalimus I and the former chalimus III and IV stages classed as chalimus II (Hamre *et al.*, 2013).

To generate a pool of samples for *L. salmonis* transcriptome sequencing 21 samples were taken from key stages of the salmon louse life cycle (Appendix 2). Samples from earlier stages of the life cycle (nauplius – chalimus II) consisted of pools of multiple individuals due to the small size of individuals at these stages (0.5 – 2.8 mm) (Johnson and Albright, 1991a). All samples were collected and preserved in an

RNA stabilisation solution (4.54 M ammonium sulphate, 25 mM trisodium citrate, 20 mM EDTA, pH 5.4; Appendix 1) prior to storage at -70 °C. The *L. salmonis* samples collected for inclusion in this study were sampled at different times due to logistical reasons. Light and dark coloured egg strings, containing immature and mature nauplii respectively, were removed from gravid adult females using a sterile scalpel blade. The two nauplius stages develop relatively quickly (30.5 hours from nauplius 1 to 2 at 10 °C) (Johnson and Albright, 1991b) and are difficult to differentiate by size. Atlantic *L. salmonis* are 0.54 – 0.56 mm in size (Johnson and Albright, 1991a), although it has been demonstrated that Pacific *L. salmonis* are larger than those originating from the Atlantic ocean (Schram, 1993). In order to obtain samples containing both stages, nauplii were collected by hatching egg strings in aerated sea water at two temperatures (8 and 10.5 °C) and collecting samples at 24 and 48 hours after egg string collection. Nauplii were collected by filtration using 0.22 mm mesh and transferred directly to RNA stabilisation solution. Similarly, free-living copepodids (0.7 mm) were filtered from culture after 5 days of incubation (10.5 °C). To obtain host-attached copepodids, fish were infected as detailed above and attached copepodids were collected using forceps under low magnification microscopy from host Atlantic salmon at 24 and 48 hours post infection. Chalimus stage lice were collected by the same process, with each stage being differentiated under low magnification microscopy by size and morphology (Johnson and Albright, 1991a; Schram, 1993). Preadult (I and II) and adult male and female salmon lice can also be differentiated by size and morphology. Preadult I males are smaller than preadult I females (total length 2.9 and 3.7 mm respectively for Atlantic *L. salmonis*) and also differ by size and shape of the genital complex. Adult male and preadult II female Atlantic *L. salmonis* are approximately the same size (total length ~5.4 mm) but can easily be distinguished under low magnification microscopy,

using common morphological features (Johnson and Albright, 1991a; Schram, 1993). The abdomen of adult male salmon lice is shorter, with an ovoid genital complex, whereas the preadult II female genital complex is larger with cuticular folds and distinct lobes, and a narrowing of the abdomen as it meets the genital complex. Similarly, adult females have larger genital complex than males, and also have a larger more developed genital complex in comparison to preadult II females (Johnson and Albright, 1991a). The samples of different salmon louse stages were stored at -70 °C and processed for total RNA extraction and purification as detailed above.

2.2.5.2 Library construction and sequencing

A total RNA pool was created that included samples from the egg string, nauplii, copepodid, chalimus, preadult (I and II) and adult stages for male and female salmon lice, by incorporating 2.5 µg total RNA from each of 21 salmon louse samples (Appendix 2). Transcriptome sequencing of this 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.

2.2.5.3 Transcriptome assembly and quality filtering

Transcriptome sequencing requires high quality sequence reads for optimal assembly as sequencing errors can often create difficulties for short-read assembly algorithms. We therefore performed stringent filtering to remove low-quality reads containing ambiguous nucleotides ("N") or with a Phred score under 20. The genome sequences for both the Pacific and Atlantic *L. salmonis* lineages (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, transcript expression levels were estimated using the Fragments Per Kilobase of transcript per Million (FPKM) values by the Cufflinks v2.0.2 (Trapnell *et al.*, 2012).

2.2.5.4 SNP and Indel identification

The sequence reads were aligned to the Atlantic *L. salmonis* reference genome [http://sealouse.imr.no/] (Accessed: July 2012) using the Bowtie2 v2.1.0 alignment software (Langmead *et al.*, 2009). Samtools v0.1.19 (Li *et al.*, 2009) software was then used to identify any Single Nucleotide Polymorphisms (SNPs) or insertions and deletions (indels) that differed between the Atlantic *L. salmonis* reference genome sequence and the new sequence resource. The original sequence generated in this study was returned, to retain any polymorphisms that may have been discovered in this new sequence resource.

2.2.5.5 Gene annotation

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 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 $< 1 \times 10^{-4}$ and 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, 03/03/2013 release) databases, and BLASTx searches against the non-redundant proteins (nr), UniprotKB/Swiss-Prot (Swissprot) and refseq_protein databases (03/03/2013 release), with an e-value cut-off $< 10^{-4}$ and minimum alignment length of 100 nucleotides being considered significant.

2.2.6 KEGG and GO annotation

Functional annotation of subtracted cDNA library sequences was performed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and the KEGG Automatic Annotation Server (KAAS) [www.genome.jp/tools/kaas]. The annotation of salmon louse transcriptome sequences were used to retrieve Gene Ontology (GO) annotation for molecular function, biological process and cellular component (Ashburner *et al.*, 2000) as well as biological pathway assignment according to the KEGG pathway database [http://www.genome.jp/kegg/pathway.html] (Kanehisa and Goto, 2000; Kanehisa *et al.*, 2012). A custom software pipeline converted transcriptome sequence GO terms to GO Slim terms, using the Protein Information resource, Generic GO Slim and Metagenomics Slim files [http://www.geneontology.org/GO.slims.shtml] (Accessed 18/03/2013).

2.2.7 Salmon louse oligonucleotide microarray design

Oligonucleotide probes (60mers) were designed to target contig sequences using the eArray Gene Expression (GE) probe design tool (Agilent Technologies, UK), employing the base composition and best probe methodologies, and implementing a 3' bias. For each sequence without a significant BLASTx based annotation two probes were designed, one of which was designed to the forward sequence while the other was designed to the reverse complement. Standard expression microarrays were designed using the eArray custom microarray design wizard (Agilent Technologies, UK), using

either an 8 × 15K or 4 × 44K design format. Microarray designs 1 and 2 comprised 15,744 features including 536 obligatory controls (Table 2.1). An initial design was used for analysis of EMB induced gene expression (Chapter 4; Agilent AMADID No 033382; EBI ArrayExpress design A-MEXP-2284). This 15K microarray design 1 incorporated oligo probes designed to 10,056 annotated and 5,052 unannotated contig sequences that were assembled from existing *L. salmonis* ESTs as detailed in section 2.2.3 of this chapter. With additional data becoming available, a minor redesign was implemented (Agilent AMADID No 039612; EBI ArrayExpress design A-MEXP-2285) for analysis of constitutive gene expression (Chapter 3). This minor redesign incorporated new probes designed to target 2,699 sequences that were identified when sequencing the subtracted cDNA libraries enriched for transcripts differentially expressed between the EMB-resistant (PT) and drug-susceptible (S) salmon louse strains as detailed in section 2.2.4 of this chapter. Two probes were designed for each of the new targets. The new probes were used to replace unannotated probes present on the original array. The two 15K microarray designs shared 10,251 identical features. A third microarray was designed as detailed above but using a 4 × 44K design format (Agilent AMADID No 048507), that incorporated new salmon louse transcriptome sequence and comprised 45,220 features including 1,417 obligatory controls (Table 2.1). This 44K microarray design incorporated oligo probes designed to 28,695 contig sequences originating from the new salmon louse transcriptome, 9,429 contig sequences assembled from existing *L. salmonis* ESTs (section 2.2.3) and 5,429 sequences generated from sequencing *L. salmonis* subtracted cDNA libraries (section 2.2.4).

Table 2.1 Composition of the features included on custom *L. salmonis* oligo microarrays.

Probe type	*15K Microarray (Design 1) (AMADID 033382)*	*15K Microarray (Design 2) (AMADID 039612)*	*44K Microarray (Design 1) (AMADID # 048507)*
Annotated	10,056	13,542	26,887
Unannotated	5,052*	1,566*	16,666*
Control probes	100	100	250
Agilent controls	536	536	1417
Total	15,744	15,744	45,220

***** Oligo probes were designed to both forward and reverse complement strands for all unannotated target sequences.

2.3 Results

2.3.1 *L. salmonis* EST assembly

A total of 129,225 *L. salmonis* ESTs were obtained from GenBank that had been generated from four sequencing projects (Table 2.2). The 'Nilsen Norway EST resource consisted of 35,577 sequences of which 34,064 were > 100bp and found to originate from the sequencing of 14 separate *L. salmonis* cDNA libraries (Appendix 4). The four EST groups were each assembled into contig sequence to give 19,279 contig and 9,693 (> 100bp) singleton sequences (Table 2.2). Manual quality control and functional annotation of these sequences provided 13,582 annotated (BLASTx e-value < 10^{-4}) and 5,616 unannotated target sequences suitable for the design of oligo probes to be used in the design of a microarray.

Table 2.2 Summary of *L. salmonis* ESTs and assembled contig sequences.

EST group ID	Original EST sequences	Quality filtered EST sequences	Assembled contig sequences	Singleton sequences*
Nilsen Norway	35,577	28,941	3,644	2,307
Atlantic Norway	19,406	18,299	4,370	1,386
Atlantic Canada	9,577	9,075	2,644	1,051
Pacific	64,665	61,331	8,621	4,949
Total	129,225	117,646	19,279	9,693

A total of 129, 225 ESTs, originating from four *L. salmonis* sequencing projects, were available for download from the NCBI GenBank database during 2010. These ESTs are described in full by Yasuike *et al.*

* Singleton sequences are original ESTs that were not assembled into contig sequence.
Four EST groups were assembled into contig sequence using the TGIC sequence assembly software. Short sequences and contaminating vector sequence were removed prior to the assembly process.

2.3.2 Salmon louse subtracted cDNA library sequencing

To obtain a pool of salmon louse cDNA sequences enriched for transcripts differentially expressed between salmon louse strains S and PT, two SSH libraries were constructed, corresponding to subtractions between strains in both directions. A pool of both libraries was subjected to Roche 454 sequencing, producing a total of 94,834 reads with an average read length of 235 nucleotides (N50 value of 289 nucleotides) (Figure 2.1). GS *de novo* sequence assembly software (Newbler v2.5.3) assembled these raw Roche 454 sequence reads into 3,242 contigs that were further categorised into isogroups according to sequence homology criteria. The contig sequences within each isogroup were further assembled into isotigs, with between 1 and 13 contigs being assembled into each isotig (Figure 2.2). The assembly of contigs from sequence reads provided 1,916 annotated (BLASTx e-value $< 10^{-4}$) and 783 unannotated target sequences. For a number of these sequences, annotation suggested affiliation to gene families potentially associated with reduced EMB susceptibility. These gene families include ABC (ATP-binding cassette) transporters, cytochrome P450 monooxygenases (CYPs), and Cys-loop receptors. In particular, a multidrug-resistance ABC transporter (Accession XP_001862061.1) and two CYPs (15a1, Accession AAS13464.1 and 18a1, Accession XP_393885.1) were identified (Table 2.3) Moreover, isolated Cys-loop receptor subunits include a ligand-gated ion channel (LGIC) receptor subunit (nAChRα-3, Accession ADD38711.1) and a γ-aminobutyric acid (GABA) -receptor associated protein (Accession ACO11910.1) that could potentially constitute pharmacological targets for EMB (Table 2.3) The longest contigs generated from the subtracted library sequencing were annotated as constituents of cytoskeleton proteins, such as myosin and tropomyosin, whereas contig sequences assembled from the highest number of sequence reads were annotated as metalloproteinase enzymes (Table 2.4).

KEGG functional analysis of the annotated sequences revealed a large representation (53 %) of genes involved in metabolism (Figure 2.3).

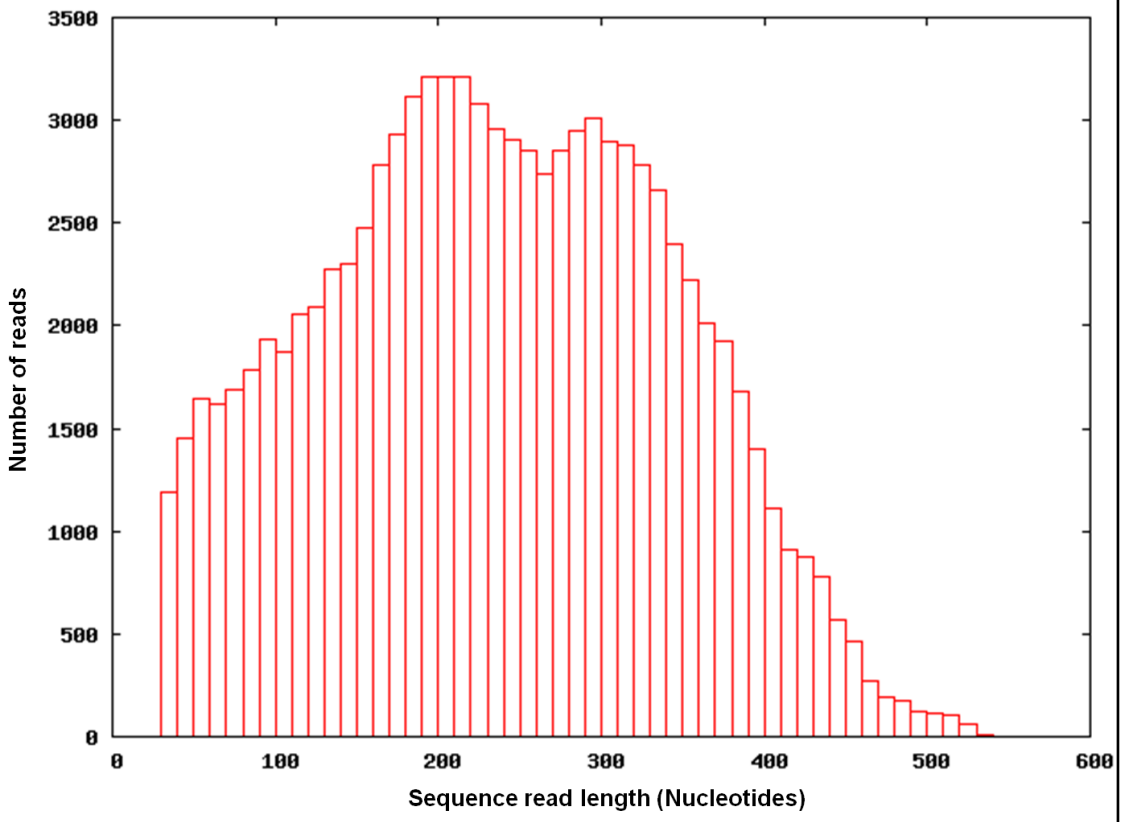


Figure 2.1 Salmon louse subtracted cDNA library sequence read length distribution.

Subtracted cDNA libraries were generated in both directions between drug susceptible (S) and EMB-resistant (PT) laboratory-maintained *L. salmonis* laboratory strains. A pool containing equalised amounts of these cDNA libraries was sequenced using a Roche Genome Sequencer (GS) FLX sytem with Titanium sequencing chemistry. Standard Flowgram Format (SFF) files were generated by sffinfo software, that also determined sequence read length distribution for the 94,834 raw sequence reads as a quality control (QC) metric.

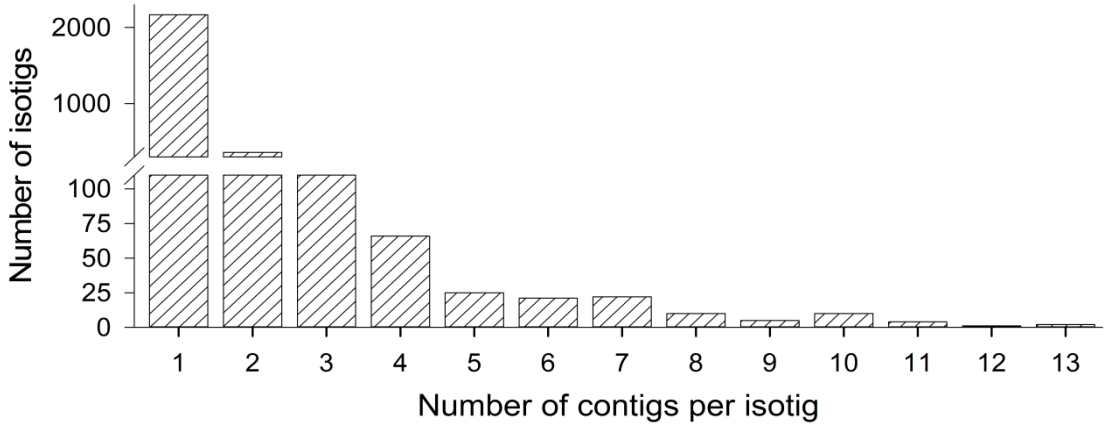


Figure 2.2 Contig sequence inclusion in assembled isotig sequences.

GS *de novo* sequence assembly software (Newbler v2.5.3) assembled raw Roche 454 sequence reads into contigs, categorised these contigs into isogroups and subsequently assembled each contig group into isotigs.

Table 2.3 ***L. salmonis*** **subtracted cDNA library sequences with annotation suggesting affiliation to gene families putatively associated with reduced EMB sensitivity.**

Sequence ID	Sequence length	Accession No.	Annotation	BLASTx e-value	Contig inclusion
ABC transporter					
isotig00897	848	XP_001862061.1	Multidrug resistance-associated protein 14 [*Culex quinquefasciatus*]	$1.00E^{-94}$	1
Cytochrome P450s					
isotig00748	1,141	AAS13464.1	Cytochrome P450 CYP15A1 [*Diploptera punctata*]	$5.00E^{-50}$	1
isotig02142	436	XP_393885.1	Cytochrome P450 CYP18A1 [*Apis mellifera*]	$2.00E^{-18}$	1
isotig02028	453	ACO10681.1	Cytochrome P450 3A24 [*Caligus rogercresseyi*]	$2.00E^{-35}$	1
isotig01206	641	XP_002400171.1	Cytochrome P450 [*Ixodes scapularis*]	$2.00E^{-49}$	1
LGIC receptor proteins					
isotig01382	570	ADD38711.1	Neuronal acetylcholine receptor subunit alpha-3 [*Lepeophtheirus salmonis*]	$5.00E^{-96}$	1
isotig00533	445	ACO11910.1	Gamma-aminobutyric acid receptor-associated protein [*Lepeophtheirus salmonis*]	$9.00E^{-58}$	2
isotig00534	271	ACO11910.1	Gamma-aminobutyric acid receptor-associated protein [*Lepeophtheirus salmonis*]	$2.00E^{-18}$	2

Table 2.4 Sequences highly represented in *L. salmonis* subtracted cDNA library resource.

	Contig ID	Contig length	Number of reads	Isogroup	Isotigs	Number of Isotigs	Isotig Annotation	Accession number	Blastx e-value
A	contig01161	1,782	46	255	isotig00728	1	Leucine rich protein, putative	XP_001658446.1	$9e^{-63}$
	contig01162	1,693	55	256	isotig00729	1	Myosin heavy chain, muscle	EFN74639.1	$1e^{-142}$
	contig01163	1,675	64	257	isotig00730	1	Acheron	AAN76709.1	$3e^{-35}$
	contig01164	1,545	39	258	isotig00731	1	Hypothetical protein	EFX77428.1	0
	contig01165	1,543	324	259	isotig00732	1	Cytochrome oxidase subunit I	AAT39730.1	0
	contig01166	1,504	31	260	isotig00733	1	Low-density lipoprotein receptor related protein 1b	XP_002916019.1	$1.48e^{-12}$
B	contig00151	466	4,151	2	isotig00001 - 49	50	Metalloproteinase	ABU41019.1	$3e^{-32}$
	contig00170	440	1,339	3	isotig00050 - 52	3	Metalloproteinase	ABU41053.1	$1e^{-139}$
	contig00166	459	1,313	2	isotig00001 - 49	50	Metalloproteinase	ABU41019.1	$3e^{-32}$
	contig00273	294	985	7	isotig00083 - 90	8	Trypsin 1a	AAP55756.1	$1e^{-149}$
	contig00176	207	775	3	isotig00050 - 55	5	Metalloproteinase	ABU41053.1	$1e^{-139}$
	contig00576	666	731	46	isotig00292 and isotig00293	2	Elongation factor 1-alpha	ABU41064.1	$1e^{-15}$

Contig sequences were sorted according to either contig length (A) or raw sequence read count used to assemble the contig (B). The six longest contig sequences and six contigs with the highest raw sequence read count are detailed.

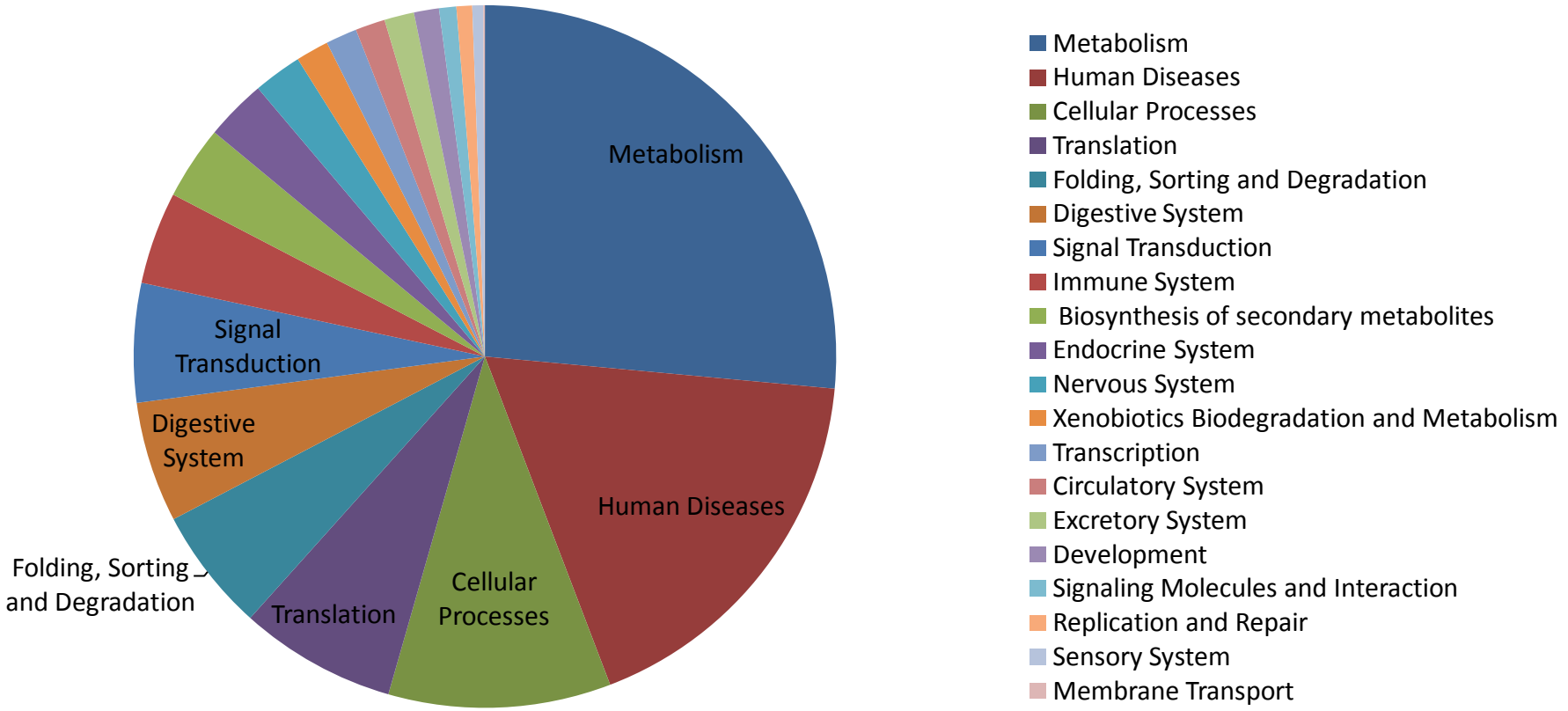


Figure 2.3 KEGG functional classification of annotated sequences from *L. salmonis* subtracted cDNA library sequencing.

Functional classification of 2,809 isotigs and 6 large contig sequences, assembled from *L. salmonis* subtracted cDNA library sequencing. Classification was performed according to the KEGG orthology database, using KAAS (KEGG Automatic Annotation Server) and SBH (single-directional best hit) methods.

2.3.3 Salmon louse transcriptome sequencing

To create a salmon louse transcriptome sequence resource representing all key stages of the life cycle including major metamorphosis steps, a total RNA pool was generated that included every stage of the salmon louse life cycle. This total RNA pool was subjected to Illumina 100 bp paired end sequencing using the Illumina HiSeq 2000 platform. A total of 389,406,444 raw sequence reads were generated with an average read length of 101 bp, 82 % of these reads having a 99.9 - 99.99 % base calling accuracy (Phred score 30 - 40) (Figure 2.4). A total of 266,447,466 reads (68.4 %) passed quality control and filtering, which were then used by TopHat and Cufflinks to generate a reference based transcriptome assembly of 33,537 transcripts using the Atlantic *L. salmonis* reference genome from the salmon louse genome project website [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 698 reference based transcripts. Based on the high quality reads, 37,681 transcripts were assembled (EBI Sequence Read Archive (SRA) study ERP002482), consisting of 30,581 unique transcripts and 3,112 alternative spliced transcripts, with an average length of 1338.19 bp ranging from 101 to 24,684 bp. Of these, 96.6 % and 43.6 % transcripts had a length more than 200 bp and 1000 bp, respectively. Finally the 33,706 unique genes have an average length of 1271.58 bp and a total length of 42,843,289 bp (Table 2.5). Each gene had an average of 1 splicing form per gene ranging from 1 to 5. To evaluate the quality of the assembled transcripts, all the usable sequencing reads were realigned to the transcripts. The sequencing depth ranged from 1 to 519,299 reads, with an average of 795 reads across whole transcripts. About 77 % of the transcripts were realigned by more than 10 reads and 35 % were remapped by more than 100 reads.

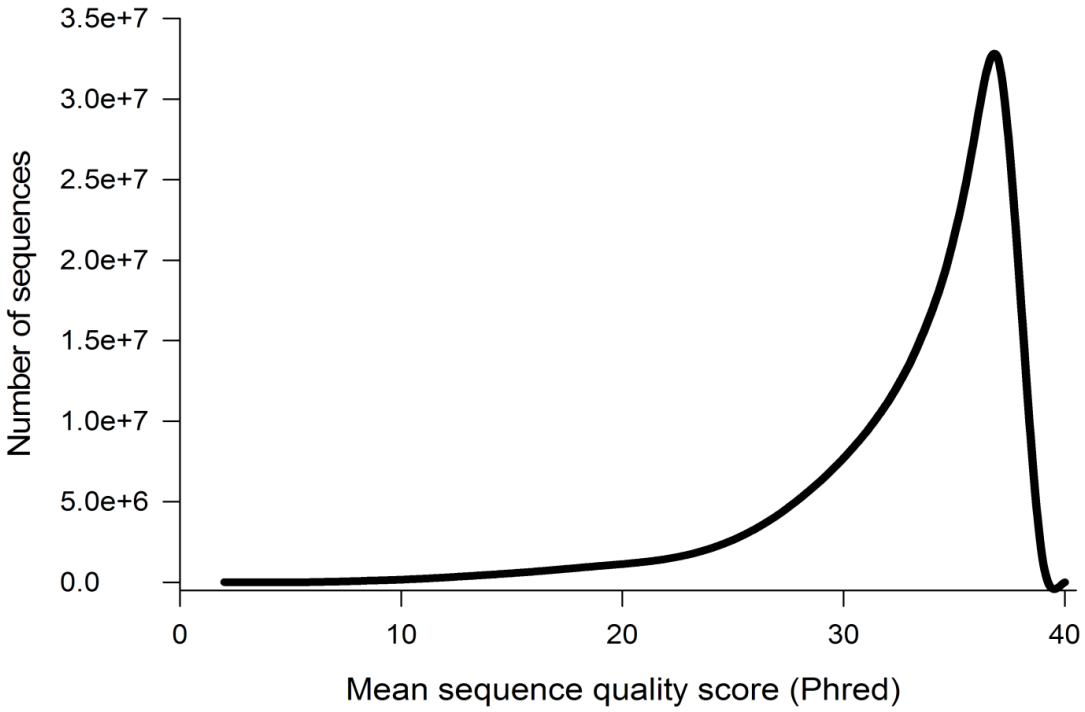


Figure 2.4 Mean quality score distribution for the salmon louse transcriptome raw sequence reads.

Quality control assessment of Illumina raw sequence reads using FastQC software (Brabraham Bioinformatics, UK) reported the mean sequence quality scores (Phred). Phred scores of 30 and 40 indicate a 99.9 % and 99.99 % base calling accuracy respectively.

Table 2.5 Transcriptome sequence set characterisation.

Transcripts		37,681
Unique genes		33,693
# consisting of single transcript		30,581
# consisting of multiple transcripts		3,112
Transcript sequence length	Unique genes	Complete transcriptome
Total sequence length (nt)	42,843,289	50,424,461
Average transcript length (nt)	1271.58	1338.19
Minimum transcript length (nt)	101	101
Maximum transcript length (nt)	21,935	24,684
Median transcript length (nt)	821	867

The assembled transcripts were annotated using BLASTp and BLASTn searches against the refseq-protein and EST/UniGene databases respectively. The results indicated that out of 33,693 genes, 28,547 (84 %), 13,194 (39 %) and 8,640 (26 %) showed significant similarity to known proteins or gene transcripts in refseq_protein, EST and UniGene databases, respectively.

Additionally, BLASTx annotation revealed 13,999 (41.5 %) well annotated transcripts (BLASTx e-value < 10^{-9}), and an additional 2,748 (8.2 %) sequences annotated with a BLASTx e-value of 10^{-4} - 10^{-9}. To evaluate the *L. salmonis* genome coverage of the assembled transcripts, the 129,225 ESTs (> 100bp) available for *L. salmonis* (Yazawa *et al.*, 2012) were aligned to the transcript sequence generated in this study and vice versa. 97,785 (76 %) of the *L. salmonis* ESTs reported by Yazawa *et al*. aligned to at least one transcript, whereas 15,507 (41 %) of the newly sequenced transcripts had at least one corresponding *L. salmonis* EST within the set reported by Yazawa *et al.*

GO annotation was assigned to the assembled *L. salmonis* transcripts/genes on the basis of refseq_protein annotation. In total, 28,547 genes had similarity to known gene products; however only 4,954 (17 %) were assigned GO annotation, with the assignment of 3,009 different functional terms and protein binding (25 %) and nucleic acid binding (24 %) being identified as highly represented biological functions (Figure 2.5).

The KEGG pathway database records the networks of molecular interactions within cells and the differences in these networks between organisms. Again, on the basis of refseq_protein annotation, the KEGG pathways of the associated genes were obtained for each transcript. In total, 28,547 transcripts were similar to known gene products; however only 3,273 (11 %) were assigned KEGG annotation that covered 247 different pathways. Of the transcripts that were assigned KEGG functional annotation, general metabolism was the largest group (19 %) with a total of 46 % annotated sequences being assigned metabolic functions (Figure 2.6).

Transcripts were identified as members of gene families putatively associated with reduced drug susceptibility in ecdysozoan invertebrates based on the BLASTx annotation (Table 2.6). These gene families include LGICs (GluCl, GABA-Cl, nAChR and Glycine-gated chloride channels), ABC transporters and detoxification enzymes (CYP, glutathione-s-transferase (GST) and esterases).

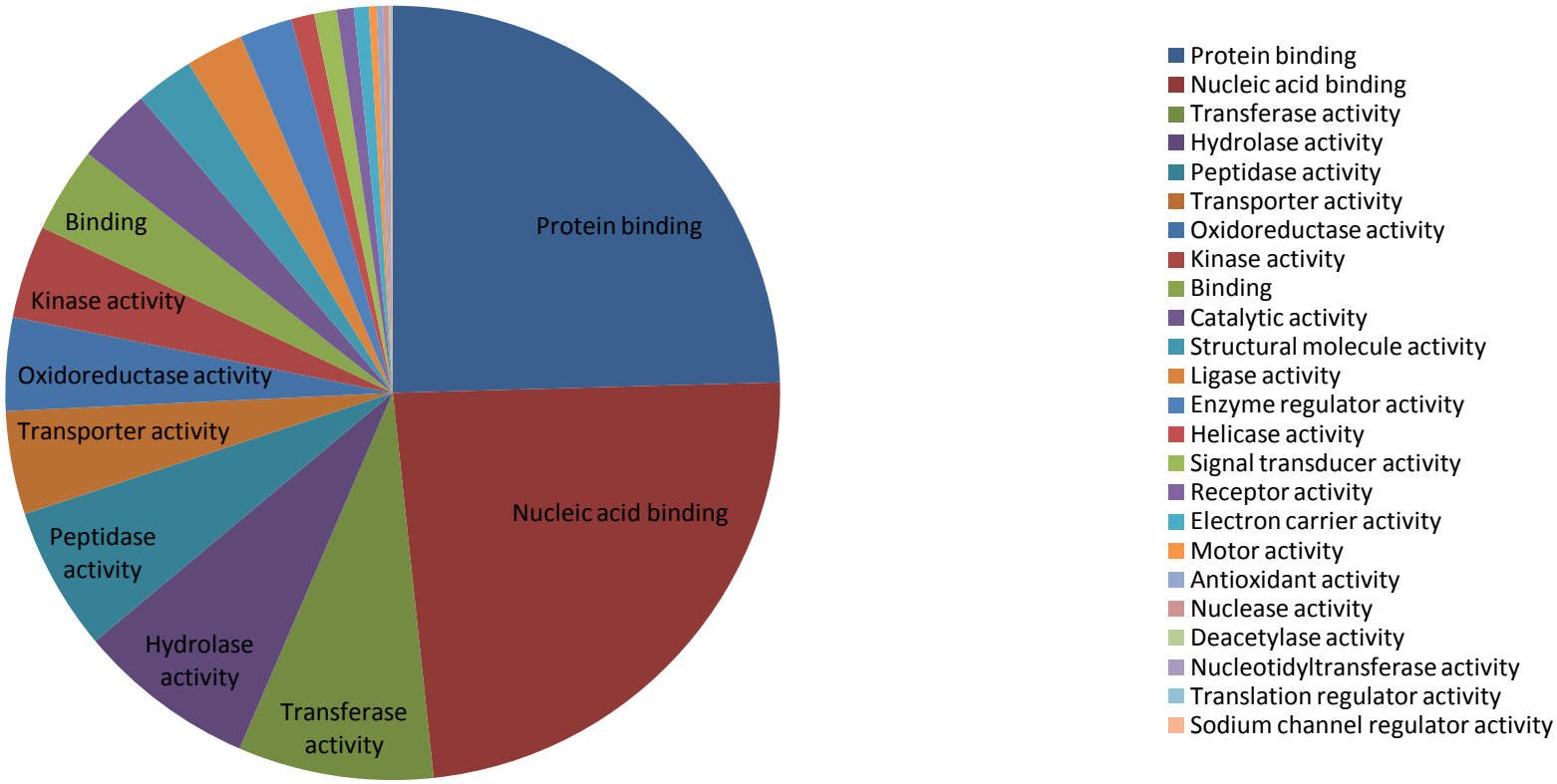


Figure 2.5 GO Slim functional classification of *L. salmonis* transcriptome sequence.

Gene Ontology (GO) terms were assigned to 4,954 (17 %) of the 28,547 annotated transcripts based on refseq_protein annotation. The distribution of GO Slim molecular function terms for the *L. salmonis* transcriptome is shown.

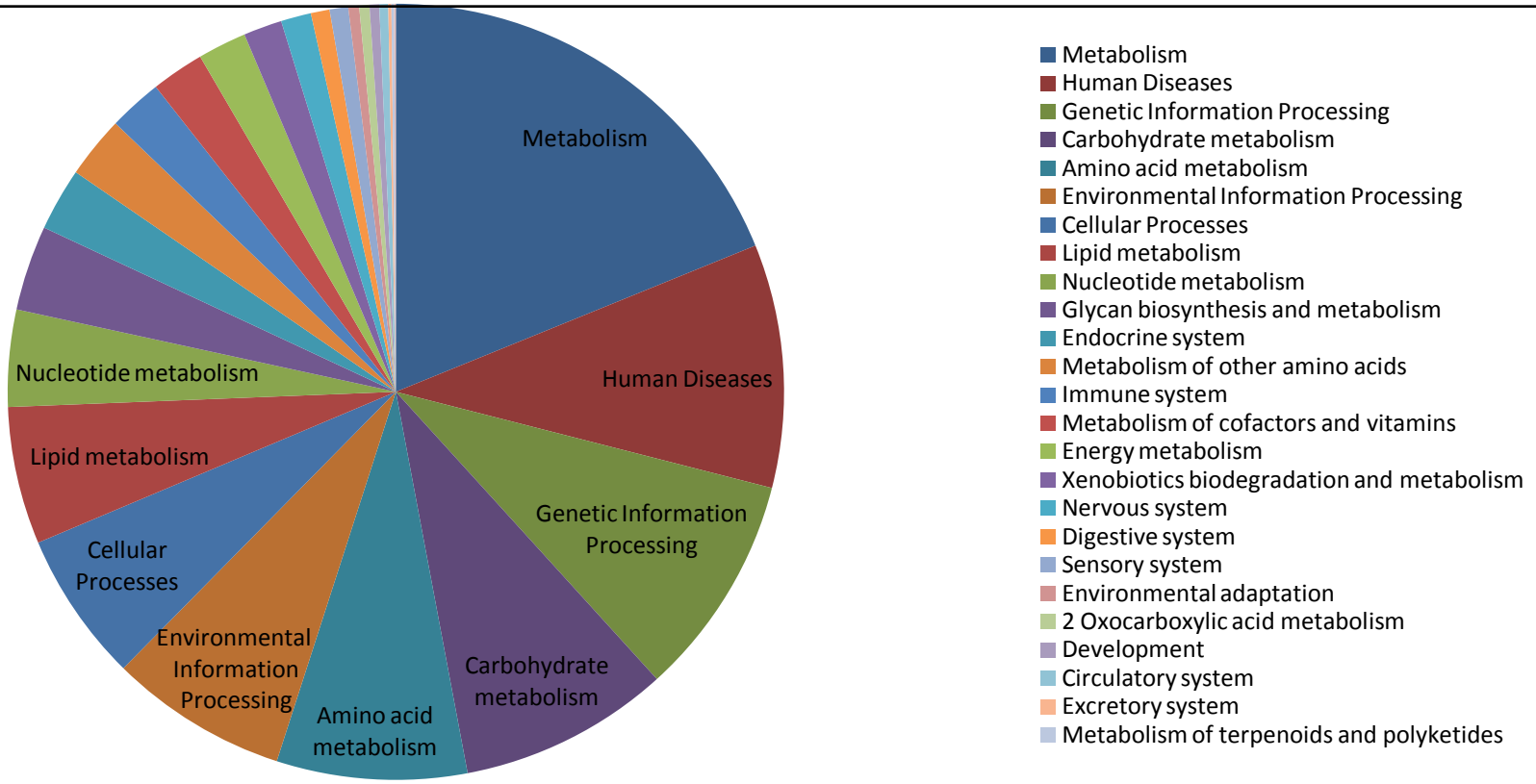


Figure 2.6 KEGG functional classification of *L. salmonis* transcriptome sequence.

KEGG pathway classification was assigned to 3,273 (11 %) of the 28,547 annotated transcripts based on refseq_protein annotation. The distribution of KEGG pathways for the *L. salmonis* transcriptome is shown.

Table 2.6 Summary of sequences putatively associated with reduced EMB susceptibility.

	EST contigs		SSH sequences		Transcriptome sequences	
Subgroup	Number of sequences	Group Total	Number of sequences	Group Total	Number of sequences	Group Total
ABC transporters						
A					1	
B	2				3	
C	6		1		7	
F					1	
G					4	
Other	5	13		1	2	18
Neuronal Acetylcholine receptors						
Alpha subunit	11		1		22	
Beta subunit	1				3	
Delta subunit					1	
Other		12		1	5	31
GluCl receptor subunits*						
Alpha subunit					6	
Other	2	2		0	2	8
GABA-Cl receptor subunits*						
Alpha subunit					2	
Beta subunit					6	
Other	5	5	2	2	4	12
Glycine receptor subunits						
Alpha subunit					8	
Beta subunit		0		0	9	17
Cytochrome P450						
2J	2				3	
3	4		1		7	
15a1	1		1		3	
18a1			1		2	
Mitochondrial	3				2	
Other	4	14	1	4	4	21
Glutathione-s-transferase						
All	17	17		0	13	13
Esterase						
All	1	1		0	7	7

The full sequence annotation, accession numbers and BLASTx e-values are detailed in Additional file 2.1.

* GluCl = Glutamate-gated chloride channel; GABA-Cl = Gamma-aminobutyric acid (GABA) chloride channel.

2.3.4 Salmon louse custom oligo microarray development

For transcriptomic analyses, custom Agilent 15K feature oligo microarrays were designed using EST sequences, publicly available in GenBank (Section 2.3.1), and sequences derived from salmon louse SSH libraries (Section 2.3.2). A larger 44K oligo microarray was designed that incorporated 60mer oligo probes designed to transcriptome sequences (Section 2.3.3), in addition to probes from the two previous 15K microarrays. On all three microarrays, oligo probes were designed to both forward and reverse complement strands for all unannotated sequences (Table 2.1).The GenePix array list (GAL) format files for the three microarray designs are detailed in Appendix 5 and supplied as Additional files 2.2 to 2.4.

2.3.4.1 L. salmonis 15K oligo microarray design 1

The initial 15K custom oligo microarray was created through designing 60mer oligo probes to 13,542 annotated and 1,566 unannotated target sequences that were assembled from ESTs (Table 2.2).

2.3.4.2 L. salmonis 15K oligo microarray design 2

A second *L. salmonis* 15K oligo microarray was created that included 60mer probes designed to 10,056 annotated (BLASTx e-value $<10^{-4}$) and 5,052 unannotated target sequences (Table 2.1). Microarray designs 1 and 2 share 10,251 identical features with the new probes included in design 2 used to replace unannotated probes from microarray design 1. Probes designed to annotated target sequence included 6,224 probes that had been included on the previous 15K microarray (design 1), and 3,832 probes designed to annotated target sequence from *L. salmonis* subtracted cDNA library sequencing (Section 2.3.2). These additional 3,832 probes comprised 2 unique probes designed to each of 1,916 annotated target sequences. The 5,052 probes designed to unannotated target sequence consisted of 3,486 probes from the previous 15K

microarray design and 1,566 probes designed to unannotated target sequence from *L. salmonis* subtracted cDNA library sequencing.

2.3.4.3 L. salmonis 44K oligo microarray

An *L. salmonis* 44K oligo microarray was created that included 60mer probes designed to 26,887 annotated (BLASTx e-value < 10^{-4}) and 16,666 unannotated target sequences (Table 2.1). The 43,553 probes represented on this microarray includes 28,695 probes designed to salmon louse transcriptome sequence, 11,970 designed to EST contig sequences and 2,888 designed to SSH sequence.

2.3.4.4 Validation of gene expression analysis

Salmon louse 15K microarray designs 1 and 2 were used to analyse differential gene expression responses between two laboratory-maintained salmon louse strains (S and PT). Microarray design one was used to measure gene expression in response to EMB exposure (see Chapter 4) and design two was used to measure constitutive gene expression between untreated salmon lice from these strains (see Chapter 3). To confirm these microarray results, transcript abundances of samples from each experiment were determined by reverse transcription quantitative PCR (RT-qPCR), and expression profiles measured using microarray analysis and RT-qPCR were compared (Table 2.7). The RT-qPCR methodology is detailed in Chapters 3, 4 and 5 of this thesis. A high degree of correlation was observed between expression values measured by both methods, for the majority of genes analysed (Pearson correlation coefficients (r) of 0.66 to 0.99; $p < 0.0001$).

Table 2.7 Correlation of relative expression ratios measured using microarray and RT-qPCR.

Accession No.	Annotation	r	p-value
	15K Microarray design 1		
NP_001136346.1	Cuticular protein	0.99	<0.0001
BAG74353.1	Metalloproteinase	0.99	<0.0001
ADD24462.1	Cerebellin-3	0.96	<0.0001
AAS91796.1	Intestinal trypsin 5 precursor	0.94	<0.0001
ADD38289.1	Gamma-crystallin A	0.77	<0.0001
ADD38711.1	Neuronal acetylcholine receptor subunit α3	0.71	<0.0001
ACM68948.1	Selenium-dependent glutathione peroxidase	0.69	<0.0001
BAI79321.2	Duplex-specific nuclease	0.66	<0.0001
	15K Microarray design 2		
XP_797271.2	Maltase-glucoamylase	0.99	<0.0001
ADD38289.1	Gamma-crystallin a	0.96	<0.0001
AAS13464.1	Cytochrome P450 15a1	0.95	<0.0001
XP_003494528.1	Cytochrome P450 18a1	0.95	<0.0001
ADD24187.1	Neuronal acetylcholine receptor subunit α3	0.92	<0.0001
EFN73916.1	GABA receptor subunit alpha	0.89	0.0001

Pearson correlation (r) of relative expression ratios (RER) calculated using *L. salmonis* 15K oligo microarray and RT-qPCR analysis. Correlation was calculated using 36 and 12 test samples for 15K microarray designs 1 and 2 respectively. (Significance $p < 0.05$).

2.4 Discussion

The establishment of commercial Atlantic salmon production sites in the north Atlantic has provided coastal regions with a high-density of salmonids, which facilitates survival and reproduction of the salmon louse that normally parasitises these fish. Pest management strategies utilised by the aquaculture industry to control this ectoparasite have very often involved chemical intervention, using a single medicinal agent that rapidly becomes ineffective as resistance develops in *L. salmonis* populations after repeated exposure. There is currently limited understanding of the general biology and reproduction of *L. salmonis* and of the molecular mechanisms underpinning reduced susceptibility to anti-sea louse medicines. A number of studies have used candidate gene approaches to investigate specific aspects of salmon louse development and reproduction (Kvamme *et al.*, 2004; Tribble *et al.*, 2007a,b; Skern-Mauritzen *et al.*, 2007; Dalvin *et al.*, 2009; Campbell *et al.*, 2009; Dalvin *et al.*, 2011). However, few studies have employed broad-scale 'omics' strategies, such as transcriptomic analysis using oligonucleotide microarrays, which compared to candidate gene approaches offer the advantage of not being based on previous assumptions regarding the biological mechanisms involved. This study demonstrates the use of high-throughput sequencing strategies to generate sequence resources for the non-model species *L. salmonis*, which supplemented existing EST resources and provided target sequence for the construction of custom oligonucleotide microarrays, used in transcriptomic analysis of *L. salmonis*. The sequencing of transcripts enriched between *L. salmonis* strains, which differ in EMB susceptibility, identified a number of candidate genes putatively associated with reduced EMB susceptibility that required further investigation using gene expression studies, *e.g.* using the custom microarrays provided here and supplementary approaches such as functional studies. The transcriptome sequence generated in this study, which

considered all life stages of the salmon louse, provides an extensive collection of sequences that is expected to facilitate investigations into a wide range of subjects related to *L. salmonis* biology and reproduction.

The assembly of contig sequences from existing *L. salmonis* ESTs generated 19,279 contigs and 9,693 singleton sequences, which was similar to the 21,035 contigs and 9,331 singletons reported recently by another group (Yasuike *et al.*, 2012), although the raw EST sequence quality filtering procedure differed in the present study. It was possible to annotate 47 % (BLASTx e-value $<10^{-4}$) of the contig and singleton sequences that were used as target sequence for the design of 60mer oligo probes in the construction of custom 15K microarrays for the study of EMB susceptibility in *L. salmonis*. The specificity and sensitivity of these oligos was confirmed through RT-qPCR validation of the two microarray experiments that utilised the 15K microarrays, with good correlation of expression ratios reported between analysis methods, for genes that exhibit differential expression within the limits of microarray technology (Fold change ≥ 1.3). There have been few published studies concerning transcriptomic analysis of *L. salmonis*, with the first study utilising a 7000 cDNA probe microarray to study post moult maturation and egg production (Eichner *et al.*, 2008). This cDNA microarray was designed using contig sequences assembled from a collection of *L. salmonis* ESTs described by Yasuike *et al.* (Yasuike *et al.*, 2012). Similarly, Sutherland *et al.* used these ESTs to construct a 38K oligo microarray to study transcriptomic responses of *L. salmonis* larvae to abiotic stress (Sutherland *et al.*, 2012). The present study demonstrates the development of a 44K oligo microarray resource that utilises an expanded sequence resource with representation of major metamorphosis steps throughout the life cycle of a laboratory maintained *L. salmonis* strain sourced from the West coast of Scotland.

The enrichment of cDNA by SSH generated a high percentage of sequences that were annotated with metabolic functions, which may indicate key metabolic differences between *L. salmonis* strains S and PT that differ in EMB susceptibility. In addition, a number of genes were identified in gene families that have previously been associated with reduced EMB susceptibility. These candidate genes include LGIC receptor subunits, CYPs and a multidrug resistance associated protein (ABC transporter subfamily C). The principal pharmacological target for avermectin (AVM) compounds such as EMB is thought to be LGICs, more specifically glutamate-gated chloride channels (GluCl), which are members of the ecdysozoan specific 'Cys loop' family of LGICs. The association of GluCl with AVM binding originated from work carried out on the model nematode (*Caenorhabditis elegans* (Maupas, 1900)) and the fruit fly (*Drosophila melanogaster*, Meigen, 1830) (Cully *et al.*, 1994; Cully *et al.*, 1996). Functional Cys-loop' LGICs are formed through the arrangement of protein subunits in a pentameric structure, which can be formed from identical subunit types or a combination of two to three different subunit isoforms. A GluClα subunit has been cloned in *L. salmonis* (Tribble *et al.*, 2007b), but was not identified in this study, when subtracted cDNA libraries enriched between *L. salmonis* strains with differential EMB susceptibility were sequenced (Table 2.3).

Reduced AVM susceptibility in nematodes has also been associated with molecular mechanisms that decrease cellular and/or tissue-specific drug accumulation and thus decreases internal drug exposure. The main mechanisms in this category include members of the ABC transporter gene superfamily. ABC proteins are located in the cell membrane and mediate the ATP-dependent active efflux transport of structurally and functionally unrelated organic compounds, including therapeutic drugs (Leslie *et al.*, 2005; Jones *et al.*, 2009). The salmon louse P-glycoprotein (P-gp)

homologue SL-PGY1 (Heumann *et al.*, 2012) was not identified among cDNAs putatively expressed differentially between *L. salmonis* strains differing in EMB susceptibility that were enriched in this study using the SSH approach. However, a multidrug resistance-associated protein (MRP) was identified in these SSH libraries that could represent a potential drug efflux pump (Kruh and Belinsky, 2003; Deeley and Cole, 2006). Several CYPs were also identified in these libraries, which have also been implicated in reduced susceptibility of invertebrate pests to a variety of antiparasitic drugs (Heckel, 2012), although further research would be required to determine if these candidates are actually associated with reduced EMB susceptibility.

The novel transcriptome sequence resource, generated as part of this study, consists of 33,693 unique transcripts of which ~50 % were functionally annotated (BLASTx e-value < 10^{-4}). These sequences represent a 54 % increase in the number of available transcript sequences for *L. salmonis*, with only 28,972 contig and singleton sequences being assembled from existing ESTs. These transcript sequences include several putative candidate genes that have previously been associated with resistance of ecdysozoan pest species to antiparasitic compounds. These include ABC transporter proteins from subfamilies B, C and G, target LGIC receptor subunits and detoxification enzymes such as CYPs and esterases. KEGG and GO annotation of these *L. salmonis* transcripts identified a high representation of genes involved in metabolism, protein binding and nucleotide binding. Major biological functions, processes and cellular components are adequately represented in the transcriptome, providing an improved resource for the study of *L. salmonis*.

Conclusions

The research reported in this chapter has provided a significant expansion of the genomic resources currently available for *L. salmonis* in the absence of a fully annotated salmon louse genome sequence. The creation of transcriptome sequence for *L. salmonis* from samples that represent key stages of the life cycle provides a sequence resource that can be used to study all aspects of *L. salmonis* biology and reproduction. The additional creation of sequence from a subtracted cDNA library, constructed using *L. salmonis* strains with differing susceptibility to EMB, provides a resource specific to the most commonly used antiparasitic agent in control of *L. salmonis* infection of Atlantic salmon, which is becoming increasingly ineffective. The assignment of suitable annotation to a high percentage of these sequences demonstrates that this resource contains adequate biological information to allow further interrogation in relation to associations with reduced EMB susceptibility and general *L. salmonis* biology. This study also reports successful use of this sequence resource, in addition to existing *L. salmonis* EST resources, in the construction of a custom *L. salmonis* 44 K oligo microarray. In the process of developing this custom microarray, two 15 K microarray designs were successfully employed in the study of EMB susceptibility of the salmon louse, showing accurate measurement of mRNA levels when compared to RT-qPCR. The resources created in this study will therefore contribute to research into a commercially important parasite (*L. salmonis*) but may also contribute to research into other copepod species or ecdysozoan pests.

Chapter 3
Comparisons of constitutive gene expression between two salmon louse strains with differing susceptibilities to emamectin benzoate.

3.1 Introduction

The development of synthetic insecticides and parasiticides has revolutionised the control of arthropod pests and parasites of agricultural, medical and veterinary importance. However, the overuse of chemical control agents tends to promote the development of heritable insecticide or drug resistance (ffrench-Constant *et al.*, 2004; Li *et al.*, 2007; Labbé *et al.*, 2007; Bass and Field, 2011; Heckel, 2012), with life-cycle traits of targeted organisms often favouring the ability to adapt genetically, such as high reproductive potential and short generation span. Drug resistance can follow from pre-existing mutations in resistance genes, termed resistance alleles, which under normal conditions are rare and have a negative or neutral effect on fitness but which, under conditions of exposure to control agents, afford fitness benefits and can become enriched in the target species' gene pool given persisting selection pressure from the control agent (Wolstenholme *et al.*, 2004). By the time resistance becomes apparent as treatment failure, resistance alleles have usually already reached high frequencies in the gene pool (Wolstenholme *et al.*, 2004).

The annual cost of sea louse infection to the global salmon farming industry has been estimated at €300 million, with the majority of this accounted for through expenses accrued from treatments with veterinary medicines (Costello, 2009). Only a limited range of anti-sea louse drugs are available and licensed for the treatment of fish (Burridge *et al.*, 2010), and the continued use of a relatively small number of compounds creates a situation potentially favouring the development of drug resistance (Denholm *et al.*, 2002). In the salmon louse, losses of efficacy have been reported for a number of control agents including organophosphates (Jones *et al.*, 1992), pyrethroids (Sevatdal and Horsberg, 2003), hydrogen peroxide (Treasurer *et al.*, 2000) and avermectins (AVMs) (Lees *et al.*, 2008a; Igboeli *et al.*, 2012).

The commonly used anti-sea louse treatment SLICE® (Merck Animal Health) contains the avermectin compound emamectin benzoate (EMB) (Stone *et al.*, 1999). SLICE® is administered orally and a one-week treatment provides prolonged protection against all host-attached life stages of sea lice (Stone *et al.*, 1999). Avermectins are also used against external and internal parasites of humans and livestock, including parasitic nematodes causing the human diseases onchocerciasis (River blindness) and lymphatic filariasis, as well as gastrointestinal parasites of sheep, cows and horses (Geary, 2005). The selective toxicity of avermectins against ecdysozoans is believed to be based on the binding and blockage of glutamate-gated (GluCl) and γ-aminobutyric acid (GABA)-gated (GABA-Cl) chloride channels in the invertebrate nervous system (McCavera *et al.*, 2007). Several molecular mechanisms have been suggested to contribute to the resistance of parasitic nematodes to the AVM compound ivermectin (IVM) (Beech *et al.*, 2011). Functional studies revealed that AVM resistance in nematodes can be based on single amino acid mutations in subunits of GluCl and GABA-Cl that decrease the channels' sensitivities to the drug (Njue and Prichard, 2004; McCavera *et al.*, 2009). Furthermore, resistant nematodes may show increased expression of ABC (ATP-binding cassette) transporters, a group of membrane proteins with members capable of mediating the cellular efflux of drugs (Ardelli and Prichard, 2004; James and Davey, 2009). Finally, avermectin resistance in insects has been connected to alterations in drug metabolism (Chen *et al.*, 2011).

Previous studies on potential molecular mechanisms of EMB resistance in salmon lice have used the candidate gene approach, *i.e.* the study of genes that have previously been linked to drug resistance in other organisms. In particular, such studies have investigated salmon louse ABC transporters (Heumann *et al.*, 2012; Tribble *et al.*, 2007a) and GABA-Cl and GluCl subunits (Tribble *et al.*, 2007b). However, potential

outcomes resulting from such candidate approaches are limited by the current knowledge of the biological process being studied. To avoid this limitation, a transcriptomic approach was followed in the present study, in which microarray analysis was used to compare transcriptomic profiles between drug susceptible and moderately (~7-fold) EMB resistant laboratory strains of salmon lice.

3.2 Materials and methods

3.2.1 Salmon louse strains

Two laboratory-maintained *Lepeophtheirus salmonis* strains (S and PT) differing in susceptibility to EMB (Heumann *et al.*, 2012), were used in this study. For more information on these strains please see the materials and methods section in Chapter 2 of this thesis.

3.2.2 Salmon louse exposure and sampling

In order to confirm the EMB susceptibility levels of salmon louse strains S and PT, 24 hour bioassays (Sevatdal *et al*., 2005) were performed. EMB (technical grade, a gift from Merck Animal Health) was solubilised in seawater with PEG_{300} (final concentration 0.01% (v/v)). EMB concentrations used in bioassays were 32.5, 75, 150, 300 and 600 µg L^{-1} with S lice, and 200, 400, 800, 1,600 and 3,200 µg L^{-1} with PT lice. Duplicate glass dishes containing 10 salmon lice and 200 mL of exposure solution were used per EMB concentration, control (seawater) or solvent control (seawater with 0.01% (v/v) PEG_{300}). At the end of 24 hours of exposure, salmon lice were recorded as normally motile or immotile upon visual examination and stimulation with a fine brush.

Adult male salmon lice were collected from anaesthetised host fish as described in Chapter 2 and allowed to recover for 2 hours in aerated filtered seawater at ambient sea temperature. To analyse transcript expression in salmon louse strains S and PT in the absence of drug exposure (constitutive expression microarray experiment), six pools

of four adult males were collected and preserved in an RNA stabilisation solution (4.54 M ammonium sulphate, 25 mM trisodium citrate, 20 mM EDTA, pH 5.4; Appendix 1) prior to storage at -70 °C. In order to replicate the analysis of strain-dependent differences in constitutive transcript expression using an additional set of biological samples, gene expression analyses were performed on seawater controls originating from a second experiment (Response to EMB exposure microarray experiment, see Chapter 4). The adult male salmon lice were collected 17 and 20 days after the introduction of infective copepodids to the Atlantic salmon hosts, for the constitutive expression and EMB exposure microarray experiments respectively. Additionally, for logistical reasons sea louse sampling was carried out in February and May for the constitutive expression and EMB exposure microarray experiments respectively. Infective copepodid lice were produced in bulk, with egg strings being removed from gravid adult female lice and incubated in aerated sea water for ~ 10 days prior to the infection of salmonid hosts, depending on water temperature. An assessment of the infective copepodid and adult male *L. salmonis* developmental stage was performed by visual examination under low magnification microscopy and was based on the size and shape of the salmon louse. In the experiment described in Chapter 4, salmon lice of either strain were subjected to short term (1 and 3 hours) EMB exposures as well as solvent (0.01 % (v/v) PEG_{300}) or seawater control treatments. The effects of EMB treatment in the EMB response microarray experiment will be described in Chapter 4 of this thesis. The use of seawater control data from the 'response to EMB exposure' microarray experiment (Chapter 4) as a quasi-replication of the constitutive expression microarray experiment in the present chapter was based on the assumption that different durations of salmon lice exposure to seawater, following collection from host fish and prior to sampling (2 h in constitutive expression microarray experiment, 3-5 h in

'response to EMB exposure' experiment), would only have minor or undetectable effects on the levels of transcripts having a role in EMB susceptibility. Previously collected bioassay data from the Marine Environmental Research Institute (MERL) support this contention, indicating that maintenance of salmon lice for up to 12 h after collection from host fish does not significantly affect the results of EMB immotility bioassays (Dr W. Roy, MERL, personal communication). No effects on sea louse motility were observed in either of these experiments. In the response to EMB exposure microarray experiment, for each combination of strain, exposure period and treatment, three pooled samples consisting of four salmon lice each were collected and stored at -70 °C for later RNA extraction as above. The data for one and three hour sea water controls from the response to EMB exposure microarray experiment were pooled for each strain and used in this study of differential constitutive gene expression, thus resulting in the same level of biological replication (n = 6 biological replicates, i.e. pools of four *L. salmonis*) as in constitutive expression microarray experiment.

3.2.3 RNA extraction and purification

For microarray and RT-qPCR experiments, samples comprised pools of four adult male salmon lice. Frozen samples were ground in liquid nitrogen and total RNA was extracted using TRI Reagent® (Sigma-Aldrich, UK), resuspended in nuclease-free water and further purified using RNeasy columns (Qiagen, UK) then assessed for purity and concentration as detailed in Chapter 2 of this thesis.

3.2.4 Microarray analyses

Labelling protocols are described in detail elsewhere (Morais *et al.*, 2011). Briefly, for each test sample 250 ng total RNA was used as template for the amplification of antisense RNA with the incorporation of the modified nucleotide 5-(3-aminoallyl)-UTP (aaUTP) into the amplified RNA (aRNA) during the in vitro

transcription step (TargetAmp™ Aminoallyl-aRNA Amplification Kit 101; Epicentre®, Cambio Ltd. UK). A common reference pool was created through pooling equal amounts of all aRNA test samples to be used in the experiment. The individual test samples were labelled with cyanine 3 (Cy3) and the common reference pool labelled with Cy5 mono-reactive dye (GE Healthcare, UK) in dye coupling reactions. Unincorporated dye was removed by column purification (Illustra Autoseq™ G-50 spin columns; GE healthcare, UK), and then dye incorporation was assessed by spectrophotometry (NanoDrop ND-1000, Thermo Scientific, USA) and fluorescent gel electrophoresis. Three hundred nanograms of each Cy 3 labelled test sample was competitively hybridised with 300 ng Cy5 labelled common reference pool on a 15K feature custom microarray, following the manufacturer's instructions (Agilent Technologies, UK). The hybridisation reactions were incubated at 65 °C with 10 rpm rotation for 17 hours in an Agilent rotary hybridisation oven and then washed with Gene Expression Wash Buffers 1 and 2, with a final wash using Stabilisation and Drying solution, again following the manufacturer's instructions (Agilent Technologies, UK). The hybridised microarrays were scanned using an Axon Genepix 4200A scanner with Genepix Pro 6.1 image acquisition software (Molecular Devices, UK) using 40 % laser power, 5 µm pixel size resolution and auto photo-multiplier tube (auto-PMT) function with 0.05 saturation tolerances. The raw microarray images were processed using Agilent Feature Extraction (FE) software version 9.5.3.1 that performed feature grid alignment, extraction and quantification. The fluorescence intensity results files from the FE software were imported into the GeneSpring GX version 12 software (Agilent Technologies, UK) for differential gene expression analysis. Data were normalised using Lowess normalisation of log2-expression ratios without baseline transformation. Features showing low quality according to Agilent quality control

metrics were discarded to provide a final feature set for analysis. Details of microarray experiments 1 and 2 have been submitted to ArrayExpress and assigned accession numbers E-MTAB-1484 (Constitutive expression microarray experiment) and E-MTAB-1478 (Response to EMB exposure microarray experiment). The recording of the microarray experimental metadata complies with Minimum Information About a Microarray Experiment (MIAME) guidelines (Brazma *et al*., 2001).

3.2.5 RT-qPCR

To validate gene expression results from microarray experiments, the abundance of selected transcripts found to be differentially expressed in microarray analyses was determined by reverse transcription quantitative PCR (RT-qPCR). Per experiment, three targets that showed stable expression levels in microarray hybridisations were selected as reference genes. (Constitutive expression microarray experiment: 60S ribosomal protein S20, 40S ribosomal protein L44 and RMD-5 homolog; Response to EMB exposure microarray experiment: hypoxanthine-guanine phosphoribosyltransferase (HGPRT), RMD-5 homolog and elongation factor 1α). For each target sequence, primers were designed with a melting temperature (Tm) of ~60 °C using Primer 3 software (Appendix 6 and 7). Aliquots (1µg) of total RNA samples previously used in microarray analyses were reverse transcribed (Superscript III, Invitrogen, UK) using random hexamers and anchored oligo-dT primers in a 3:1 molar ratio. No-template controls and controls omitting RT enzyme were included on each assay plate to detect potential DNA contamination. A cDNA pool containing equal amounts of all samples was made and included on each assay plate, serving as a calibration sample (20-fold dilution) and for derivation of a standard curve from serial dilutions. RT-qPCR reactions were performed in duplicate in a total volume of 20 µL containing 5 µL sample cDNA (20-fold dilution), 0.3 µM of each primer and 10 µL Absolute SYBR

Green I mix (ThermoFisher Scientific, UK), using the Mastercycler ep realplex2 (Eppendorf, UK) with the following amplification conditions: 95 °C for 15 minutes, followed by 40 cycles of 94°C for 30 seconds, 15 seconds at the specific primer pair annealing temperature (Ta; Appendix 6 and 7) and 72 °C for 30 seconds. After amplification a melt curve was generated to ensure that a single product was amplified in each reaction. To this end, fluorescence was monitored while heating each sample from 55 °C to 95 °C at 0.5 °C increments for 15 seconds each. Threshold cycles were analysed using the PCR cycler software. Standard curves were derived from plots of the threshold cycle against the logarithm of the relative concentration of cDNA pool. Primer efficiency (E) was derived from linear fits to the standard curve according to the equation E = 10(-1/slope). The BestKeeper tool (Pfaffl *et al.,* 2004) was employed to analyse expression stability of three reference genes and determine a robust BestKeeper expression index as a geometric mean for the three reference genes, which was in turn used to establish relative gene expression ratios using the ΔΔCt method (Ratio = $(E_{target})^{\Delta Ct\ target\ (control\ -\ sample)}$ / $(E_{reference})^{\Delta Ct\ reference\ (control\ -\ sample)}$) using the Relative Expression Software Tool (REST) Multiple Condition Solver (MCS) (Pfaffl, 2001).

3.2.6 Statistical analysis

Microarray gene expression data was analysed using GeneSpring GX version 12 (Agilent Technologies). The analysis of constitutive differential gene expression in experiments 1 and 2 used students T-test adapted for samples with unequal variance (Welch) using a fold change threshold of 1.3. Multiple testing corrections were not applied to any statistical analysis of this gene expression study as this can often be over-conservative when studying potentially subtle gene expression responses to stimuli (Leaver *et al.,* 2008; Morais *et al.,* 2011). This decision is supported by confirmation of differential expression by RT-qPCR in the current study. Gene enrichment analysis was

performed on lists of features chosen based on differential gene expression patterns using default settings of the FuncAssociate 2.0 web application (Berriz *et al.*, 2009). Gene enrichment was calculated according to the significance ($p < 0.05$) of the association between the list of features and the GO attributes represented on the microarray. Relative expression ratios from RT-qPCR experiments were log transformed and tested for normality and equal variance to allow assumptions to be satisfied before being subjected to one way ANOVA using MiniTab 16.1 software (MiniTab Inc., UK). The significance level was set at 0.05 in all tests.

3.3 Results

3.3.1 Experimental design

Two laboratory-maintained strains of salmon lice were used in the present study. Strain S is susceptible to all currently licensed anti-sea louse treatments including EMB, while strain PT is moderately resistant to EMB (Figure 3.1) (Heumann *et al.*, 2012). Adult male salmon lice were used for the transcriptomic analyses as they are considered to provide a more steady physiological state than adult females, which undergo considerable morphological change following fertilisation and which are subject to repeated cycles of egg production. Salmon lice were collected from host fish anaesthetised in 100 mg L^{-1} 2-phenoxyethanol and were then allowed to recover in aerated seawater for 2 hours before use.

In the constitutive expression microarray experiment, RNA expression profiles were analysed in lice sampled directly after recovery in order to reveal differences in constitutive gene expression between strains. The response to EMB exposure microarray experiment investigated the effects of short-term (1 to 3 hours) exposure to 200 µg L^{-1} of EMB, compared to seawater and carrier controls. In addition to being investigated for effects of EMB (see Chapter 4), the data from the response to EMB exposure microarray experiment were also analysed with respect to differences in constitutive transcript expression between strains. For this purpose, data for the one and three hour sea water controls from the response to EMB exposure microarray experiment were pooled for each strain in order to obtain the same level of replication as used in the constitutive expression microarray experiment (n = 6 biological replicates, i.e. pools of four *L. salmonis*). This pooling of control data was regarded as justified, as we had previously observed that exposure of salmon lice to seawater for up to 12 hours after collection from the host has no effect on responses to EMB in water-borne bioassays (Dr W. Roy, MERL, personal communication).

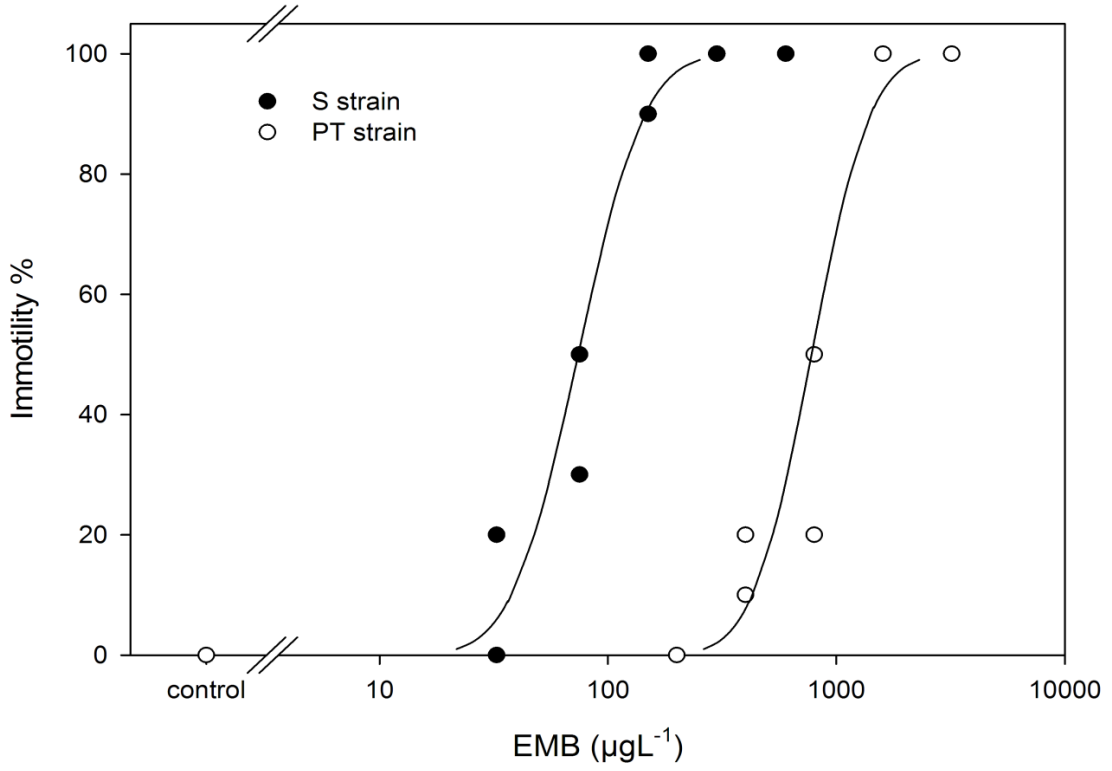
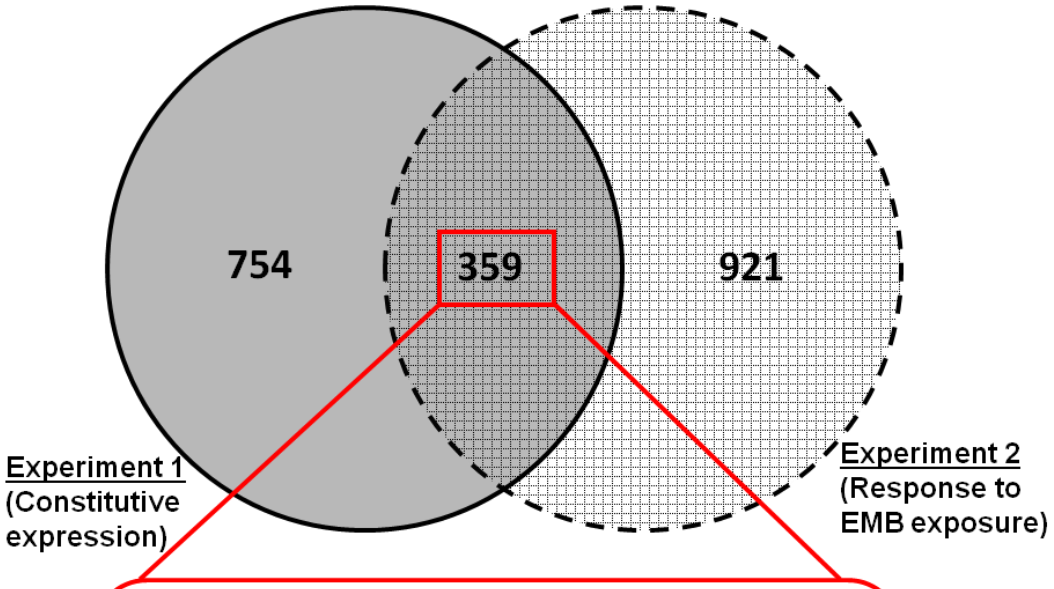


Figure 3.1 Susceptibility of salmon louse laboratory strains to emamectin benzoate (EMB).

Toxicity of EMB to adult male salmon lice (*L. salmonis*) from laboratory strains S and PT in 24 hours immotility bioassays. Symbols represent the immotility response observed in one of duplicate beakers of ten individuals included for each combination of strain and treatment. Dose-response relationships (solid lines) were established using probit analysis, and yielded EC50 values of 73.9 µg L^{-1} (95 % confidence intervals: 58.9 - 92.0 µg L^{-1}) for the S strain and 642.3 µg L^{-1} (642.3 - 957.4 µg L^{-1}) for the PT strain.

3.3.2 Analysis of differences in constitutive gene expression

To determine constitutive differences in gene expression between the PT and S strains, mRNA expression profiles were analysed in adult male salmon lice sampled in February 2012 (Constitutive expression microarray experiment) and May 2011 (seawater controls of the response to EMB exposure microarray experiment). When data from each experiment were analysed, including only features present on both microarrays, similar numbers of features were found to be differentially expressed between strains in both the constitutive and the response to EMB exposure microarray experiments (1,113 and 1,280 features respectively; Figure 3.2). Comparison of these two feature lists revealed that only 359 features were reported as being significantly differentially expressed between strains in both experiments. Of these, 294 (82 %) showed the same direction of strain differences in the two experiments (Figure 3.2) and represented 226 genes of which 57 % were annotated. These 226 genes were arranged by significance of the expression differences determined in the constitutive expression microarray experiment. Genes that were represented in the top 100 most significantly differentially expressed transcripts are detailed in Table 3.1, which includes a substantial number of cytoskeleton proteins (26 %) and proteases (12 %).



Experiment 1 Expression change (PT/S)	Experiment 2 Expression change (PT/S)	Number of features	Number of genes	
Down	Down	235 (*66%*)	180	226*
Up	Up	59 (*16%*)	46	
Down	Up	40 (*11%*)	37	
Up	Down	25 (*7%*)	21	
Total		359	284	

Figure 3.2 Comparison of microarray features differentially expressed across salmon louse strains between experiments.

Genes differentially expressed between two salmon lice strains differing in EMB susceptibility (S, PT) were determined by microarray analysis in two independent experiments. Features that showed differential expression between strains (fold change ≥ 1.3, $p < 0.05$) were derived for both experiments. The Venn diagram only includes features studied in both experiments.

*A total of 226 genes showed comparable differential expression between salmon louse strains in two independent microarray experiments and were therefore selected for further analysis of biological function.

Table 3.1 Genes showing differential expression between salmon louse strains differing in EMB susceptibility.

Annotated genes (57 %) were sorted by the significance of differential expression between strains in constitutive expression microarray experiment and arranged by biological function. Features with identical annotation were removed prior to categorising biological function. Also indicated is the percentage representation for each functional category in the total number of annotated genes (129 genes).

Accession No.	Annotation	**Constitutive expression experiment** Fold change (PT/S)	p-value	**Response to EMB exposure experiment** Fold change (PT/S)	p-value
Ligand gated ion channel (<1 %)					
ADD24187.1	Neuronal acetylcholine receptor subunit alpha-3	-3.21	7.59E-05	-2.52	4.29E-03
Metabolism of xenobiotics (<1 %)					
ACO15001.1	Cytochrome P450 3A24	-1.48	1.95E-03	-1.31	4.13E-02
Regulation of synapse development (<1 %)					
ADD24462.1	Cerebellin-3	-1.86	3.60E-04	-4.91	3.53E-06
Eye lens proteins (<1 %)					
ADD38111.1	Beta-crystallin A1	2.33	6.79E-05	1.37	1.35E-02
Transporters (2 %)					
NP_001116712.1	Solute carrier family 8 (sodium/calcium exchanger)	-2.10	3.63E-04	-4.38	1.74E-05
EFX88361.1	Alpha subunit of Na+/K+ ATPase	-1.67	2.21E-04	-2.08	5.34E-05
ACO12613.1	Excitatory amino acid transporter 3	-1.42	1.10E-04	-1.34	4.96E-02
Cytoskeleton proteins (26 %)					
ADD38332.1	Troponin I	-1.77	4.85E-05	-2.61	1.17E-06
XP_001950563.1	Muscle LIM protein	-1.67	1.57E-04	-2.30	1.32E-06
AAA17371.1	Fast myosin heavy chain	-2.11	1.73E-03	-6.23	1.68E-06
ACO13186.1	Myosin light chain alkali	-2.65	1.69E-05	-3.38	8.46E-06
ACO12924.1	Myosin light chain alkali	-2.06	1.16E-05	-3.69	9.66E-06
ABU41018.1	Collagen alpha-1 chain	-1.38	1.51E-03	-6.85	2.61E-05
EFV61840.1	Smoothelin	-1.43	4.03E-04	-1.85	6.25E-05
ACO12887.1	Troponin T	-1.73	1.04E-03	-2.93	8.12E-05
ADV40202.1	Troponin 1	-1.94	1.87E-03	-2.93	3.23E-04
AAW22542.1	Myosin light chain	-1.76	1.00E-03	-2.23	3.73E-04
ACO10528.1	Troponin C, isoform 1	-1.91	1.69E-03	-2.22	5.12E-04
ACO12630.1	Troponin C, isoform 1	-1.61	3.01E-05	-1.51	5.92E-04
ACO12421.1	Tropomodulin	-1.51	1.17E-04	-2.37	1.01E-03
ACO12794.1	Troponin C, isoform 1	-2.07	6.14E-05	-1.60	2.85E-03
ACO14751.1	Troponin C, isoform 1	-2.12	4.94E-05	-1.56	4.18E-03
ACO11818.1	Torso-like protein precursor	-1.83	5.11E-05	-1.82	9.67E-03
ACO11077.1	Troponin C, isoform 1	-1.92	6.27E-05	-1.48	1.56E-02
Regulation of actin cytoskeleton (<1 %)					
XP_002407362.1	Paxillin	-2.79	8.32E-07	-1.48	2.28E-03
Calcium transport (<1 %)					
NP_001032719.1	Sarco/endoplasmic reticulum calcium transporting ATPase	-2.15	8.58E-07	-2.46	2.15E-04
Calcium binding (1 %)					
XP_002734090.1	Calmodulin-like	-1.60	6.41E-05	-2.46	3.25E-04
ACO11757.1	Sarcoplasmic calcium-binding protein, beta chain	-3.18	2.05E-06	-3.58	1.28E-03
Cuticle proteins (4 %)					
ADD24515.1	Cuticle protein 6	-2.29	1.79E-03	-12.28	4.75E-04
ABU41025.1	Cuticle protein	-2.57	2.78E-04	-12.91	1.88E-03
ACO14885.1	Cuticle protein CP14.6 precursor	-1.77	7.50E-04	-3.40	4.67E-03
Proteolysis (12 %)					
ADD38666.1	Matrix metalloproteinase-9	-2.29	1.07E-05	-7.35	1.84E-06
ADD38283.1	Kunitz/BPTI-like toxin	-2.45	5.43E-06	-1.79	2.50E-04
ABU41053.1	Metalloproteinase	-5.61	7.00E-06	-4.38	3.99E-04
BAG74353.1	Metalloproteinase	14.83	8.20E-05	18.61	4.64E-04
ACO11096.1	Serine carboxypeptidase CPVL precursor	1.58	2.44E-04	1.36	1.21E-02
ABU41117.1	Metalloproteinase	-2.72	6.62E-05	-1.41	1.82E-02
AAS91793.1	Intestinal trypsin 2 precursor	7.73	1.90E-06	1.82	2.52E-02
AAS91795.1	Intestinal trypsin 4 precursor	1.48	6.69E-04	1.42	2.79E-02

Enrichment analysis of the 294 features resulting from comparison of expression between strains was performed with respect to the gene ontology (GO) annotation representation on the microarray. Nine GO attributes were found to be significantly over-represented (Table 3.2), with calcium ion binding, structural constituent of muscle and actin binding being shown to be the most significantly over-represented GO terms. To confirm findings from microarray analyses, transcript abundance was analysed for a sub-set of significantly differentially expressed genes using RT-qPCR. Genes were selected on the basis of potential significance as pharmacological targets of EMB (GABA-Cl subunit alpha and neuronal acetylcholine receptor subunit α3), or detoxification mechanisms (cytochrome P450 isoforms, carboxylesterase). Maltase-glucoamylase was further included because of its high level of differential expression (105-fold) between salmon louse strains. RT-qPCR analysis found that transcripts of nAChR α-3 were ~3.1-fold and ~2.6-fold less abundant in the PT than the S strain in experiments 1 and 2 respectively, which confirmed trends observed in the microarray analyses (Table 3.3). Similarly, RT-qPCR demonstrated that PT lice showed significantly lower levels of GABA-Cl α-subunit mRNA expression compared to the S strain (1.4-fold and 1.6-fold in experiments 1 and 2, respectively; Table 3.3), although differences were marginal. Isoforms of cytochrome P450 and carboxylesterase, *i.e.* enzymes potentially involved in detoxification, were found to show higher mRNA expression levels in the PT compared to the S strain in the constitutive expression microarray experiment, but not in the response to EMB exposure microarray experiment. Transcript levels of maltase-glucoamylase were much lower in PT than S lice in constitutive expression microarray, whereas in the response to EMB exposure microarray experiment the mRNA expression was moderately increased in PT compared to S lice (Table 3.3).

Table 3.2 Enrichment of GO classes in the list of features showing differential expression between salmon louse strains.

GO attribute ID	GO attribute name	LOD[1]	adjusted p-value[2]
GO:0005509	Calcium ion binding	0.82	< 0.001
GO:0008307	Structural constituent of muscle	1.25	< 0.001
GO:0003779	Actin binding	0.79	< 0.001
GO:0003774	Motor activity	0.92	< 0.001
GO:0008061	Chitin binding	0.87	< 0.001
GO:0000146	Microfilament motor activity	1.18	0.006
GO:0008474	Palmitoyl-(protein) hydrolase activity	2.00	0.012
GO:0016290	Palmitoyl-CoA hydrolase activity	2.00	0.012
GO:0004099	Chitin deacetylase activity	1.31	0.037

[1]LOD is the Log_{10} of the GO attribute representation ratio (query list: entire gene entity list)

[2]The p-value is adjusted using 1000 null hypothesis simulations to test that the attribute single t-test p-value is significant (Adjusted p-value < 0.05).

Table 3.3 Gene expression measured by RT-qPCR in salmon lice from two strains differing in EMB susceptibility.

Accession No.	Annotation	Constitutive expression microarray experiment						Response to EMB exposure microarray experiment					
		Microarray		RT-qPCR		Pearson correlation		Microarray		RT-qPCR		Pearson correlation	
		p-value	Fold change (PT/S)	p-value	Fold change (PT/S)	r	p-value	p-value	Fold change (PT/S)	p-value	Fold change (PT/S)	r	p-value
ADD24187.1	Neuronal acetylcholine receptor subunit α3	7.59E-05	-3.21	0.000	-3.08	0.93	<0.0001	4.29E-03	-2.52	0.009	-2.64	0.89	0.0001
EFN73916.1	GABA receptor subunit alpha	NS	-1.19	0.015	-1.35	N/A	N/A	2.05E-04	-1.75	0.005	-1.56	0.89	0.0001
XP_003494528.1	Cytochrome p450 18a1	3.42E-08	3.34	0.000	3.14	0.95	<0.0001	N/A	N/A	NS	1.00	N/A	N/A
AAS13464.1	Cytochrome p450 15a1	8.93E-04	2.14	0.000	1.82	0.95	<0.0001	N/A	N/A	NS	1.09	N/A	N/A
NP_001136104.1	Carboxylesterase	1.13E-04	1.38	0.041	1.21	0.65	0.02	NS	1.12	NS	1.12	N/A	N/A
XP_797271.2	Maltase-glucoamylase	9.12E-05	-104.50	0.000	-194.85	0.99	<0.0001	7.10E-03	1.72	0.017	2.00	N/A	N/A

For selected genes, mRNA expression was measured in salmon lice from two strains differing in EMB susceptibility (see Figure 1 for information on strains), results compared to findings obtained in microarray analyses. Significance ($p < 0.05$) assessed by t-test (Welch) for microarray analysis and one-way ANOVA for RT-qPCR analysis. Fold changes ≥ 1.3 are underlined.

3.4 Discussion

Using transcriptional profiling in comparative studies of a drug-susceptible and an EMB-resistant salmon louse strain, this study demonstrated the reduced constitutive mRNA expression of subunits of certain ligand-gated ion channels (LGIC) in the EMB resistant strain, namely a GABA-gated chloride channel subunit (GABA-Cl, ~1.4-fold decreased) and a neuronal acetylcholine receptor subunit (nAChR α-3; ~2.8-fold decreased). The toxicity of EMB and other AVMs against ecdysozoan invertebrates is reported to be based mainly on their interaction with another class of LGICs, the GluCls (Dent *et al.*, 2000; Kane *et al.*, 2000; Bloomquist, 2003), although GABA-Cls are also believed to be pharmacological targets of AVMs (Feng *et al.*, 2002). While nAChRs are traditionally not considered to be implicated in the toxic action of AVMs in ecdysozoans, they can be allosterically modulated by IVM (Krause *et al.*, 1998). This study's finding that mRNA levels of GABA-Cl and nAChR subunits are decreased in EMB-resistant salmon lice suggests that these LGICs may represent potentially additional target sites for AVMs in sea lice. AVM compounds such as EMB may have a lower affinity to target sites on these alternative LGICs, leading to a response that is tolerable in *L. salmonis*. The chloride ion influx resulting from such lower binding affinity may therefore not be strong enough to elicit nervous impulse disruption in the parasite compared to that resulting from normal AVM receptor binding of natural LGIC targets. The present study only considered levels of mRNA. Molecular mechanisms underlying differential susceptibilities between the studied strains could also include post-transcriptional regulatory mechanisms, such as mRNA processing and degradation, translation and protein degradation (Vogel and Marcotte, 2012); however, investigation of these factors lay outside the scope of the present study. Additionally, should reduced *L. salmonis* EMB susceptibility result from coding region mutations of genes

responsible for target LGICs or detoxification enzymes that do not lead to gene expression changes, then these mechanisms will not be detected using a transcriptomic approach (Beech and Silvestre, 2010).

The experimental design used during this study included the measurement of constitutive differential gene expression between one drug susceptible and one EMB-resistant salmon louse strain. The experimental design thus includes only one *L. salmonis* strain for each EMB susceptibility phenotype, which is a significant limitation of the study, but reflects the current availability of laboratory strains of *L. salmonis* in our laboratory. Atlantic *L. salmonis* populations appear to have very little genetic variation (Glover *et al.*, 2011) which suggests that differential gene expression changes observed in strains S and PT should reflect field *L. salmonis* populations, although it is possible that a genetic bottleneck could have occurred when these strains were established (Kramer and Sarnelle, 2008) reducing genetic diversity of strains S and PT. Despite the limitation of an experimental design based on a restricted number of drug resistant and susceptible pests or parasites, studies using similar designs have succeeded in identifying molecular determinants of IVM resistance in mosquitoes (Bariami *et al.*, 2012) and the nematodes *Caenorhabditis elegans* (James and Davey, 2009) and *Teladorsagia circumcincta* (Dicker *et al*., 2011).

Comparison of transcriptomic profiles for strains S and PT identified reduced expression of many transcripts in strain PT compared to strain S which were enriched for functions such as calcium ion binding and chitin metabolism. The functions of these transcripts show no obvious association with EMB susceptibility but could be related to the process of cuticle formation. In terrestrial arthropods, one mechanism of drug resistance involves cuticle thickening in order to reduce the penetration of topically applied insecticides (Wood *et al.*, 2010; Lin *et al.*, 2012). Such a mechanism is less

likely to be involved in reduced EMB susceptibility of *L. salmonis* strain PT as normal EMB exposure in field situations is considered to be through food consumption. An alternative hypothesis is that disparity in the expression of genes involved in calcium binding and chitin metabolism reflects strain differences in growth rate and metabolism. Drug resistance mechanisms often include the reallocation of metabolic resources to detoxification mechanisms, which often has associated fitness costs (Kliot and Ghanim, 2012). It could be possible that strain PT salmon lice have reduced metabolic rates to compensate for the fitness costs associated with reduced drug susceptibility, resulting in the reduced mRNA levels of various transcripts associated with metabolic homeostasis that were found in the current study. Alternatively, the finding of differential expression between strains of genes with roles in calcium ion binding and chitin metabolism may suggest there was a problem with synchronisation of the cohorts of the strains used in the current study. Crustaceans undergo moulting cycles (ecdysis) throughout their lifecycle that involves physiological changes where the cuticle is replaced. This requires considerable gene expression changes before and after ecdysis to facilitate the resorption of old cuticle and generation of new cuticle (Terwilliger *et al.*, 2005; Stillman *et al.*, 2008; Seear *et al.*, 2010b). Adult male salmon lice that had completed the final moult were used in the current study to limit the amount of moulting related gene expression changes, although it is difficult to accurately determine when individual *L. salmonis* have completed their last moult. The adult male salmon lice used in this study will have recently developed into adults; therefore a considerable amount of the genes relating to cuticle turnover that show differential expression may be associated with post-moult growth and cuticle thickening that occurs as these adult salmon lice mature. These gene expression changes may have been avoided through sampling the lice at later stages of development at which point the adults should have

completed any post-moult maturation. It may be possible that random differences in salmon louse development may have contributed to the differences in gene expression profiles observed between strains S and PT. In order to mitigate potential random effects, differences in constitutive gene expression between strains S and PT were analysed for data generated from two experiments. The salmon louse sampling time, after removal from the salmonid host, differed slightly between these two experiments (2 h in constitutive expression microarray experiment, 3-5 h in 'response to EMB exposure' experiment). While previous bioassay data does suggest that this difference in sampling time would have a negligible influence on EMB susceptibility, analysis of the 'response to EMB exposure' experiment (Chapter 4) indicates that time may be a significant factor affecting gene expression. This discrepancy in sampling time may therefore account for some of the differences found between these two experiments when comparing microarray features that were differentially expressed across salmon louse strains (Figure 3.2). Similarly, as salmon lice were sampled in February and May for constitutive expression and EMB exposure microarray experiments respectively, the seasonal difference in sea water temperature may also have an influence on the differential gene expression observed between the experiments.

AVMs are used against parasitic and pest species of ecdysozoans including nematodes, insects and mites, and more recently crustaceans. While the molecular target sites of AVMs in crustaceans are unknown, GluCls are generally considered to be the main pharmacological targets of IVMs in nematodes and insects (Cully *et al.*, 1994; Cully *et al.*, 1996). Consistent with the role of GluCl as the main target of AVMs, IVM-resistant strains of invertebrates can show mutations changing the expression levels or the peptide sequence of channel subunits (Dent *et al.*, 2000; Kane *et al.*, 2000). A GluClα subunit has been cloned in *L. salmonis* (Tribble *et al.*, 2007b) and while

GluClα was represented amongst the microarray targets used for this study, no difference in mRNA expression was observed between salmon lice of the two studied strains.

In addition to GluCl, further LGICs are known to interact with AVMs. For instance, IVM modulates the activity of nematode GABA-Cl (Feng *et al.*, 2002), and can exert direct activating or potentiating effects on vertebrate glycine-gated chloride channels (Shan *et al.*, 2001). Moreover, AVMs can modulate the activity of cation-LGICs such as the α-7 nAChR (Krause *et al.*, 1998) and the ATP-gated $P2X_4$ receptors (Silberberg *et al.*, 2007). A number of observations involving drug-resistant insects and nematodes support the hypothesis that LGICs other than GluCl constitute further toxicologically relevant targets of AVMs in invertebrates. Cyclodiene-resistant fruit flies having a single amino acid mutation in a GABA-Cl showed a moderate degree of cross-resistance to IVM (Kane *et al.*, 2000). A null mutation in a histamine-gated chloride channel also conferred moderate IVM resistance in *Drosophila melanogaster* (Yusein *et al.*, 2008), and a novel dopamine-gated ion channel (HcGGR3) was significantly down-regulated in an AVM-selected strain of the nematode *Haemonchus contortus* (Rao *et al.*, 2009). The observation in this study that EMB-resistant salmon lice show decreased mRNA levels of nAChR and GABA-Cl is consistent with findings in the literature cited above, and suggests a role for nAChR and GABA-Cl as potential additional pharmacological targets of EMB in salmon lice. It is worth noting in this context that observed changes in nAChR expression could also relate to previous exposure of PT lice to compounds interfering with cholinergic neurotransmission such as the organophosphate (OP) anti-sea louse drug azamethiphos (Burridge *et al.*, 2010). However, decreases in nAChR expression are not among typical molecular responses associated with OP resistance in insects (ffrench-Constant *et al.*, 2007; Labbé *et al.*,

2007). While decreased expression of nAChR has been observed in nematodes resistant to imidazothiazoles and other drugs that directly target nAChR channels (Beech *et al.*, 2011), these are classes of compound that have not been used against sea lice.

Apart from modifications of the molecular targets, biocide resistance in pests and parasites can result from increased elimination of the chemical as a result of up-regulation of biotransformation enzymes and/or drug transporters. The superfamily of cytochrome P450s (CYPs) contains heme-thiolate proteins that function as monooxygenases, many of which are involved in drug metabolism (Nerbert and Gonzalez, 1987; Bernhardt, 1995). CYPs play important roles as chemical resistance genes in insects (ffrench-Constant *et al.*, 2007; Heckel, 2012), but their roles in the biochemical defence against toxicants in crustaceans are less well understood. The microarrays used in the present study included probes representing a number of target sequences that are annotated as CYPs (Constitutive microarray experiment: 18 probes; response to EMB exposure microarray experiment: 14 probes). These partial *L. salmonis* CYP sequences could not be unequivocally attributed to specific CYP families, as this would require establishing the overall degree of amino acid similarity to CYP family members (Nelson, 1998). However, based on BLASTx annotation (e-values of $\leq 10^{-7}$), most of these sequences could be provisionally allocated to CYP clans, which are higher-order groupings that combine phylogenetically related CYP families (Nelson, 1998) (Clan 2: 6 sequences; clan 3: 5 sequences; mitochondrial clan: 3 sequences). Two targets showing similarity to CYP15A1 and CYP18A1 (both clan 2) differed in mRNA expression in *L. salmonis* from the constitutive expression microarray experiment, but not the response to EMB exposure microarray experiment. Moreover, compared to the EMB susceptible strain a target showing similarity to CYP3A24 (clan 3) had lower expression levels in the EMB resistant strain, with a

moderate (1.48-fold) difference in transcript abundance found between the strains. In insects, CYP15A1 is involved in juvenile hormone synthesis (Helvig *et al.*, 2004), whereas CYP18A1 functions to inactivate ecdysteroids (Guittard *et al.*, 2011). Crustaceans possess homologues to both these clan 2 CYPs (Baldwin *et al.*, 2009), but little is known of their function. In the green shore crab (*Carcinus maenas*) expression levels of two CYPs from clan 2 were affected by both the moulting cycle and previous exposure to xenobiotics (Dam *et al.*, 2008). The differences in CYP mRNA levels found in this study were relatively small and/or variable between experiments and therefore do not provide clear evidence for an involvement of CYPs in the differential EMB susceptibility found in the salmon louse strains that were studied.

Carboxylesterases are another class of enzymes that can confer insecticide resistance (ffrench-Constant *et al.*, 2007). In this study, the expression of one carboxylesterase was moderately enhanced in EMB-resistant salmon lice in the constitutive expression microarray experiment, but no significant differences in expression were observed between strains in the response to EMB exposure microarray experiment. Accordingly, the data provides no evidence for a role of carboxylesterase in EMB resistance of salmon lice.

ABC proteins are a family of membrane bound transporters mediating the transport of a diverse array of substrates across biological membranes (Jones *et al.*, 2009). Certain ABC proteins are drug efflux transporters located in the cell membrane, and have roles in the biochemical defence against toxicants (Leslie *et al.*, 2005). The ABC transporter P-glycoprotein transports IVM (Bain and LeBlanc, 1996) and has relevance as a biochemical factor limiting the drug's toxicity in mice and nematodes (Schinkel *et al.*, 1994; James and Davey, 2009). It has been suggested that P-glycoprotein could be implicated in the resistance of pests and parasites to AVMs

(Lanning *et al.*, 1996b; Xu *et al.*, 1998; Ardelli *et al.*, 2006), and a salmon louse homologue of P-glycoprotein called SL-PGY1 has been cloned (Heumann *et al.*, 2012). In the present study, mRNA expression of SL-PGY1 did not differ between S and PT strains (data not shown), confirming similar data previously reported for these strains (Heumann *et al.*, 2012).

Conclusions

The profiling of constitutive transcription in drug susceptible and EMB resistant laboratory strain of salmon lice revealed reduced mRNA expression of a number of LGIC subunits in the EMB resistant lice. GluCl, considered to be the major target site of AVM drugs in ecdysozoan invertebrates, displayed similar mRNA expression levels in EMB-resistant and reference strains. In contrast, subunits of GABA-Cl and nAChR showed decreased mRNA abundances in the EMB resistant compared to the reference strain. While GABA-Cl is considered a secondary target of AVMs in invertebrates, nAChR is not traditionally considered a target site for AVMs, even though it has been shown to interact with AVMs in vertebrates. It is possible that nAChR and GABA-Cl represent additional EMB target sites in salmon lice, and that the down-regulation of these channel subunits in this EMB-resistant strain could be related to the resistance phenotype. In the present study no changes were seen in the expression levels of biotransformation enzymes and drug transporters, both of which classes have been suggested to contribute to AVM resistance in other species. Further studies are needed to investigate potential relationships between the transcriptional changes observed and the susceptibility phenotype.

Chapter 4
Transcriptomic responses to emamectin benzoate exposure.

4.1 Introduction

A variety of chemical compounds have been developed for the control of undesired invertebrate species from the nematoda and arthropoda phyla (Yu, 2008). The emergence of resistance to antiparasitic agents has become a major issue for the control of ecdysozoans with medicinal, veterinary and agricultural importance. As the antiparasitic compounds currently available only interact with a limited number of molecular targets, there is a high likelihood of resistance development unless treatment frequency is not closely controlled (ffrench-Constant *et al.*, 2004; Labbé *et al.*, 2007; Li *et al.*, 2007; Bass and Field, 2011; Heckel, 2012). Accordingly, exposure of successive parasite generations to a toxin can lead to the selection of rare genetic mutations that confer improved fitness upon exposure. In parasite species with short generation times and extensive offspring production, the development of resistance is enhanced under conditions of persistent chemical selection (Wolstenholme *et al.*, 2004).

AVM resistant in insects can involve the enhanced activity of detoxification enzymes such as Cytochrome P450 monooxygenase (CYP) (Schmidt *et al.*, 2010; Liu *et al.*, 2011; Tao *et al.*, 2012) and carboxylesterase (Raymond *et al.*, 1998; Hawkes and Hemingway, 2002; Cui *et al.* 2006) and/or increased activity of ATP Binding Cassette (ABC) proteins (James and Davey, 2009), which include membrane bound transporters that move structurally and functionally different compounds across membranes. Drug resistance mechanisms in pests and parasites that are based on increased activity of detoxification pathways can involve constitutive changes in gene expression, and/or implicate gene regulation in response to exposure to the drug (Li *et al.*, 2007). It has been demonstrated that CYP gene expression is induced by pyrethroid insecticide exposure of the mosquito *Culex quinquefasciatus* (Komagata *et al.*, 2010; Liu *et al.*, 2011) and *Aedes aegypti* (Poupardin *et al.*, 2008) in addition to constitutive

overexpression of CYP genes, suggesting that both mechanisms may be involved in insecticide detoxification. In the phytophagous insect *Helicoverpa zea* plant allelochemical exposure can induce CYP gene expression (Li *et al.*, 2002), with these genes being capable of metabolising both plant allelochemicals and insecticides (Li *et al*., 2004; Sasabe *et al*., 2004). There is also evidence that exposure of the cotton bollworm *Helicoverpa armigera* to the phytotoxin gossypol and pyrethroid insecticide deltamethrin induces similar CYP gene expression profiles (Tao *et al*., 2012). Glutathione-s-transferase and esterase detoxification enzymes have also been found to be involved in the tolerance of insect pests to plant allelochemicals, but are also often implicated in cases of insecticide resistance (Li *et al*., 2007).

In the nematodes *Caenorhabditis elegans* (James and Davey, 2009) and *Haemonchus contortus* (Prichard and Roulet, 2007) exposure to ivermectin (IVM) has been shown to induce the overexpression of P-glycoprotein (P-gp) ABC transporters. It has also been reported that exposure of *Lepeophtheirus salmonis* to emamectin benzoate (EMB) can induce increased expression of P-gps (Tribble *et al*., 2007a; Igboeli *et al*., 2012; Igboeli *et al*., 2013). These studies would suggest that P-gp may be an important mechanism in resistance of *L. salmonis* to avermectin compounds, although other studies were unable to confirm this (Heumann *et al*., 2012) indicating that other mechanisms may be involved in reduced avermectin susceptibility of the salmon louse.

Chapter 3 of this thesis analysed constitutive differences in the transcriptomic profiles between one moderate EMB-resistant and one drug susceptible strain of *L. salmonis* in order to understand the differences in EMB between these strains. The main objective of this thesis chapter is to investigate transcriptomic responses to EMB treatment in the same *L. salmonis* strains. A global transcriptomic approach similar to

that used in Chapter 3 was followed in the current study to measure mRNA responses of drug susceptible and moderately (~7-fold) EMB resistant *L. salmonis* laboratory strains to short-term EMB exposure. It is hoped that the results of this study will help unravel the complex interactions that may be responsible for reduced EMB susceptibility in *L. salmonis*.

4.2 Materials and methods

4.2.1 Salmon louse strains

Two laboratory-maintained *L. salmonis* strains (S and PT) differing in susceptibility to EMB (Heumann *et al.*, 2012), were used in this study. For more information on these strains please see the materials and methods section in Chapter 2 of this thesis.

4.2.2 Salmon louse exposure experiments

Adult male salmon lice were collected from anaesthetised host fish as described in Chapter 2 and allowed to recover for 2 hours in aerated filtered seawater at ambient sea temperature. On the basis of results from salmon louse acute toxicity assays described in Chapter 3, a concentration of 200 µg L^{-1} EMB was selected for short-term exposure experiments (1 and 3 hour) performed to investigate EMB effects on transcript expression profiles. An EMB concentration of 200 µg L^{-1} results in > 95 % immotility in S lice after 24 hours but has no apparent effects in PT lice (See Figure 3.1, Chapter 3). In addition, previous studies investigating the time course of EMB action revealed that the first toxic responses in the S strain became apparent after 6 hours of exposure (Data not shown). In view of these findings it was argued that if EMB tolerance in the PT strain required transcriptional regulation in response to drug exposure, then these should be detectable at early time points, and 1 and 3 hour exposure times were subsequently selected for this study. In addition to EMB treatments, the experiment

comprised seawater and solvent (0.01 % (v/v) PEG300) controls (Figure 4.1). For each combination of strain, exposure period and treatment, three pooled samples consisting of four salmon lice each were collected and preserved in an RNA stabilisation solution (4.54 M ammonium sulphate, 25 mM trisodium citrate, 20 mM EDTA, pH 5.4) prior to storage at -70 °C. None of the treatments had effects on louse motility. At the end of the experiment (3 hours), water samples were taken and sent to a commercial laboratory (Eclipse Scientific, Chatteris, UK) for EMB residue analysis (liquid chromatography with detection by MSMS). The measured EMB concentration in the nominal 200 µg L^{-1} EMB treatment was 99.5 ± 5.2 µg L^{-1} EMB. This depletion of solubilised active ingredient may be attributed to EMB adsorption to the glass containers used for exposure assays (Helgesen and Horsberg, 2013b).

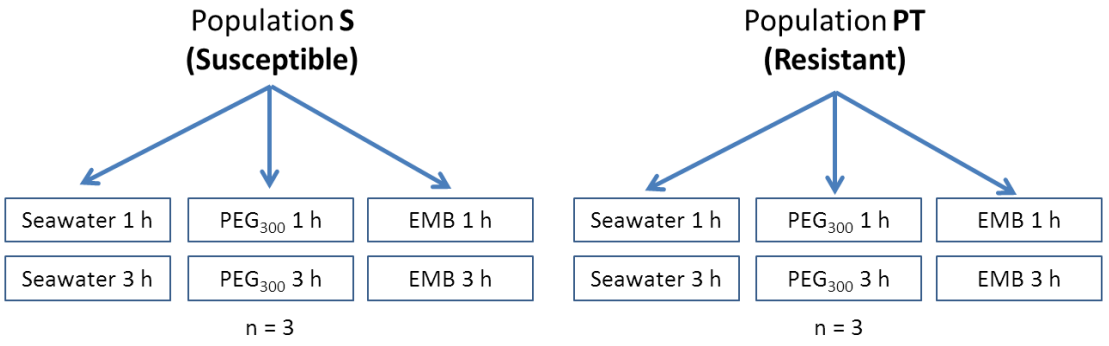


Figure 4.1 Design of microarray experiment.

Microarray analysis was used to measure transcriptomic profiles in drug-susceptible (S) or EMB resistant salmon louse strains (PT). Salmon lice were removed from hosts and then subjected to exposure of seawater containing 200 µg L^{-1} of EMB for 1 or 3 hours before sampling. Control treatments included exposures to seawater, or seawater containing the carrier PEG_{300}, which was used to solubilise EMB. The number of pools of four salmon lice sampled for each combination of treatment and strain is given as n.

4.2.3 RNA extraction and purification

In microarray and RT-qPCR experiments, samples comprised pools of four adult male salmon lice. Total RNA was extracted and purified from these samples, then assessed for purity and quantified, as described in detail in Chapter 2 of this thesis.

4.2.4 Microarray analyses

The protocols used in this chapter for generating amplified RNA (aRNA), test sample and common reference pool labelling with mono-reactive dyes (cyanine 3 (Cy3) and Cy5 respectively), and microarray hybridisation and washing are described in detail in Chapter 3 of this thesis. Similarly, microarray image capture and processing and GeneSpring analysis are also described in Chapter 3. Functional annotation of the genes found to be differentially expressed upon EMB exposure of *L. salmonis* was performed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) as detailed in Chapter 2 of this thesis. Details of these microarray experiments have been submitted to ArrayExpress and assigned accession number E-MTAB-1478. The recording of the microarray experimental metadata complies with Minimum Information About a Microarray Experiment (MIAME) guidelines (Brazma *et al.*, 2001).

4.2.5 RT-qPCR

To validate gene expression results from microarray experiments, the abundance of selected transcripts found to be differentially expressed in microarray analyses was determined by reverse transcription quantitative PCR (RT-qPCR). Three targets that showed stable expression levels in microarray hybridisations were selected as reference genes. (Hypoxanthine-guanine phosphoribosyltransferase (HGPRT), Required for meiotic nuclear division 5 (RMD-5) homolog and Elongation factor 1α). For each target sequence, primers were designed with a melting temperature (Tm) of ~60 °C using Primer 3 software (Appendix 6 and 8). Total RNA samples previously used in

microarray analyses were reverse transcribed and analysed in RT-qPCR reactions as detailed in Chapter 3 of this thesis. Similarly, relative gene expression ratios were established using the ΔΔCt method as detailed in Chapter 3.

4.2.6 Statistical analysis

The analysis of EMB induced differential gene expression employed three-way ANOVA to compare the effects of the factors salmon louse strain, exposure time and treatment on transcript expression. Multiple testing corrections were not applied to any statistical analysis of this gene expression study for the reasons detailed in Chapter 3 of this thesis. Hierarchical clustering of entities and conditions using normalised intensity values was performed using the GeneSpring GX version 12 software (Agilent Technologies, UK) and employed Euclidean similarity measure and Wards linkage rule. Principal component analysis (PCA) of experimental conditions using four components was also performed using the GeneSpring software. Network analysis of the gene expression data was performed using the BioLayout Express^{3D} application (Theocharidis *et al.*, 2009). A network graph was constructed using the Pearson correlation coefficient (threshold of 0.94) to determine similarities between expression profiles, which were then arranged into groups of features with similar profiles using the Markov clustering algorithm (MCL) with the default inflation setting (2.2) for optimal clustering. Gene enrichment analysis was performed on lists of features chosen based on differential gene expression patterns using default settings of the FuncAssociate 2.0 web application (Berriz *et al.*, 2009). Gene enrichment was calculated according to the significance ($p < 0.05$) of the association between the list of features and the GO attributes represented on the microarray. Relative expression ratios from RT-qPCR experiments were tested for normality and equal variance and log transformed to allow assumptions to be satisfied before being subjected to one way

ANOVA using MiniTab 16.1 software (MiniTab Inc., UK). The significance level was set at 0.05 in all tests.

4.3 Results

4.3.1 Effects of short-term exposure to EMB on transcript profiles in salmon lice

Three-way ANOVA of the microarray expression data from the EMB response microarray experiment showed that of the total number of features that passed quality filtering (n = 10,804), a large proportion (55 %) was affected by the factor strain. In contrast, the factor treatment had a comparatively small influence on gene expression (Table 4.1).

Table 4.1 Numbers of differentially expressed features identified by microarray analysis.

Experimental factor	Number of features
Strain	5940 (55 %)
Treatment	369 (3 %)
Time	968 (9 %)
Strain x Treatment	406 (4 %)
Strain x Time	950 (9 %)
Time x Treatment	1309 (12 %)
Strain x Treatment x Time	1701 (16 %)

Significance (p-value < 0.05) assessed by three-way ANOVA. Also indicated is the percentage of the total number of features (10,804) used in the analysis.

Following hierarchical clustering of the normalised expression values for this list of features, the group of samples from strain S clustered together distinctly from those of the PT strain (Figure 4.2). Relatively little gene expression profile variation was observed among samples from strain PT regardless of exposure. Within strain S, the transcriptomic profiles of salmon lice from strain S that were exposed to seawater for 3 hours (S,SW,3H) and EMB for 1 hour (S,EMB,1H) clustered separately from the other

treatment groups within this strain (Figure 4.2). Principal component analysis (PCA) identified two main principal components explaining 51.2 % and 10.3 % of the variation, respectively. The results from PCA confirmed the findings from the hierarchical clustering, with the different groups of PT lice grouping together in the PCA scatter plot, whereas within strain S the group exposed to EMB for one hour were positioned in the PCA scatter plot relatively far away from the other groups (Figure 4.3).

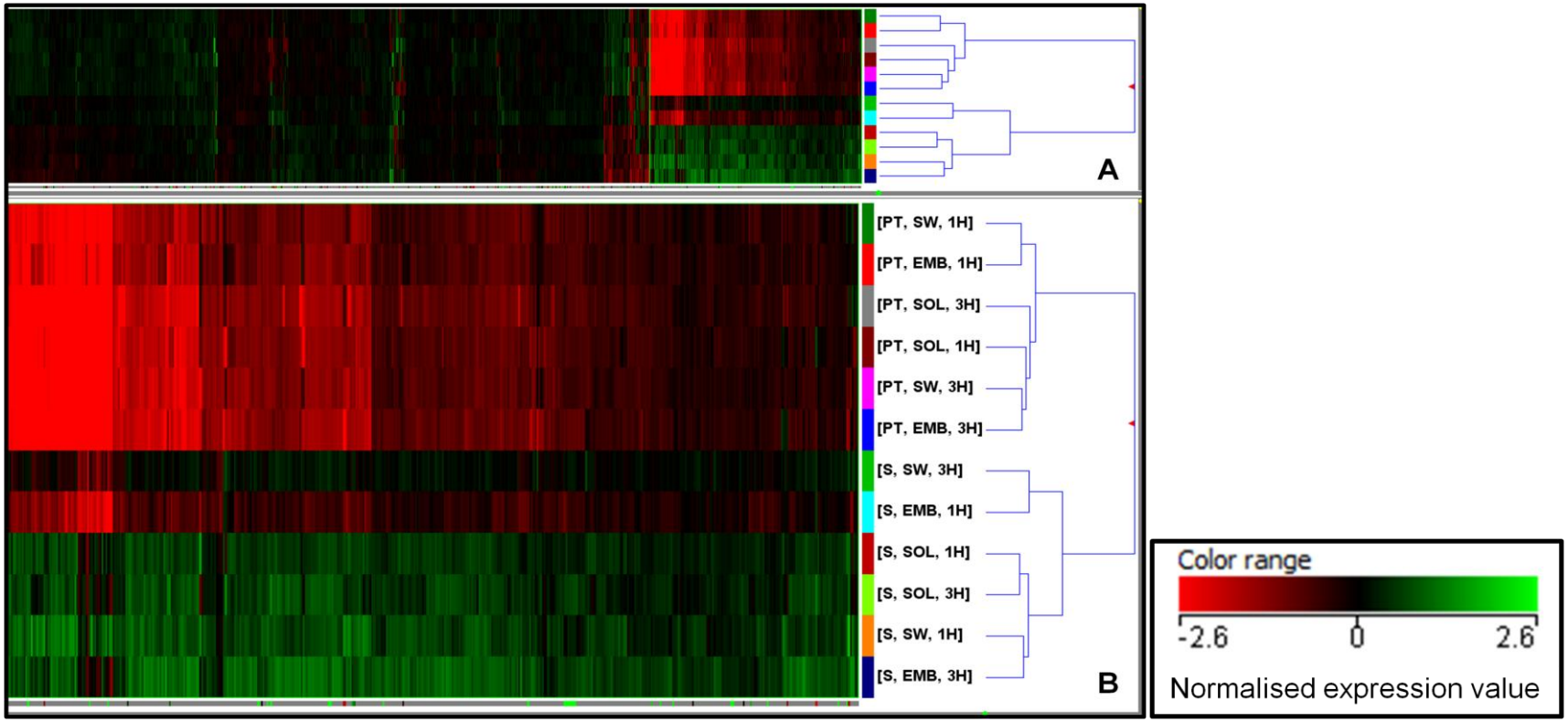


Figure 4.2 Hierarchical clustering of features significantly affected by EMB exposure.

A. Hierarchical clustering of the 2,020 microarray features displaying significant differential expression in response to EMB exposure or through the interaction of EMB treatment with the other factors analysed. Hierarchical clustering was performed on both conditions and entities.

B. Enlarged view of the hierarchical cluster map showing treatment group clustering.

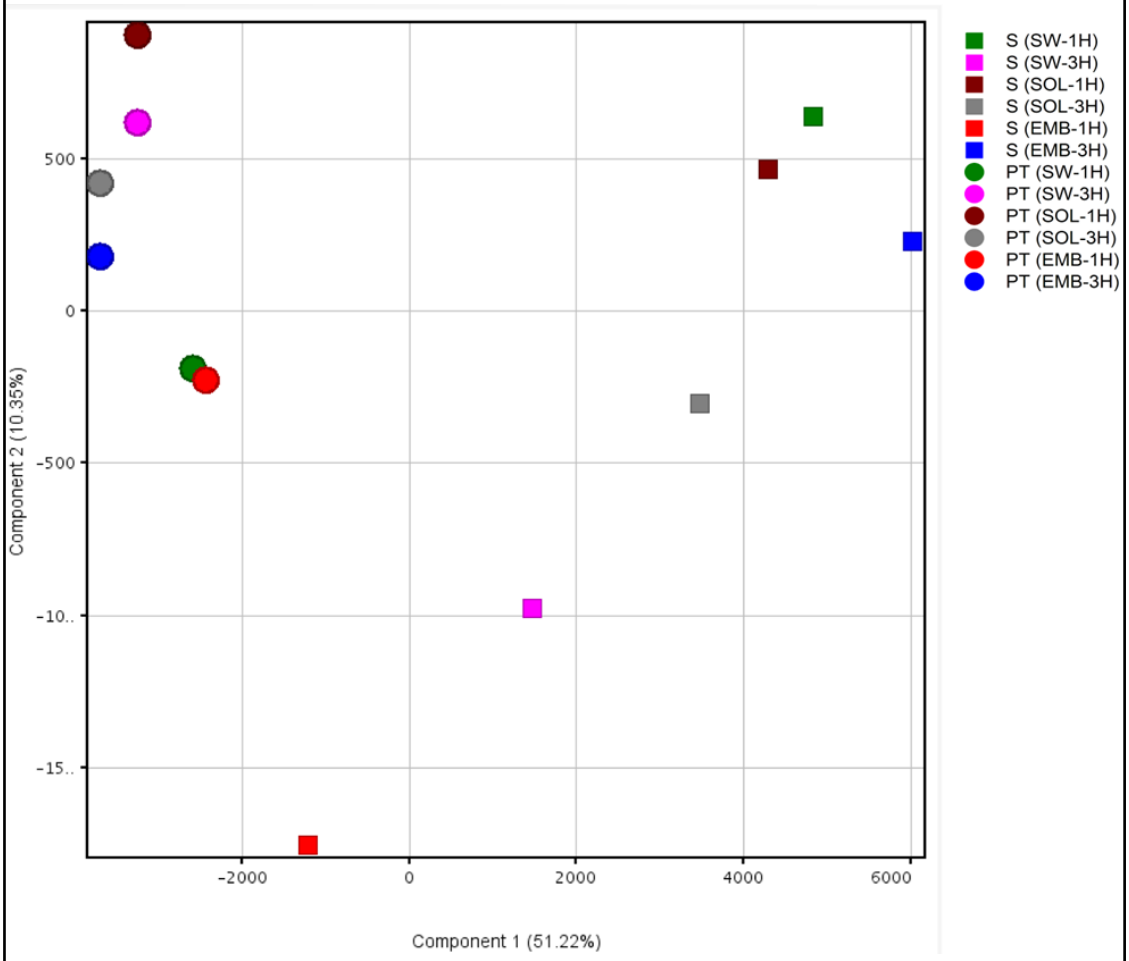


Figure 4.3 Principal component analysis (PCA) of features significantly affected by EMB exposure.

PCA of the 2,020 microarray features displaying significant differential expression in response to EMB exposure or through the interaction of EMB treatment with the other factors analysed.

To confirm these microarray results, transcript abundances were determined by RT-qPCR for six selected genes. Genes were selected so as to include a number of qualitatively different expression profiles, detectable fold-changes and selected candidate genes (γ-aminobutyric acid gated chloride channel (GABA-Cl) and neuronal acetylcholine receptor α3 (AChR-α3) subunits) (Figure 4.4). A high degree of correlation was observed between expression values measured by both methods (Pearson correlation coefficients (r) of 0.71 to 0.99; $p < 0.0001$) (Table 4.2). To further investigate the effects of EMB exposure, a list of those features that were significantly affected by EMB treatment or for which significant interactions between treatment and other factors were observed (treatment × strain; treatment × time; treatment × strain × time) was compiled. This list comprised a total of 2,020 features, of which at least 35 % of those which could be matched to KEGG hits by BLASTx, were involved in metabolism (Figure 4.5).

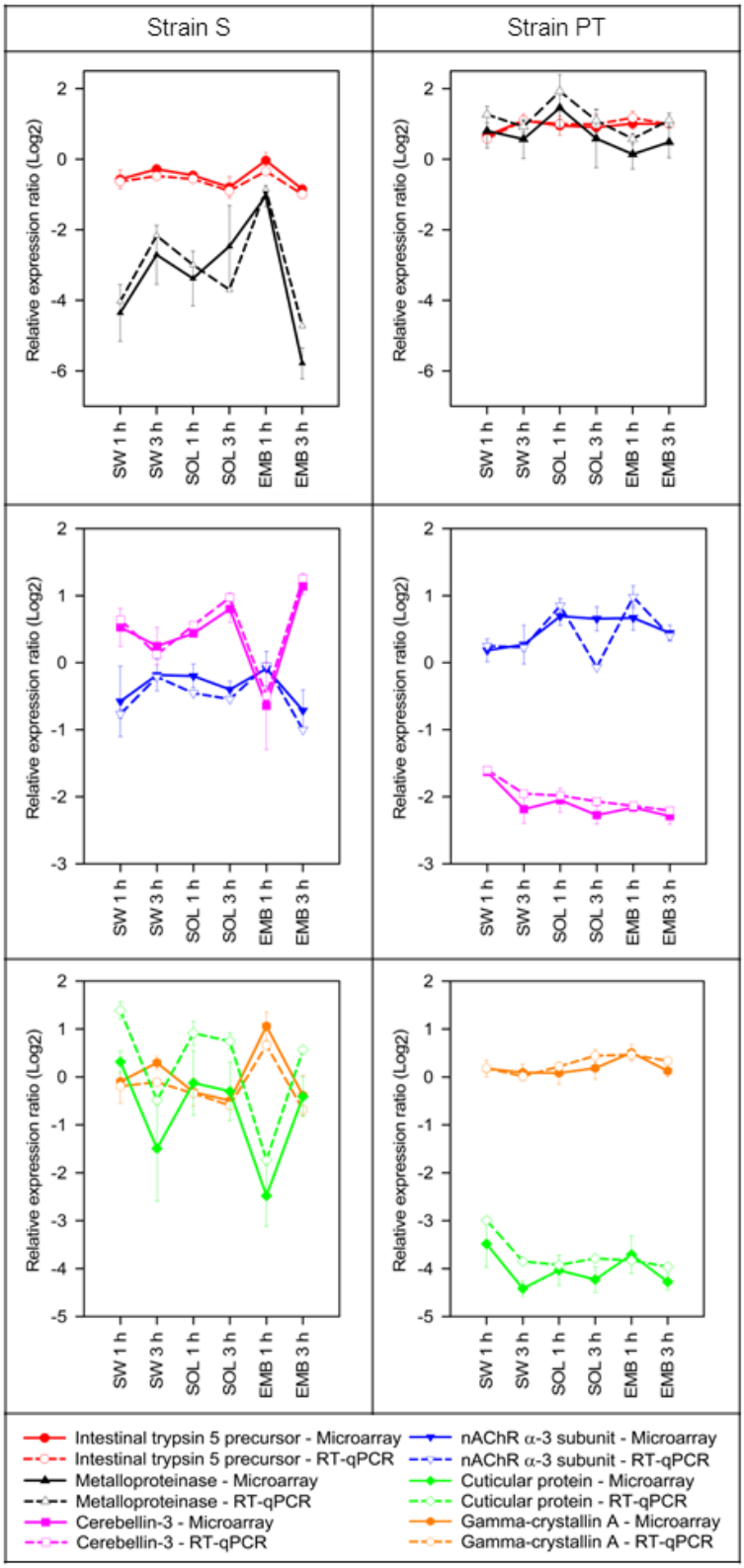


Figure 4.4 Gene expression responses at early time points of exposure to EMB.

Relative expression ratios (RER) measured by microarray and RT-qPCR analysis of S and PT salmon louse strains (graphs displayed side by side) after 1 and 3 hours exposure to 200 µg L^{-1} EMB, seawater (SW), or the solvent PEG_{300} (SOL). Data are Log_2 RER ± SE (n = 3).

Table 4.2 Correlation of relative expression ratios measured using microarray and RT-qPCR.

Accession No.	Annotation	r	p-value
NP_001136346.1	Cuticular protein	0.99	<0.0001
BAG74353.1	Metalloproteinase	0.99	<0.0001
ADD24462.1	Cerebellin-3	0.96	<0.0001
AAS91796.1	Intestinal trypsin 5 precursor	0.94	<0.0001
ADD38289.1	Gamma-crystallin A	0.77	<0.0001
ADD38711.1	Neuronal acetylcholine receptor subunit α3	0.71	<0.0001

Pearson correlation (r) of relative expression ratios (RER) calculated using *L. salmonis* 15K oligo microarray and RT-qPCR analysis. Correlation was calculated using 36 test samples. (Significance $p < 0.05$).

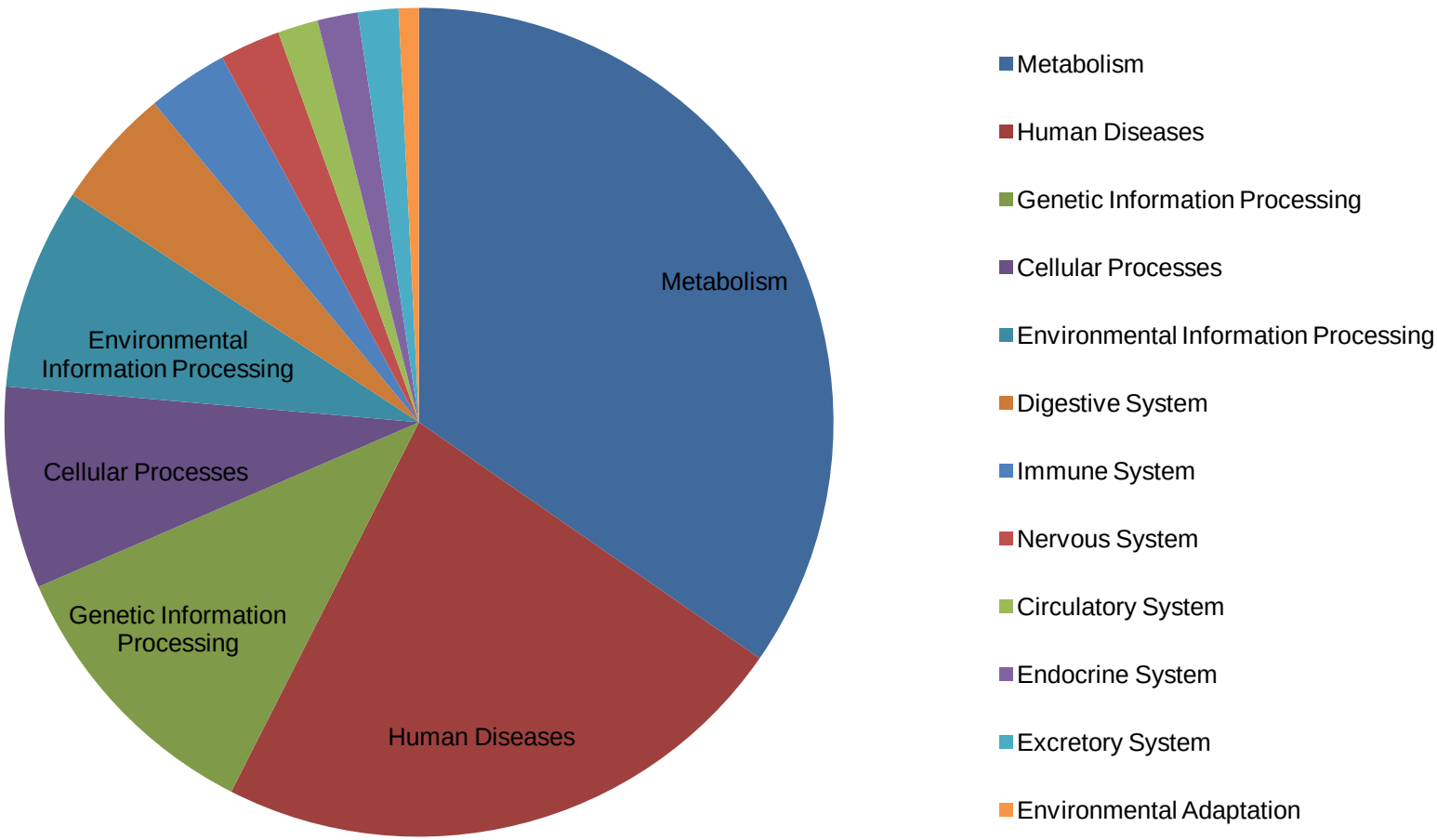


Figure 4.5 KEGG functional classification of genes differentially expressed on exposure of salmon lice to EMB.

Transcript abundance profiles for the 2,020 features were further subjected to network clustering using the BioLayout Express^{3D} application (Theocharidis *et al.*, 2009). This resulted in the resolution of 59 clusters with a minimum cluster size of four features. The two main clusters 1 and 2 contained 418 and 62 features, respectively, that showed fold changes > 1.3 across all conditions (Figure 4.6 A & B). Within the two clusters, expression profiles were characterised by pronounced responses in S lice following 1 h of EMB exposure (down-regulation in cluster 1, up-regulation in cluster 2), and a full or partial return to basal expression levels after 3h of EMB exposure. Moreover, for genes in both clusters, few if any responses to EMB exposure were observed in the PT strain.

The most significantly differentially regulated genes from cluster 1, and the responses to EMB exposure in both salmon louse strains are summarised in Additional file 4.1. An analogous selection of genes from cluster 2 is provided in Additional file 4.2. Genes in cluster one include the GABA-Cl and nAChR α-3 subunits which are potential targets for EMB, with nAChR α-3 also having been identified as constitutively differentially expressed between salmon louse strains. Genes in cluster two included a glutathione-S-transferase isoform and a nAChR α-3 precursor. Enrichment analysis of the features in cluster one is detailed in Table 4.3, showing significant over representation of twelve and under representation of four GO attributes. Chitin binding, calcium ion binding and hydrolase activity were the most significantly over-represented attributes and nucleic acid binding was identified as the most significant under represented attribute. KEGG functional analysis also shows a high representation of cytoskeleton proteins in cluster 1 (Figure 4.7), whereas proteins involved in protein digestion and absorption were the largest group identified in cluster 2 (Figure 4.8).

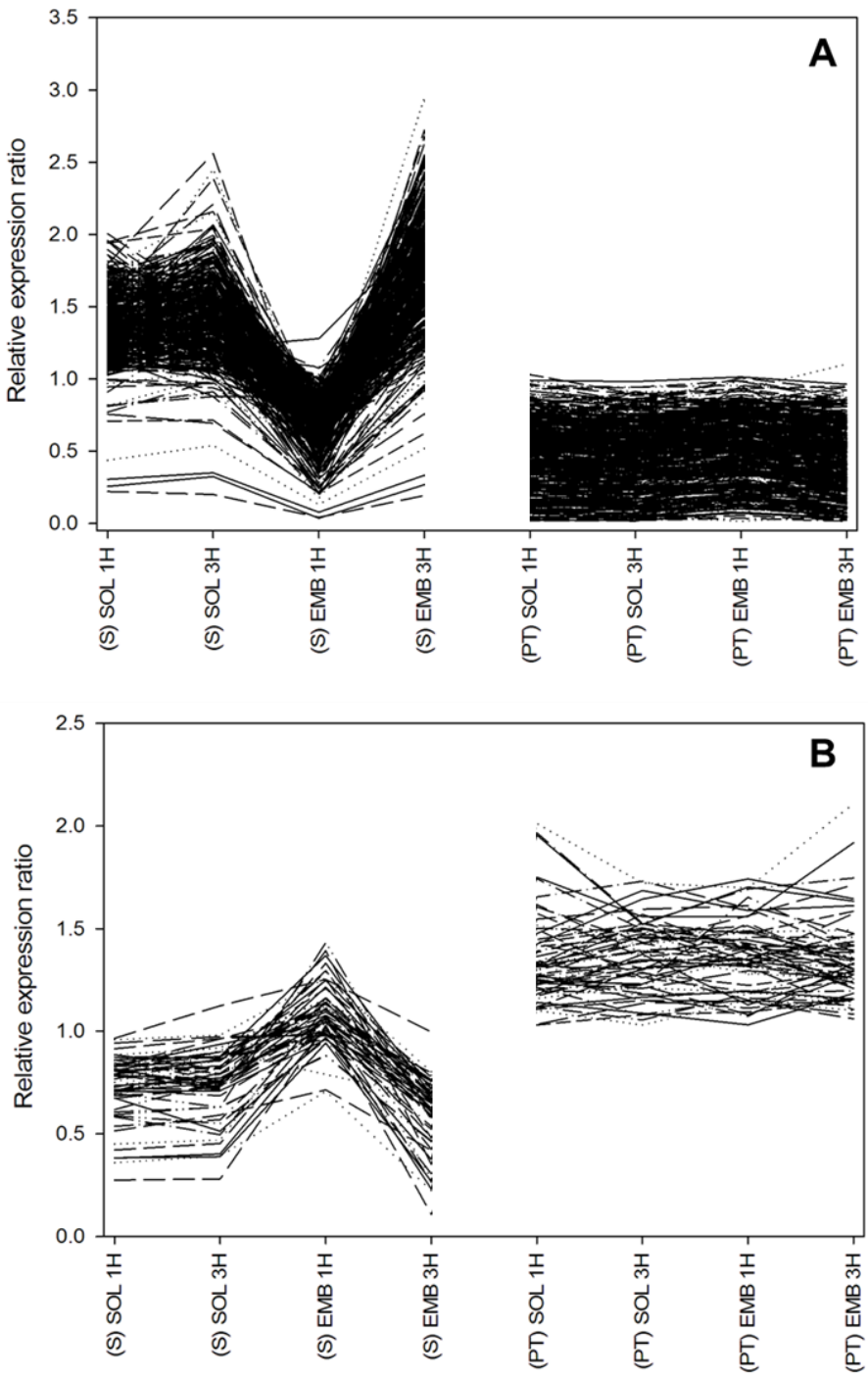


Figure 4.6 Relative expression profiles for the features in network clusters one (A) and two (B).

A total of 418 (cluster 1) and 62 (Cluster 2) features (fold change ≥ 1.3 in S strain) were clustered using network analysis. The similarity of expression profiles were measured using the Pearson correlation coefficient and clustered using the Markov clustering algorithm (MCL).

Table 4.3 Enrichment of GO classes among features of network cluster 1.

GO Attribute ID	GO Attribute Name	LOD	Adjusted p-value
GO:0008061	Chitin binding	0.95	< 0.001
GO:0005509	Calcium ion binding	0.64	< 0.001
GO:0016810	Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	1.03	< 0.001
GO:0042302	Structural constituent of cuticle	0.87	0.001
GO:0005201	Extracellular matrix structural constituent	1.08	0.001
GO:0050998	Nitric-oxide synthase binding	1.40	0.002
GO:0019894	Kinesin binding	1.16	0.006
GO:0005471	ATP:ADP antiporter activity	2.32	0.016
GO:0048407	Platelet-derived growth factor binding	2.32	0.016
GO:0004099	Chitin deacetylase activity	1.28	0.019
GO:0004017	Adenylate kinase activity	1.07	0.02
GO:0019205	Nucleobase, nucleoside, nucleotide kinase activity	1.04	0.022
GO Attribute ID	GO Attribute Name	LOD	Adjusted p-value
GO:0003676	Nucleic acid binding	-1.13	< 0.001
GO:0003677	DNA binding	-0.82	0.002
GO:0003723	RNA binding	-0.97	0.005
GO:0005515	Protein binding	-0.41	0.007

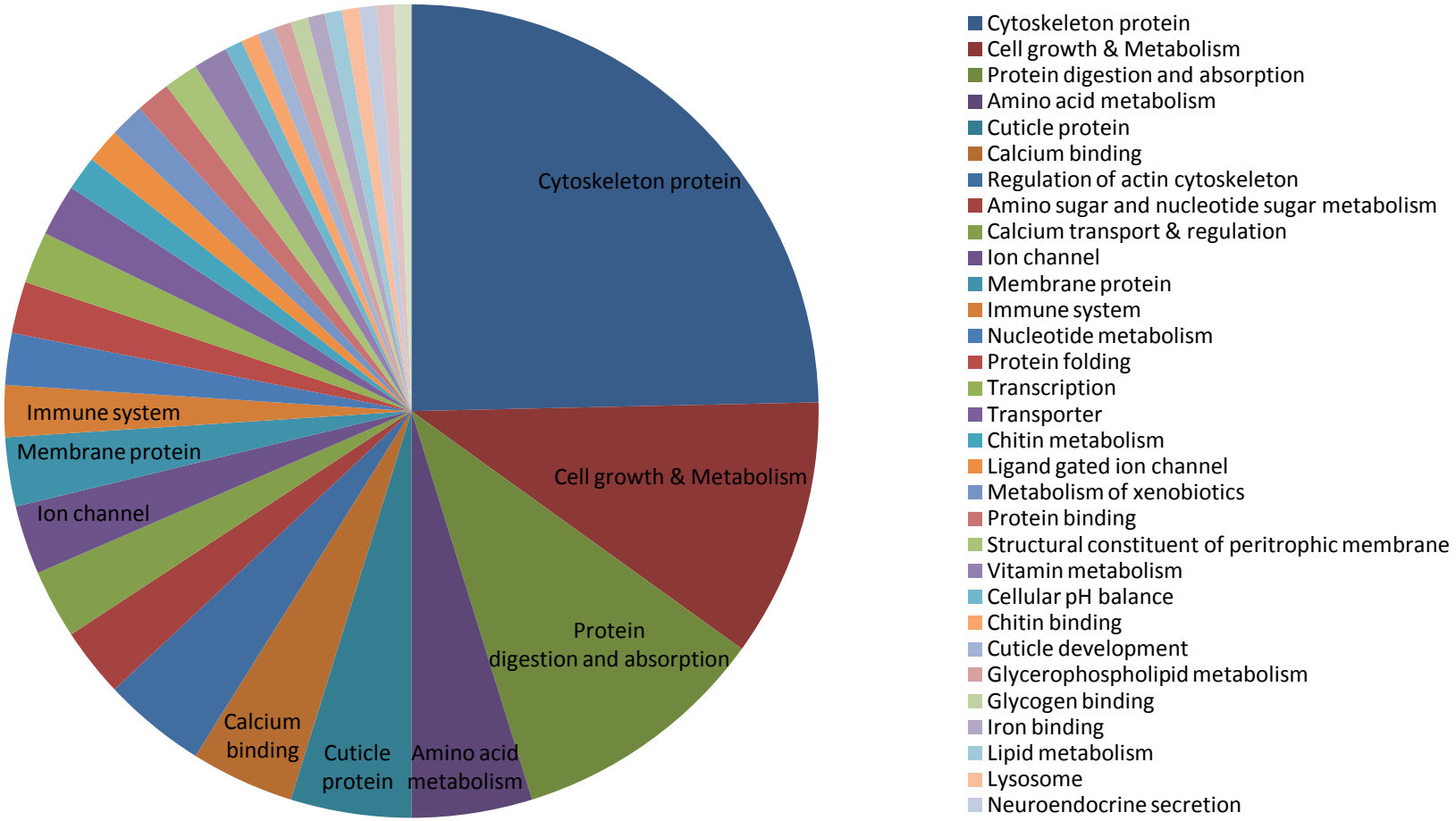


Figure 4.7 KEGG functional classification of genes of network cluster 1.

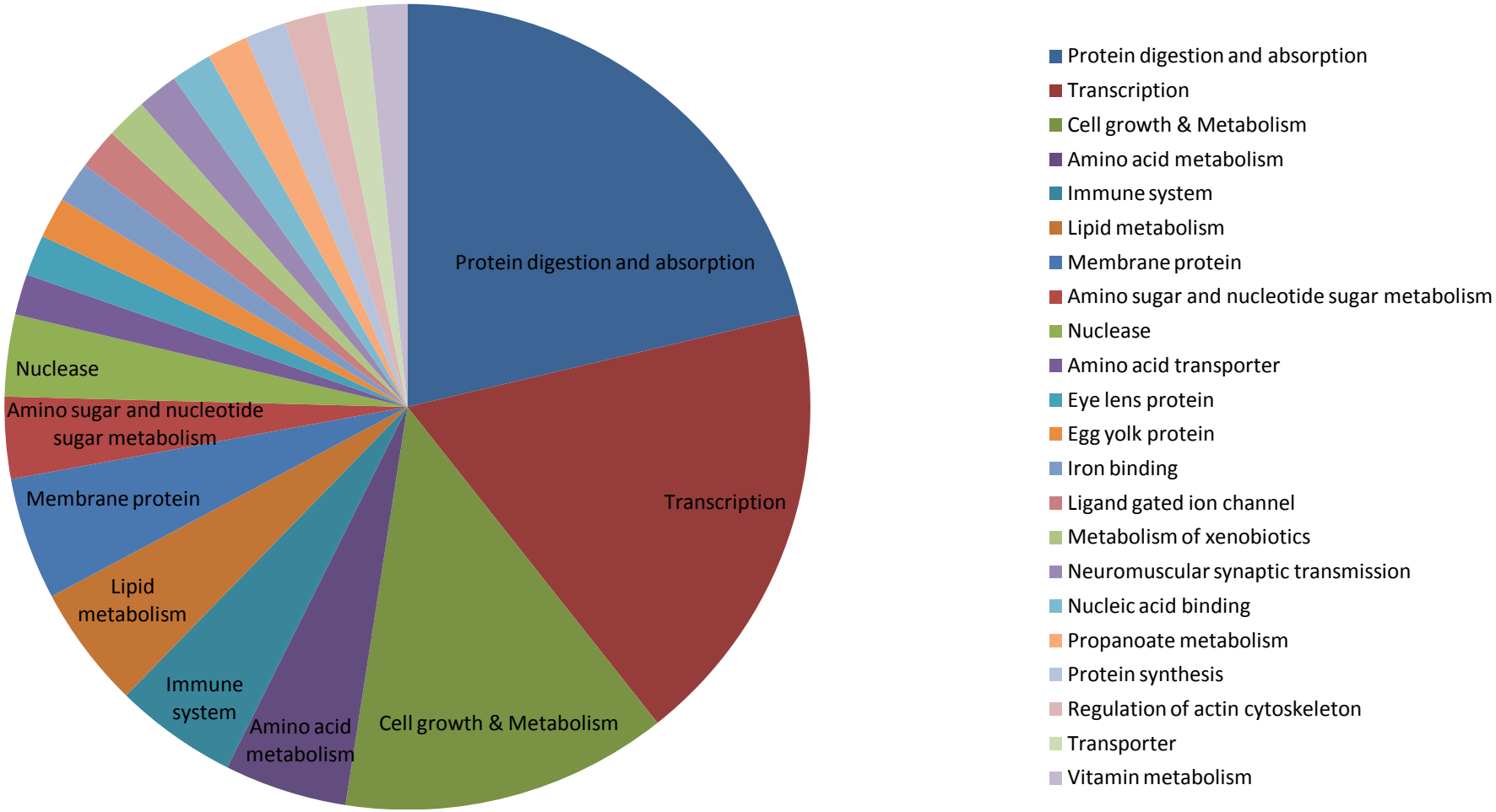


Figure 4.8 KEGG functional classification of genes of network cluster 2.

4.4 Discussion

In the present study, transcriptomic responses of salmon louse strains S and PT to short term (1-3 h) aqueous exposures to 200 µg L^{-1} of EMB were investigated. Longer exposure (24 h) to this drug concentration does not induce behavioural signs of toxicity in the moderately EMB-resistant PT strain, but results in close to 100 % immotility of *L. salmonis* from the drug-susceptible S strain. Following short term exposures, a number of transcriptional responses to the treatment were observed in lice from strain S, but few transcriptional changes were found in lice from the PT strain. While the possibility exists that EMB exposure might provoke more pronounced transcriptomic responses in PT lice at later time points, differential behavioural responses to toxicity of EMB between the louse strains is observable as early as 5 hours post-exposure (data not shown). This suggests that, at this time point at least, some of the mechanisms responsible for EMB resistance will be expressed in strain PT. Accordingly, should EMB resistance of this strain involve transcriptional changes that are expressed constitutively or in response to drug exposure; such changes were expected to be detectable at the short term exposure endpoints selected. Chapter 3 of this thesis investigated constitutive changes in *L. salmonis* strain PT compared to strain S and therefore does not provide evidence of a role for classical resistance mechanisms that require transcriptional regulation in response to EMB exposure which were investigated during the current study.

At the time of this study, the PT strain had been maintained in the laboratory for ~3 years without EMB selection, and over this time period had displayed moderate but stable (~7-fold) EMB resistance. The strategy employed, which seeks to identify global transcriptomic responses only considers changes in mRNA levels. As noted in Chapter 3, the EMB induced molecular mechanisms that may be responsible for reduced

susceptibility of PT salmon lice could involve changes in gene expression due to post-transcriptional regulatory mechanisms, such as mRNA processing and degradation, translation and protein degradation (Ménez *et al.*, 2012; Vogel and Marcotte, 2012). Additionally, molecular mechanisms may have evolved in PT salmon lice to enable a moderate (~7-fold) reduction in EMB susceptibility, constituting subtle fold-changes in numerous different proteins involved in detoxification and/or drug transport that could not be measured within the detection limits of microarray-based transcriptomic analysis. The toxicity assay used in the present study to assess salmon lice EMB susceptibility, involves the observation of reduced motility and abnormal swimming behaviour, during aqueous EMB exposure, as an indication of EMB toxicity (Sevadtdal *et al.*, 2005; SEARCH, 2006). The standard *L. salmonis* EMB exposure route on Atlantic salmon production sites is considered to be food-borne ingestion over a seven day period. Accordingly, the possibility cannot be excluded that EMB resistant mechanisms may exist that are expressed only following oral EMB exposure. However, strain PT fails to show toxic responses to both waterborne (see Chapter 3) and on-fish EMB exposure (Dr. W. Roy, personal communication), suggesting that major mechanisms of resistance that play a role in field situations are also expressed during experimental aqueous *L. salmonis* exposure.

In the drug-susceptible strain S, a complex array of transcriptional responses to EMB was observed, this being unsurprising given the recognised toxic effects of 24 hour exposure to EMB on this strain. Early transcriptional responses in strain S are therefore likely to comprise both adaptive and general stress responses, as well as transcriptional changes reflecting the toxic action of the drug. Salmon lice from strain S showed a modest reduction in gene expression after 1 hour of EMB exposure and very little response after 3 hours of exposure. These results would suggest that the EMB

concentration or exposure time at which salmon lice from strain S exhibit the greatest transcriptomic response is out-with those selected during these experiments. Moreover, it may be the case that the exposure conditions selected during this study were not suitable for capturing transcriptomic responses that may be associated with reduced EMB susceptibility in strain PT. The main response observed in PT salmon lice was significant down regulation of several genes including cytoskeletal proteins and associated binding proteins. This may represent a mechanism adopted by less susceptible salmon lice involving the down regulation or cessation of non-essential cellular processes, as was suggested by Dicker *et al.* when they studied global transcriptomic responses of multi-drug resistant *Teladorsagia circumcinta* to IVM exposure (Dicker *et al.*, 2011). Alternatively, this response may relate to stress responses resulting from the EMB exposure of PT lice, which masks any subtle changes in drug transport or detoxification that facilitate survival. Laboratory-maintained strains S and PT may not share a common genetic background and have been laboratory-maintained for different periods of time (3 and 10 years for PT and S, respectively). Differences in genetic background may be responsible for differential transcriptional stress responses to removal from the host fish and/or drug exposure, which could mask the transcriptomic responses responsible for survival of EMB exposure. Additionally, differences in gene expression found in this study may be unrelated to EMB susceptibility and reflect the different genetic backgrounds of the strains.

Drug resistance of ecdysozoans has often been associated with gene amplification, over expression or coding sequence mutations of three gene families responsible for detoxification (Li *et al*, 2007), which include CYP (Giraudo *et al.*, 2010; Puinean *et al.*, 2010; Bariami *et al.*, 2012; Tao *et al.*, 2012), glutathione-s-transferase (GST) (Che-Mendoza *et al.*, 2009; Zhou *et al.*, 2012) and esterase enzymes (Field and

Devonshire, 1998; Puinean *et al.*, 2010; Bariami *et al.*, 2012). These mechanisms can show constitutively enhanced expression, or induction following drug exposure. ABC transporters (James and Davey, 2009; Ardelli and Prichard, 2013) have also been shown to be involved in AVM resistance in some nematodes. ABC transporters (Tribble *et al.*, 2007a; Heumann *et al.*, 2012; Igboeli *et al.*, 2012; Igboeli *et al.*, 2013), CYPs (Rewitz *et al.*, 2006), GSTs, carboxylesterases and catalases (Jemec *et al.*, 2010) have all been identified as biochemical defence mechanisms in crustaceans, however, they are less well characterised than in other ecdysozoan invertebrates. Herbivorous insects often possess pre-adaptations to plant allelochemicals, which gives these pests detoxification mechanism plasticity that can facilitate the development of insecticide resistance (Schuler, 2011). These insects are either specialists that parasitise a narrow host range, or generalist species that have a wider host range and are therefore exposed to a large diversity of chemicals (Li *et al*, 2004). Generalist species require more adaptable detoxification systems and more complex regulatory mechanisms that are often inducible upon exposure to exogenous compounds (Li *et al*, 2002). Crustacean species such as *L. salmonis* are exposed to a diverse array of chemicals (LeBlanc, 2007; Lauritano *et al.*, 2012) which suggests they may possess adaptable detoxification systems. Detoxification enzyme gene family evolution in crustaceans may be similar to dipteran insects such as the mosquito *Anopheles gambiae*, where continual exposure to many different compounds is thought to be responsible for significant gene family expansions (Ranson *et al.*, 2002). Although, lifestyle differences between these species mean that toxic compound exposure of crustacean parasites such as *L. salmonis* will be different from *A. gambiae*. Additionally, specific *L. salmonis* detoxification enzymes may not be represented in the limited sequence resources used in this study. Alternatively, as detoxification enzymes are often involved in the stress response of

marine copepods (Lauritano *et al.*, 2012), any additional associations these enzymes may have with reduced EMB susceptibility in *L. salmonis* may be obscured by these stress responses.

The most well documented AVM-induced mechanisms in AVM resistant nematodes is the up-regulation of P-gp ABC transporters (Prichard and Roulet, 2007; James and Davey, 2009; Lloberas *et al.*, 2013). Recently, it was demonstrated that IVM exposure of *Drosophila* cells induced increased P-gp expression which was associated with elevated intracellular calcium via the Calmodulin/Relish (NF-κB) signalling pathway (Luo *et al.*, 2013). This emphasises the complexity of mechanisms that may be responsible for increased P-gp expression and supports the decision to perform global transcriptomic analysis of EMB-induced responses in *L. salmonis*. The P-gp homologue (SL-PGY1) in *L. salmonis* was represented on the oligo microarray used in the present study (3 probes, BLASTx e-value 10^{-20} – 10^{-86}), although P-gp mRNA expression in strains S and PT was unaffected by EMB treatment (data not shown), confirming similar data previously reported for these strains (Heumann *et al.*, 2012). Increased P-gp expression has previously been associated with reduced EMB susceptibility in *L. salmonis* (Tribble *et al.*, 2007a). However, these observations were made using sea lice originating from New Brunswick, Canada with a different drug exposure history to those used in the current study, which may account for the differences in expression of an inducible detoxification mechanism such as P-gp ABC transporters. Three transcripts representing MRP (BLASTx e-value $\leq 10^{-7}$) ABC transporters were also not significantly differentially expressed upon EMB exposure of strains S and PT (data not shown). The results from this study do not provide evidence to support the involvement of these ABC transporters in reductions of EMB susceptibility in *L. salmonis* strains S and PT, however; only two subfamilies (B and C) were represented. Additionally, post-

translational phosphorylation of ABC transporter proteins could be involved in the control of EMB induced transporter activation, which would not be detected in the current study (Stolarczyk *et al.*, 2011). Hopefully, the release of an annotated *L. salmonis* genome sequence in 2013 will allow discovery of a fuller complement of *L. salmonis* ABC transporters that can then be interrogated with regards to their involvement in EMB resistance.

CYPs have been identified as significant components in the development of drug resistant insect pests (ffrench-Constant *et al.*, 2004; Heckel, 2012), but comparable functions in crustacean species has yet to be established. The CYP target sequences represented in this study included 14 probes that were provisionally allocated to CYP clans according to BLASTx annotation (e-values of $\leq 10^{-7}$). The expression of these CYPs did not significantly change in response to EMB exposure of either strains S or PT and therefore it was not possible to identify a role for these CYPs in reduced EMB susceptibility of the *L. salmonis* PT strain. Drug resistance of some insect and mite species have been associated with gene expression changes for different CYP genes and isoforms (Schmidt *et al.*, 2010; Liu *et al.*, 2011; Tao *et al.*, 2012) with only some isoforms being inducible upon drug exposure (Liu *et al.*, 2011). It may be possible that CYP isoforms with roles in EMB detoxification in *L. salmonis* were not represented on the microarray used to measure transcriptomic responses in strains S and PT to EMB exposure. CYP gene expression changes have often been shown to be controlled by mutations in cis-acting promoter sequences or trans-acting regulatory factors (Li *et al*, 2007). Additionally, it has been shown that the insertion of transposable elements into gene promoter regions can result in increased expression of some CYPs (Aminetzach *et al*, 2005; Schmidt *et al.*, 2010). Moreover, transposon-mediated gene expression changes are thought to be highly adaptive and can be responsible for rapid changes in

expression in response to exposure to toxic agents (Li *et al*, 2007). If transposon-mediated gene expression changes were responsible for EMB-induced responses in *L. salmonis* it would be expected that such responses would be detected in the current study.

Finally, enhanced GST (Wang *et al*, 1991; Ranson *et al*, 2001; Wei *et al*, 2001) and esterase (Raymond *et al.*, 1998; Hawkes and Hemingway, 2002; Cui *et al.* 2006) production has also been associated with insecticide resistance, although these targets did not show significant changes in mRNA level upon EMB exposure of *L. salmonis* during this study. However, esterases were poorly represented in the genomic resources available for construction of the microarray and it has also been demonstrated that CpG methylation or demethylation can lead to increased or decreased esterase gene expression (Field *et al*, 2000), although this mechanism would not be detected using transcriptomic analysis in the current study.

Conclusions

The transcriptional profiling of EMB-resistant and -susceptible laboratory salmon louse strains in response to short term (1-3 h) aqueous EMB exposure was unable to provide evidence for the presence of specific resistance mechanisms requiring transcriptional regulation. The drug-susceptible S strain showed a number of transcriptional responses to treatment, but few responses were found in the EMB-resistant PT strain other than down-regulation of non-essential cellular processes. The current study found similar mRNA expression for putative LGIC target subunits, biotransformation enzymes and drug transporters, which have previously been implicated in AVM resistance of ecdysozoans. It is recognised that further work is necessary to study transcriptomic responses between salmon lice from both extremes of EMB susceptibility, using modified aqueous exposure assay methods to select highly resistant lice from the PT strain. This study does, however, demonstrate the successful use of custom *L. salmonis* oligo microarrays in the measurement of transcriptomic responses of laboratory maintained salmon lice with differing EMB susceptibilities.

Chapter 5

Use of genome-wide SNP discovery to examine complex traits including drug resistance in the salmon louse.

5.1 Introduction

Rapid development of next generation sequencing (NGS) technologies now allow genome-wide marker identification and genotyping to be performed relatively simply and economically (Davey *et al.*, 2011). The application of NGS to Restriction-site associated DNA sequencing (termed RAD-seq), provides an efficient method to generate a reduced representation of the genome and involves the large-scale discovery of Single Nucleotide Polymorphisms (SNPs) or insertions and deletions (indels) (Baird *et al.*, 2008, Hohenlohe *et al.*, 2010; Hohenlohe *et al.*, 2012). When using RAD-seq, unique barcoding allows the association of sequence reads with individual samples so that DNA from multiple individuals can be analysed in the same RAD library, permitting sophisticated experimental designs suitable for the analysis of complex traits (Davey and Blaxter, 2011; Peterson *et al.*, 2012). Moreover, RAD-seq reads can not only be examined in relation to a reference genome for genetic marker identification but can also be analysed *de novo* (Davey and Blaxter, 2011). It has therefore been possible to incorporate RAD-seq into a wide array of experimental designs and use it for more complex analyses such as population genetics (Hohenlohe *et al.*, 2010; Hohenlohe *et al.*, 2012), linkage mapping (Baxter *et al.*, 2011; Richards *et al.*, 2013; Takahashi *et al.*, 2013), Quantitative Trait Locus (QTL) analysis (Houston *et al.*, 2012) and phylogeography (Emerson *et al.*, 2010; Reitzel *et al.*, 2013).

As discussed in earlier chapters the precise mechanism responsible for emamectin benzoate (EMB) resistance in *Lepeophtheirus salmonis* is currently unknown with a role for P-glycoprotein (P-gp) ABC transporters being demonstrated in some studies (Tribble *et al.*, 2007a; Igboeli *et al.*, 2012; Igboeli *et al.*, 2013), but not others (Heumann *et al.*, 2012), suggesting a role for other mechanisms. Using comparative transcriptomic profiling of two *L. salmonis* strains differing in EMB susceptibility, the

studies reported in Chapters 3 and 4 of this thesis attempted to identify the transcripts associated with differential EMB susceptibility observed between drug susceptible (S) and EMB-resistant (PT) *L. salmonis* strains. While a majority of the differentially expressed transcripts identified between strains S and PT were related to muscle and cuticle formation, relatively subtle but significant changes in mRNA levels of ligand gated ion channels (LGIC) were detected. In contrast, no clear changes in transcript levels were found for genes involved in detoxification pathways, such as cytochrome P450s and ABC transporters. These results suggest that the mechanism responsible for EMB resistance in *L. salmonis* strain PT is not associated with large differences in transcript expression. Genetic analysis offers an alternative approach to unravelling the molecular determinants responsible for drug resistance; however the availability of a sufficiently large set of genetic markers is a prerequisite for this approach.

The aim of the research reported in this chapter was to use RAD-seq for the discovery of genetic markers in *L. salmonis* that may be suitable for analysing genetic determinants of complex traits such as drug susceptibility. RAD-seq experiments usually combine marker discovery and massive parallel genotyping. In the present study, genetic markers were identified in *L. salmonis* and subsequently used in a preliminary experiment employing bulk segregant analysis to compare salmon louse strains S and PT. Time and budget constraints did not allow for F2 crosses to be generated between these strains, which would have permitted the implementation of a superior experimental approach involving QTL analysis. For the same reason, the factors strain and EMB susceptibility were confounded in the current experimental design, *i.e.* only one strain representing high (strain S) and low (strain PT) EMB susceptibility were included. Accordingly, further studies of any candidate genetic markers that show significant association with *L. salmonis* strain will be required to

confirm any associations with EMB susceptibility. Given differences in susceptibility observed between louse sexes, a further application for these markers was to test the hypothesis that sex determination in *L. salmonis* is genetic by attempting to identify sex-linked genetic markers through the inclusion of individuals from both sexes in the RAD-seq libraries. *L. salmonis* is gonochoristic, and laboratory-maintained cohorts of this species normally show sex ratios close to 1:1 (Johnson and Albright, 1991a; Hamre *et al.*, 2009). While this observation is consistent with a genetic mechanism of sex determination, the mechanism in *L. salmonis* and other copepods is currently unknown. Environmental and genetic sex determination have both been reported in crustaceans (Juchault, 1999), and a similar situation exists for insects (Gempe and Beye, 2011). The mechanism for genetic sex determination is not conserved in crustaceans and there is evidence for a variety of male and female heterogametic systems (reviewed in: Legrand *et al.*, 1987) in crustaceans, including the XX/XY and ZW/ZZ systems that have been described in mammals and birds, respectively (Charlesworth and Mank, 2010; Nakamura, 2010).

5.2 Materials and methods

5.2.1 Salmon louse strains

Two well characterised laboratory-maintained strains of salmon lice were used for RAD library preparation. Strain S has previously been shown to be susceptible to all salmon delousing agents in current use while strain PT shows decreased susceptibility (~7-fold) to EMB, as based on 24 h waterborne bioassays (Heumann *et al*., 2012) which is illustrated in Chapter 3, Figure 3.1 of this thesis. Verification of SNP marker association with sex was performed using salmon lice from these two strains and from an unrelated strain that had recently been established from a farm isolate (FI). These strains have all been cultured under identical laboratory conditions, as described in detail elsewhere (Heumann *et al*., 2012). For more information on these strains please see the materials and methods section in Chapter 2 of this thesis.

5.2.2 Salmon louse selection

L. salmonis engage in complex courtship behaviour between adult males and late preadult II stage females, which culminates in the formation of pre-copula pairs (Ritchie *et al.*, 1996). Copulation takes place soon after the female moults into the adult stage, and females retain spermatophores from the mating in order to fertilise egg strings produced over their lifetime (Ritchie *et al.*, 1996). Adult male and preadult female (n = 24) *L. salmonis* from strains S and PT were used for RAD library preparation. Preadult females were selected in preference to adult females to avoid the possibility of sample contamination with stored sperm. Adult female salmon lice were used for the verification of SNP sex association in strain FI, after genital segments had been removed to avoid male DNA contamination. Adult male and preadult female *L. salmonis* are approximately the same size (total length ~5.4 mm) but can easily be distinguished at these stages of development under low magnification microscopy,

using common morphological features (Johnson and Albright, 1991b). The abdomen of adult male salmon lice is shorter than that of females with an ovoid genital complex, whereas the preadult II female genital complex is larger with cuticular folds and distinct lobes, and a narrowing of the abdomen as it meets the genital complex. Similarly, adult females are much larger than adult males, have a larger genital complex than males and are also larger and, following mating, have a more developed genital segment in comparison to preadult II females (Johnson and Albright, 1991b).

5.2.3 Determination of salmon louse EMB susceptibility phenotype

While salmon lice from the laboratory maintained strains S and PT have shown stable EMB susceptibilities over multiple generations, EMB susceptibilities among individuals within either of these strains may show a degree of variation. The individual EMB susceptibility of male salmon lice used to prepare RAD libraries was characterised by exposing them to a relatively high EMB concentration (800 µg L^{-1}), and establishing the time at which an immotility response became apparent in each individual. This time-until-response served as confirmation of the EMB susceptibility for male salmon lice included in RAD libraries. It was planned to obtain EMB susceptibility phenotypes for female salmon lice, however, preadult females were not available in sufficient numbers when this logistically elaborate experiment had been scheduled, which involved overnight drug exposure with regular observations at the Marine Environmental Research Laboratories (MERL) of the Institute of Aquaculture. Female lice were collected at a later date, but without characterisation of EMB susceptibility phenotypes. In order to establish individual EMB susceptibility phenotypes of males, salmon lice were collected from anaesthetised host fish as described in Chapter 2 and allowed to recover for two hours in aerated, filtered seawater at ambient sea temperature. EMB (technical grade, a gift from Merck Animal

Health) was solubilised in seawater with PEG_{300} (final concentration 0.01 % (v/v)) to a concentration of 800 µg L^{-1}. Bioassays were set up in glass dishes containing 22 salmon lice and 200 mL of exposure solution (800 µg L^{-1} EMB). At regular intervals (Appendix 9) throughout the 24 hour bioassays salmon lice were recorded as normally motile or immotile upon visual examination and stimulation with a fine brush. Salmon lice showing an immotility response were sampled into absolute ethanol and the exposure time at which the response had been observed was noted. Visual examination was performed until all 22 salmon lice exhibited an immotility response and were sampled for analysis, or that the remaining lice did not exhibit a response after 24 hours of EMB exposure. Samples were stored at 4 °C pending extraction of genomic DNA.

5.2.4 RAD library preparation and sequencing

RAD-seq libraries were prepared from adult male and preadult female *L. salmonis* selected from strains S and PT (n = 48; 24 from each strain). For male salmon lice sampled from strain S, the individuals that responded earliest to EMB exposure were selected (*i.e.* the most sensitive), whereas for strain PT the individuals that survived the longest were selected (*i.e.* the most resistant). This strategy was used to select the extremes of EMB susceptibility between strains S and PT. Genomic DNA was extracted from individual *L. salmonis* using the REAL-Pure genomic DNA extraction kit (Durviz S.L., Spain), including removal of residual RNA through RNase A treatment of the extracts. UV spectroscopy (NanoDrop ND-1000, Thermo Scientific, USA) was used to confirm purity of the DNA samples and establish concentrations, whereas high molecular weight (MW) DNA integrity was assessed by agarose gel electrophoresis and ethidium bromide staining. Each high MW DNA sample was then diluted to a concentration of 45 ng/µl in 5 mM Tris, pH 8.5. The RAD libraries were prepared as detailed previously (Etter *et al.*, 2011) with minor modifications as detailed

in Houston *et al.* (Houston *et al.*, 2012). Sequence details for the P1 and P2 paired-end adapters and library amplification primers used in RAD library preparation are available elsewhere (Baxter *et al.*, 2011) and detailed in Appendix 10. Briefly, 200 ng of each DNA sample was digested at 37 °C for 45 minutes with 2 units of *Pst*I high fidelity restriction enzyme (recognising the CTGC|AG motif) in a 10 µL reaction containing 1 × Reaction Buffer 4 (New England Biolabs, UK). The reactions were then heat-inactivated at 80 °C for 20 minutes. Each of the *Pst*I digested DNA samples were individually identified through the ligation of specific P1 adapters each containing a unique five base nucleotide barcode (Appendix 11 and 12), at 25 °C for 30 minutes in a 12.5 µL reaction containing 100 nM P1 adapter, 200 units of T4 DNA Ligase, 1 mM rATP and 1 × Reaction Buffer 2 (New England Biolabs, UK). Ligation reactions were heat inactivated at 65 °C for 20 minutes prior to combining them in four multiplexed libraries, each containing 12 salmon louse samples. Adaptive Focus Acoustics™ (AFA™) using the S220 High Performance Ultrasonicator (Covaris® Inc., KBiosciences, UK.) was employed to randomly shear each RAD library pool to a size range of 150-700 bp. This sheared DNA was then column purified (PCR MinElute Kit, Qiagen) and size selected as described by Houston *et al.*, 2012. The RAD library construction protocol was then followed as published (Baird *et al.*, 2008; Etter *et al.*, 2011). The RAD library pools were PCR amplified using 15 - 16 cycles and 150 µL of each amplified library was column purified, size selected (300 - 550 bp) and quality checked as described by Houston *et al.*, 2012. The four RAD library pools were further quality checked and quantified by quantitative PCR (qPCR) (KAPA Library) prior to sequencing on one lane of the Illumina HiSeq 2000 platform (v3 chemistry) using 100 bp paired-end reads (EBI Sequence Read Archive (SRA) study ERP002400). Raw sequence data were processed using RTA 1.12.4.2 and Casava 1.6 (Illumina). RAD

library qualitative and quantitative checks, Illumina sequencing and processing of raw sequence reads were performed at The GenePool Genomics Facility (University of Edinburgh, UK).

5.2.5 Genotyping RAD Alleles

Sequence reads with low quality scores (quality index score under 30, while the average quality score was 37), missing the restriction site or those with ambiguous barcodes (with more than one mismatch) were discarded from the sequence set. All the remaining sequence reads were then sorted into loci and RAD markers were genotyped using the Stacks software 0.9995 (Catchen *et al.*, 2011). The likelihood-based SNP calling algorithm implemented in Stacks (Hohenlohe *et al.*, 2010) evaluated each nucleotide position for every RAD-tag from all individual samples, thereby differentiating true SNPs from sequencing errors. The processing parameters used in Stacks included; a minimum stack depth of at least 30 sequences, a maximum of 2 mismatches in each locus for each individual and up to 1 mismatch between alleles. The paired-end reads were assembled using both Stacks and Velvet (version 1.2.08) software (Zerbino and Birney, 2008), which were used to separate RAD-tag sequences, with or without potential SNPs, but belonging to separate candidate loci.

5.2.6 SNP association with sex, strain and EMB susceptibility

The genetic association of *L. salmonis* phenotypic sex, strain and EMB susceptibility with RAD marker alleles was carried out by counting the number of times each allele was associated with a particular sex, strain or EMB susceptibility respectively. These counts were compared to an ideal scenario where each allele would be specific to the particular phenotype.

5.2.7 Verification of SNP sex association

An additional twelve adult male and twelve preadult female *L. salmonis* per strain were sampled from strains S and PT and preserved in ethanol as detailed above. Similarly, twelve adult male and adult female *L. salmonis* were sampled from strain FI. Genomic DNA was extracted from each *L. salmonis* individual, quality checked and diluted as detailed above. SNP marker sex-association was verified using an allele specific PCR genotyping assay (KASP^{TM} v4.0, LGC Genomics, UK). SNP-specific primers were designed by LGC Genomics using sequence flanking RAD-marker *Lsa*101901. For each of the three strains, twelve male and twelve female salmon lice were genotyped in duplicate 10 µL reactions each containing approximately 40 ng template DNA, using the following amplification conditions: 94 °C for 15 minutes followed by 35 cycles of 94 °C for 20 seconds then touch-down cycles over 61 – 55 °C for 60 seconds (dropping 0.6 °C per cycle). Individual *L. salmonis* genotype assignment was performed through reading the fluorescence emission of the FAM (5' 6-carboxyfluorescein) and CAL Fluor Orange 560 fluorophores for each sample, in comparison to no-template control reactions, using endpoint genotyping software and the Quantica qPCR thermal cycler (Bibby Scientific, UK).

5.2.8 RT-qPCR analysis of prohibitin-2 expression

The mRNA abundance of the prohibitin-2 gene was determined in adult male (n = 10) and female (n = 8) drug susceptible (S) *L. salmonis* by reverse transcription quantitative PCR (RT-qPCR), using relative quantification with two reference genes that had shown stable expression levels in previous experiments (Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and Required for Meiotic nuclear Division 5 (RMD-5) homolog) (unpublished data). Primers were designed for these three genes with melting temperatures (T_m) of ~60 °C using Primer 3 software

(Appendix 13). Adult male and female salmon lice were collected from anaesthetised host fish as described above and allowed to recover for 2 hours in aerated filtered seawater at ambient sea temperature and then preserved in an RNA stabilisation solution (4.54 M ammonium sulphate, 25 mM trisodium citrate, 20 mM EDTA, pH 5.4; Appendix 1) prior to storage at -70 °C. Total RNA was extracted and purified from these samples, then assessed for purity and quantified, as described in detail in Chapter 2 of this thesis. Total RNA from adult male or female *L. salmonis* were reverse transcribed and analysed in RT-qPCR reactions, and relative gene expression ratios established using the ΔΔCt method as detailed in Chapter 3 of this thesis. Relative expression ratios from RT-qPCR analysis were compared between male and female *L. salmonis* using the non-parametric Mann-Whitney test as implemented in the Minitab 16.1 software package (Minitab Inc., UK). The significance level was set at $p < 0.05$. Relative expression ratios from RT-qPCR analysis were compared between male and female *L. salmonis* using the non-parametric Mann-Whitney test as implemented in the Minitab 16.1 software package (Minitab Inc., UK). The significance level was set at $p < 0.05$.

5.3 Results

5.3.1 Emamectin exposure experiment

Adult male and preadult female salmon lice from laboratory-maintained strains S and PT (Heumann *et al.*, 2012) were used for RAD library preparation. In order to establish estimates of the EMB susceptibility for individual male lice, *L. salmonis* were exposed to 800 μg L^{-1} of EMB and examined at regular time intervals to establish the exposure time required for immotility responses to become apparent. *L. salmonis* rated as immotile were removed from the test vessels and sampled pending DNA extraction, taking a note of the exposure time at which the response was observed. Salmon louse

strains S and PT clearly differed regarding the time required for an immotility response to become evident (Figure 5.1).

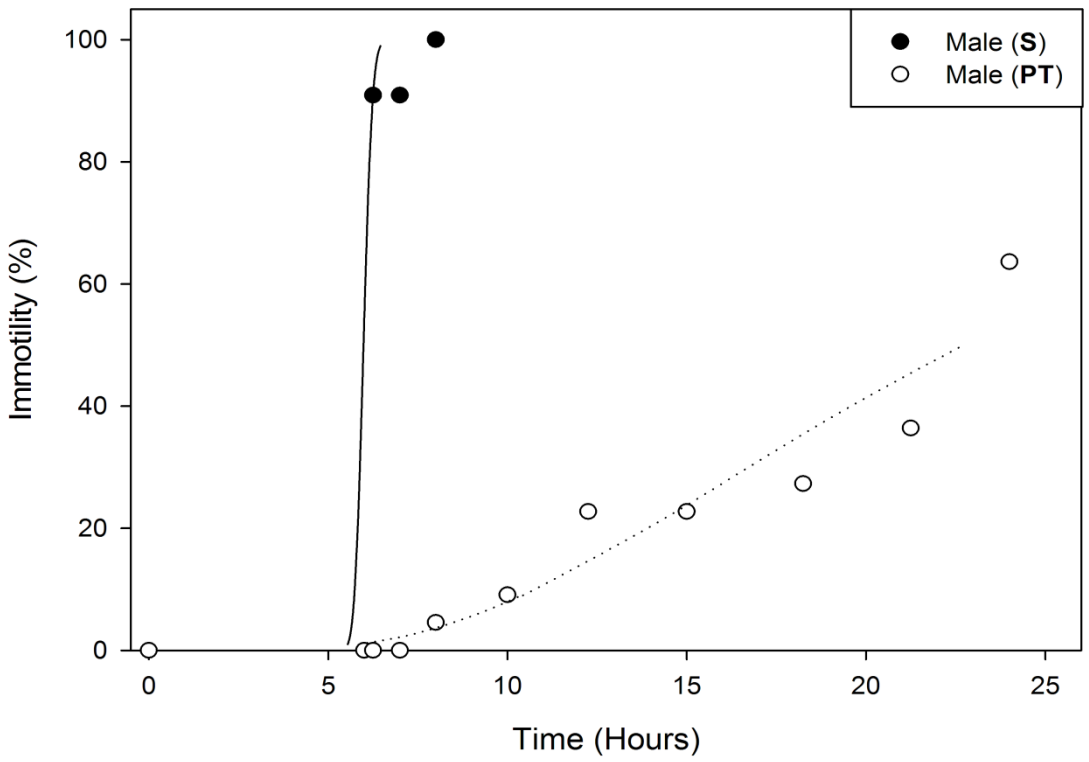


Figure 5.1 Adult male salmon louse susceptibility to acute EMB exposure for two laboratory strains.

Toxicity response of adult male salmon lice (*L. salmonis*), from S and PT laboratory strains, to acute EMB exposure (800 μgL^{-1}) using 24 hour immotility bioassays. Symbols represent the immotility response observed in a beaker of 22 individuals (Each symbol represents the percentage of the 22 lice that exhibited an immotility response at each time point).

5.3.2 RAD Sequencing

DNA from each of 12 male and 12 female individuals from both the drug susceptible (S) and EMB-resistant (PT) laboratory-maintained *L. salmonis* strains was used to generate multiplexed *Pst*I RAD libraries and sequenced at high depth using the Illumina HiSeq 2000 platform. In total, 331,642,960 raw reads (100 nt long) were produced, that comprised 165,821,480 paired-end reads (EBI Sequence Read Archive (SRA) studies ERP002400 and ERP002422 for strains S and PT respectively). After removal of low quality sequence reads (quality score under 30), sequences with ambiguous barcodes and orphaned paired-end reads, 80 % of the raw reads were retained (264,343,660 reads). The Stacks package (Catchen *et al.*, 2011) was then used to assemble loci (RAD-tags) for each individual, which produced 378,697 unique RAD-tags (Figure 5.2). The raw sequence read and RAD-tag count for each sample from strains S and PT are reported in Appendices 11 and 12 respectively.

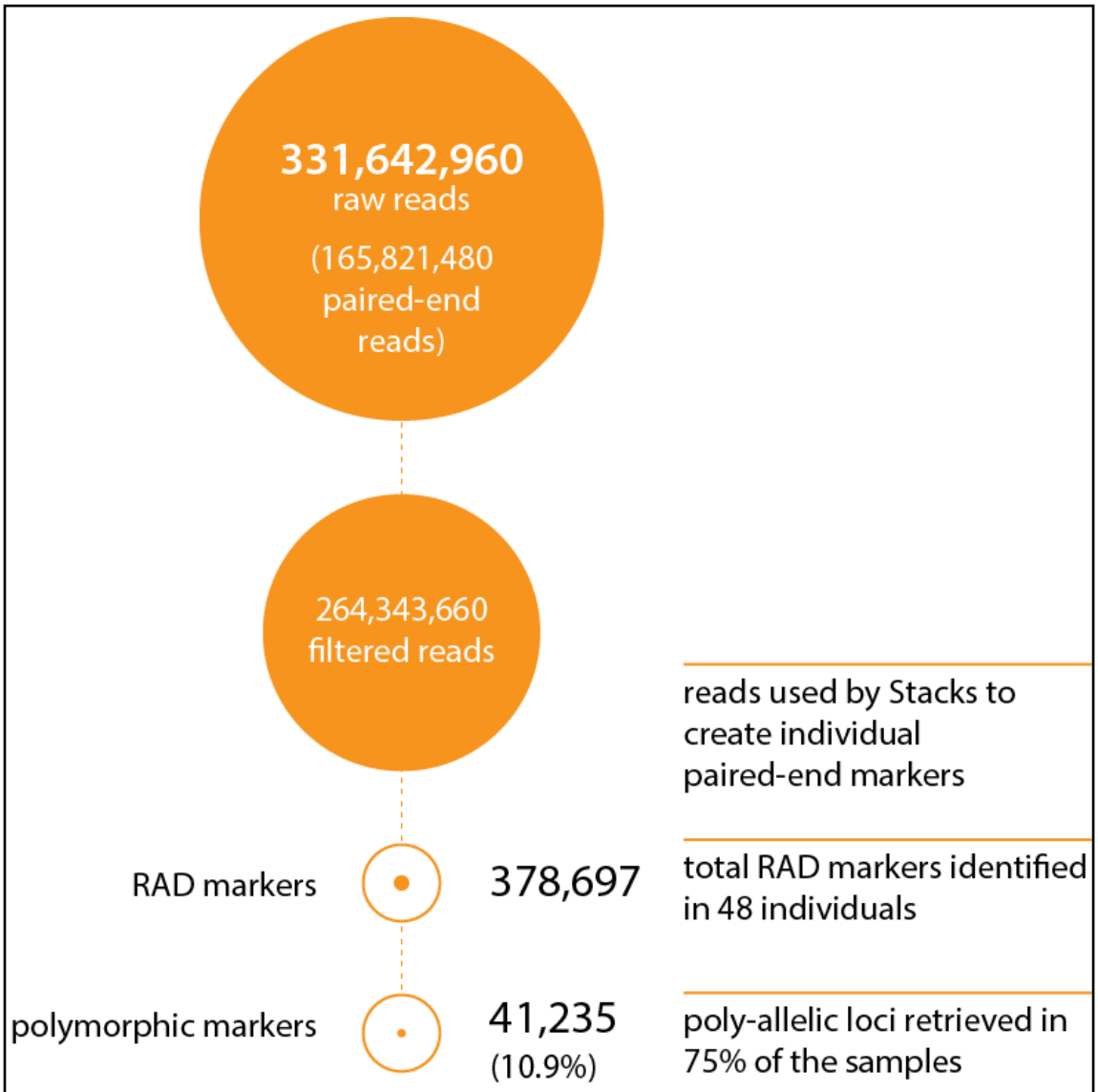


Figure 5.2 Salmon louse RAD sequencing and polymorphic marker identification.

RAD-seq technology was employed to genotype 48 individuals from drug susceptible (S) and EMB resistant (PT) laboratory maintained salmon louse (*L. salmonis*) strains, using 100 base paired end sequencing on an Illumina HiSeq 2000 platform. This produced 331, 642,960 raw sequence reads that comprised 165,821,480 paired end reads although after filtering 264,343,660 raw sequence reads remained that produced 378,697 paired-end RAD markers. 41,235 of these polymorphic markers were poly-allelic and present in 75 % of the individuals analysed.

5.3.3 SNP association with strain, sex and EMB susceptibility

Initial analysis of read number for the 378,697 RAD-tags did not reveal any sex-specific markers (*i.e.* present in only one of the sexes). To maximise the number of informative markers and minimise the amount of missing or erroneous data, only paired-end RAD-tags retrieved from at least 75 % of the samples were used, which resulted in the retention of 82,954 RAD-tags. Analysis of this filtered set of markers did not reveal any RAD-tags with twice the coverage in one gender compared to the other. However, the median sequence read coverage was 68 reads per marker for each sample which varied greatly from 20 to 200 reads per marker for each sample, meaning that it would be difficult to identify markers with twice the coverage as only highly represented markers may be evident using such an analysis strategy. Further analysis revealed that 41,235 of these RAD-tags were polymorphic (containing 1 or 2 SNPs), of which 28,041 were bi-allelic (Figure 5.2, Table 5.1). The genetic association of polymorphic markers with phenotypic sex and strain was performed by direct comparison of each allele with the sex and strain of the individuals. Evaluation of the RAD markers for association with salmon louse strain identified 27 markers, with 15 of these exhibiting complete association with strain (Table 5.2). The SNP alleles and RAD marker allele sequences for the 27 strain-linked markers are detailed in Additional file 5.1. Alignment of the RAD marker sequences to salmon louse transcriptome sequence created as part of this thesis (Chapter 2) revealed that some of the markers were located in transcribed regions (Table 5.3).

Table 5.1 Bi-allelic and poly-allelic RAD loci found in ≥ 75% of S and PT *L. salmonis* samples.

	S strain	PT strain	Common Alleles*
Total RAD loci	85,560	95,780	82,954
Total poly-allelic RAD loci	31,555	38,884	41,235
Total bi-allelic RAD loci	24,538	29,234	28,041
Bi-allelic RAD loci with both alleles fixed	-	-	1,801

Table 5.2 Identification of associations between RAD marker genotype and salmon louse strain.

RAD marker ID	Marker association (%)	S strain genotype	PT strain genotype	Genome representation	Transcriptome representation
Lsa578	100	AA	CC	No	No
Lsa4637	100	GG	AA	No	No
Lsa8868	100	GG	AA	Yes	No
Lsa17314	100	AA	CC	No	No
Lsa29463	100	TT	AA/AT	Yes	Yes
Lsa58229	100	CC	TT	No	No
Lsa72290	100	CC	TT	No	No
Lsa91273	100	GG	AA	Yes	No
Lsa100190	100	GG	AA	Yes	No
Lsa100302	100	TT	CC	No	No
Lsa103134	100	CC	TT	No	No
Lsa109508	100	CC	AA/AC	Yes	Yes
Lsa111515	100	GG	AA	No	No
Lsa112470	100	CC	TT	No	No
Lsa127075	100	TT	CC	No	No
Lsa4170	98	CC	CC/CT/TT	Yes	Yes
Lsa5470	98	CC	CC/CT/TT	Yes	Yes
Lsa38618	98	CC	CC/CG/GG	Yes	Yes
Lsa103900	98	GG	AA/AG/GG	Yes	Yes
Lsa104162	98	CC/CG/GG	GG	Yes	Yes
Lsa3006	96	TT	GG/GT/TT	Yes	Yes
Lsa3626	96	TT	CC/CT/TT	Yes	Yes
Lsa96328	96	AA	AA/AC/CC	Yes	Yes
Lsa96339	96	CC	AA/AC/CC	Yes	Yes
Lsa112687	96	GG	AA/AG/GG	Yes	Yes
Lsa77217	94	AA	AA/AG/GG	Yes	Yes
Lsa41579	93	CC	AA/AC/CC	Yes	Yes

The genetic association of polymorphic RAD marker genotype with salmon louse strain was performed by direct comparison of the marker alleles with salmon louse strain (*i.e.*, S or PT) for each of the 48 individual samples. The association (%) of each allele with strain is detailed, and also the presence or absence of each marker sequence in the draft genome and transcriptome sequence resources available for *L. salmonis*.

Table 5.3 Strain-linked RAD marker sequence alignment to salmon louse transcriptome.

RAD marker ID	RAD Marker length (bp)	Transcript Length (bp)	Transcriptome Hit Annotation	Transcriptome Hit Accession	Blastn e-value
Lsa578	666				
Lsa4637	556				
Lsa8868	557				
Lsa17314	556				
Lsa29463	612	1551	Camar1 transposase *Chymomyza amoena*	AAO12862	$3E^{-43}$
Lsa58229	560		Unannotated		
Lsa72290	419				
Lsa91273	595				
Lsa100190	641				
Lsa100302	561				
Lsa103134	604				
Lsa109508	494	2178	40S ribosomal protein S14 *Lepeophtheirus salmonis*	ADD24065	$8E^{-49}$
Lsa111515	659				
Lsa112470	579				
Lsa127075	629				
Lsa4170	504	1489	Putative uncharacterized protein	EFN85722	$5E^{-27}$
Lsa5470	602	1585	Unannotated	NA	$6E^{-19}$
Lsa38618	635	14404	Unannotated	NA	$5E^{-172}$
Lsa103900	96	877	7 transmembrane receptor *Trichinella spiralis*	XP_003377890	$2E^{-139}$
Lsa104162	215	915	Hypothetical protein *Caenorhabditis remanei*	XP_003101552	$2E^{-79}$
Lsa3006	573	472	Unannotated	NA	$8E^{-37}$
Lsa3626	299	1232	Hypothetical protein *Tribolium castaneum*	EFA06378	$1E^{-110}$
Lsa96328	541	1189	GA12899-PA *Tribolium castaneum*	XP_973981	$2E^{-11}$
Lsa96339	218	5937	Lactate dehydrogenase	P52643	$3E^{-93}$
Lsa112687	571	3895	GF11514 *Drosophila ananassae*	XP_001960432	$1E^{-20}$
Lsa77217	523	895	Unannotated	NA	$9E^{-11}$
Lsa41579	406	1800	Unannotated	NA	$2E^{-13}$

For the identification of sex-linked markers the results were then ranked in order, from maximum (complete separation between male and female or strain) to minimum association (not significantly different from random association). Evaluation of the 28,041 markers identified that only one marker (*Lsa*101901) exhibited complete association with gender, where all samples were shown to be heterozygous female (allele 'G' or 'T') or homozygous male (allele 'G' only) genotype (*Lsa*101901; NCBI dbSNP Accession: 749737482; Table 5.4). The mean read number at this locus was 29 reads; female heterozygous alleles showed a mean read number of 14.5 each, whereas the male homozygous allele had 29 reads. It was not possible to find any statistically significant associations between RAD marker genotype and EMB susceptibility levels estimated using the bioassay. This may be due to the fact that there were only 12 individuals analysed for each sample group.

Table 5.4 *L. salmonis* sex-linked SNP marker and KASP assay primer sequences.

ID	Primer allele 1	Primer allele 2	Primer common	Allele 1	Allele 2	Marker allele 1	Marker allele 2
Lsa101901	CAGATCGA GCCAGGGA TTTTAATAT C	ACAGATCG AGCCAGGG ATTTTAATA TA	GTTCCGTAAT GGCTACATC GTCCAA	G	T	TGCAGCCGCGTATTCCCGA CCAAAACTAAGTTCCGTAA TGGCTACATCGTCCAAAAT GATATTAAAATCCCTGGCT CGATCTGTCAATTGCTTTC GAATGAGCATTGACACTTG TTGACGCTGTGTAATGAGC TGAGAAGCATTGAACTTGG CCACAACGCCCTTGAGAAC TTCATTACAAATGGATGGA AGGACTTTTTCATCAAAGT CTCTTCCCA	TGCAGCCGCGTATTCCCGACCA AAACTAAGTTCCGTAATGGCTA CATCGTCCAAAATTATATTAAA ATCCCTGGCTCGATCTGTCAATT GCTTTCGAATGAGCATTGACAC TTGTTGACGCTGTGTAATGAGCT GAGAAGCATTGAACTTGGCCAC AACGCCCTTGAGAACTTCATTA CAAATGGATGGAAGGACTTTTT CATCAAAGTCTCTTCCCA

Two SNP alleles and RAD-tag allele sequences that were identified as the SNP marker *Lsa101901* are detailed, along with the allele specific primers and common primer designed for the allele specific PCR genotyping assay (KASP™, LGC Genomics, UK).

5.3.4 Verification of sex association

The association of marker Lsa101901 to phenotypic sex was further investigated using an allele specific PCR genotyping assay (KASP^{TM}, LGC Genomics, UK). Individuals genotyped for the marker first included 12 male and 12 female salmon lice unrelated to the individuals from strain S that were used to generate the RAD library. Twelve males and 12 females from each of two further laboratory maintained *L. salmonis* strains PT and FI were also analysed. In all tested individuals, a complete association of the marker with phenotypic sex was observed, with females being heterozygous (G/T) and males homozygous (G/G) (Figure 5.3A).

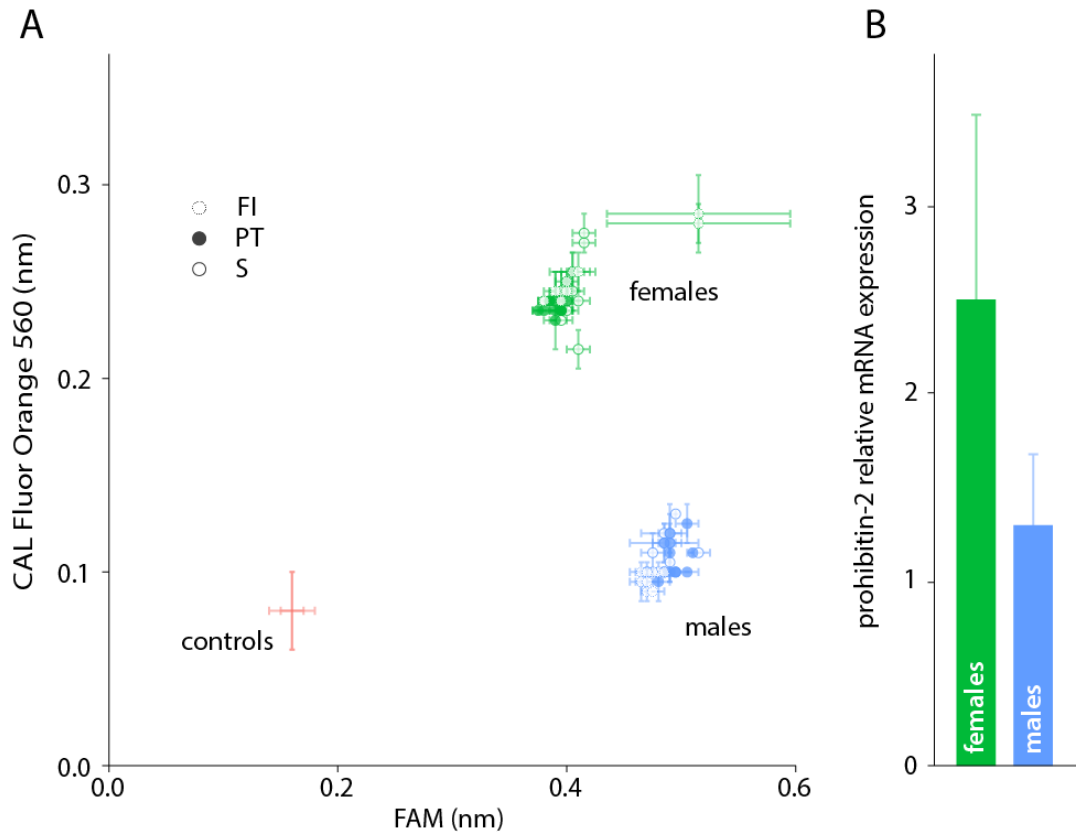


Figure 5.3 Analysis of prohibitin-2 in male and female *L. salmonis*.

(A) Genotyping using the allele specific KASP assay. A total of 72 individuals (36 male and 36 female) from three unrelated *L. salmonis* strains (S, PT and FI) were genotyped using an allele specific PCR assay (KASP™, LGC Genomics, UK). Individual *L. salmonis* genotype assignment was performed through reading the fluorescence emission of the FAM (Allele 1) and CAL Fluor Orange 560 (Allele 2) fluorophores for each sample, in comparison to no-template control reactions. The results of this PCR genotyping assay confirmed complete association of SNP genotype with *L. salmonis* sex as identified by RAD-seq analysis.

(B) Differential expression of prohibitin-2. Relative prohibitin-2 expression (Mean ± SD) is shown for adult female (n = 8) and adult male (n = 10) *L. salmonis* from the drug susceptible (S) laboratory-maintained strain. The prohibitin-2 expression differed significantly between *L. salmonis* males and females (p = 0.0117, Mann-Whitney test).

5.3.5 Sex-linked SNP marker annotation

The 218 nt marker sequence containing the *Lsa*101901 sex-linked SNP was used as a query in a nucleotide BLAST (Basic Local Alignment Search Tool) search, against the non-redundant nucleotides (nr) database available in GenBank at the National Centre for Biotechnology Information (NCBI). The marker sequence containing SNP *Lsa*101901 was identical to *L. salmonis* putative prohibitin-2 sequence (Accession BT121810.1, BLASTn e-value 2×10^{-109}).The SNP in marker *Lsa*101901 was found to be a synonymous polymorphism within the coding region of the prohibitin-2 gene. A BLASTx search against the NCBI Reference Proteins (refseq-protein) database further established the identity of the marker-containing sequence, as it showed a high similarity to a Prohibitin-2-like protein (Accession XP003746427.1) from *Metaseiulus occidentalis* (Western predatory mite): 88% identity across the whole sequence (BLASTx e-value 2×10^{-37}). The two *Lsa*101901 marker allele sequences were also identified in expressed sequence tag (EST) sequences (100 % query coverage) from Canadian and Norwegian Atlantic *L. salmonis* populations in addition to the Pacific population, using a BLASTn search against the NCBI EST database (Table 5.5).

Table 5.5 Identification of *L. salmonis* EST sequences representing marker *Lsa101901* alleles.

L. salmonis population	Allele	EST Accession
Atlantic Norway	G	GW663052.1
Atlantic Norway	T	HO677162.1
Atlantic Canada	G	GW644163.1
Atlantic Canada	T	GW642628.1, GW642629.1
Pacific	G	FK914464.1, EX486009.1
Pacific	T	FK913245.1, FK913246.1

5.3.6 Gene expression analysis of prohibitin-2

RT-qPCR analysis demonstrated that the marker sequence, containing the SNP *Lsa*101901 and annotated as *L. salmonis* prohibitin-2, was significantly differentially expressed ($p = 0.0117$, Mann-Whitney test) between male ($n = 10$) and female ($n = 8$) *L. salmonis* from a drug susceptible laboratory-maintained strain (Figure 5.3B). Relative expression analysis found that adult female *L. salmonis* expressed 1.8 fold more prohibitin-2 mRNA compared to adult males from this strain.

5.4 Discussion

The research presented in this chapter reports the identification of a library of SNP markers that were polymorphic between individuals from laboratory-maintained *L. salmonis* strains S and PT. Further analysis of these RAD markers identified 15 that exhibit complete association with salmon louse strain and therefore provide candidates for further study to establish an association with reduced EMB susceptibility in *L. salmonis*. In the present study a SNP marker was also identified in *L. salmonis* that showed complete association with sex in 96 genotyped individuals from three different strains. These results strongly suggest that sex determination in *L. salmonis* is genetic, and provide evidence for a female heterogametic ZW/ZZ system.

The experimental design used in the current study included only one drug susceptible and one EMB-resistant strain that do not originate from the same genetic background, meaning that experimental factors ‘strain’ and ‘resistance’ were confounded. It would therefore be necessary to perform more elaborate experimentation to fully test the hypothesis that these SNP markers are associated with reduced EMB susceptibility and not just genetic differentiation between strains. Firstly, an alternative RAD-seq strategy could be employed that analyses salmon lice from strains S and PT that respond at early and late EMB exposure time points, which was not logistically

possible during the current study. Secondly, as strain PT is only moderately resistant to EMB it would be beneficial to establish a highly EMB-resistant laboratory strain through repeated cycles of selecting resistant individuals using aqueous EMB exposure assay methods for breeding next generations, which has been successfully employed to establish AVM resistant ecdysozoan invertebrate populations (He *et al.*, 2009; Pu *et al.*, 2009; Shad *et al.*, 2010; Chen *et al.*, 2011). Thirdly, reciprocal crosses between a highly EMB-resistant laboratory strain and strain S would be the most informative strategy, as it would enable the construction of structured families for determining the inheritance of candidate resistance alleles and an association with EMB resistance phenotypes established using aqueous EMB bioassays. Similar strategies have been used in several studies involving AVM resistant species (He *et al.*, 2009; Pu *et al.*, 2009; Kwon *et al.*, 2010; Shad *et al.*, 2010). Initially, however, the strain-linked SNP markers identified in this study can be further validated in future analysis of stains S and PT and additional salmon louse populations that exhibit reduced EMB susceptibility, using an allele specific fluorescence PCR assay. The requirement that *L. salmonis* are cultured on the salmonid host means that establishment of targeted laboratory strains and families, as discussed above, was not achievable within the financial and time constraints of the current study.

It has been reported that the sex determination of a number of free-living and parasitic copepods can be influenced by environmental factors (Michaud *et al.*, 2004; Gusmão and McKinnon, 2009). However, the results of this study provide evidence for a genetic sex determination mechanism in *L. salmonis*, which is supported by observations of sex ratios close to 1:1 in laboratory studies with *L. salmonis* (Johnson and Albright, 1991a; Hamre *et al.*, 2009). Cytogenetic investigations and studies of sex-linked marker heritability have suggested diverse systems of genetic sex determination

in crustaceans, with the most common ones being based on male (XX/XY) or female heterogamety (ZW/ZZ) (reviewed in: Legrand *et al.*, 1987). In decapods, genetic linkage maps have provided evidence for ZW/ZZ systems in a number of penaeid shrimps and a freshwater prawn (Li *et al.*, 2003; Staelens *et al.*, 2008; Ventura *et al.*, 2011a), whereas cytogenetic studies have suggested male heterogametic systems (XX/XY or X0/XX) in brachyuran crabs (reviewed in: Lecher *et al.*, 1995). Cytogenetic data further provide evidence for the presence of both male and female heterogametic sex determination systems among Copepoda (reviewed in: Legrand *et al.*, 1987). The available data thus illustrate that mechanisms of sex determination are not conserved among crustaceans, which parallels the situation in insects (Gempe and Beye, 2011). Interestingly, the divergent sex determination systems of insects share an evolutionarily conserved pathway involving the *transformer* gene and its downstream target *doublesex*, but differ with respect to an upstream switching mechanism (Gempe and Beye, 2011). Homologues to sex determination-related insect genes (Kopp, 2012) have been reported from *Penaeus monodon* (giant tiger prawn) (Leelatanawit *et al.*, 2009) and *Macrobrachium nipponense* (oriental river shrimp) (Qiao *et al.*, 2012). Moreover, a homologue of *doublesex* has been shown to be involved in environmental sex determination in the branchiopod *Daphnia magna* (Kato *et al.*, 2011). Together, this suggests that molecular pathways of sex determination are partially conserved between insects and crustaceans.

Relatively little is known concerning sex differentiation and its endocrine control in crustaceans, and most available data have been obtained on decapods (reviewed in: Rodriguez *et al.*, 2007). In this group, the default route of sexual development is female. Male sexual differentiation requires the presence of a male endocrine organ called the androgenic gland that produces an insulin-like factor controlling testis

function (Ventura *et al.*, 2011b). Activity of the androgenic gland in males and ovaries in females is negatively controlled by the gonad-inhibiting hormone (GIH) and positively controlled by the gonad-stimulating hormone (GSH) (Rodriguez *et al.*, 2007). Ecdysteroids are insect and crustacean hormones regulating the moulting process (ecdysis), and have been shown to stimulate ovarian growth in some crustaceans (Kato *et al.*, 2011). Exposure to the ecdysteroid 20-hydroxyecdysone increased the number of male offspring in the branchiopod *Daphnia pulex* (Water flea) and the copepod *Tisbe battagliai* (Hutchinson *et al.*, 1999; Peterson *et al.*, 2001). Some studies have further suggested roles for steroids in crustacean reproduction; however, the precise identity and function of steroid hormones in crustaceans is still unknown (Rodriguez *et al.*, 2007; Mazurová *et al.*, 2008).

The sex-linked SNP marker isolated in *L. salmonis* during the present study was shown to correspond to a synonymous polymorphism in a gene encoding a homologue to prohibitin-2. Prohibitin-2 and the related prohibitin-1 are highly conserved ubiquitous eukaryotic proteins found in the mitochondria, where they have been suggested to function as chaperone proteins (Mishra *et al.*, 2006). Prohibitins are also found in the nucleus, where they may regulate gene expression through interaction with a wide variety of transcription factors including steroid receptors. Prohibitin-2, also known as repressor of oestrogen receptor activity (REA), binds directly to the oestrogen receptor (ER), acting as a negative co-regulator of transcriptional activity (Montano *et al.*, 1999). In targeted gene disruption studies with mice, the homozygous null mutation of prohibitin-2 was lethal, whereas in heterozygous knockouts increased physiological responses to oestrogens were observed in females, but not males (Park *et al.*, 2005). Gene disruption studies in the nematode *Caenorhabditis elegans* further provide evidence for roles of prohibitin-1 in gametogenesis (Artal-Sanz and Tavernarakis,

2009). A study of a prohibitin homologue in *Eriocheir sinensis* (Chinese mitten crab) suggested a role in spermatogenesis (Mao *et al*., 2012), whereas an investigation in *P. monodon* demonstrated prohibitin-2 mRNA expression in both male and female gonads (Leelatanawit *et al*., 2009). In summary, published studies provide some evidence for sex-specific roles of prohibitins, which is in accordance with this study's finding of significantly higher mRNA levels of a prohibitin-2 homologue in adult female when compared to adult male *L. salmonis*. However, no evidence exists for a role for prohibitins in sex determination and/or sex differentiation. Following from these findings, it is clear that further research will be required to elucidate the nature of the molecular determinant(s) of sex determination in *L. salmonis* and to clarify the relationships that such determinants may have to the SNP marker reported in this study. Given the clear differences in EMB susceptibility observed between male and female *L. salmonis* and gender differences in P-gp ABC transporter expression (Heumann *et al*., 2012; Igboeli *et al*, 2012; Igboeli *et al*., 2013), which is a molecular mechanism potentially associated with reduced EMB susceptibility, the identification of a robust marker for *L. salmonis* gender may help improve our understanding of EMB resistance in the salmon louse.

Conclusions

The current study identified fifteen RAD markers that show complete association with salmon louse strain when analysing salmon lice from drug susceptible (S) and EMB-resistant (PT) laboratory strains. These markers may therefore include some that are putatively associated with EMB susceptibility, although further studies are necessary to confirm marker association with EMB susceptibility. A novel sex-linked SNP marker showing complete association with sex has also been identified in the salmon louse and suggests a genetic mechanism of sex determination in *L. salmonis* based on female heterozygosity. This sex-linked SNP marker represents a synonymous polymorphism in a prohibitin-2 homologue; however, the functional relationship of prohibitin-2 to sex determination remains uncertain. These findings contribute towards an improved understanding of sex determination in sea lice and may serve to help develop improved control strategies for this species. The identification of strain-linked SNP markers also provides genetic tools that may contribute to elucidating the molecular mechanisms responsible for EMB resistance in the salmon louse.

Chapter 6 – General Discussion

The overall aim of this study was to further our understanding of the molecular mechanisms associated with reduced susceptibility of the salmon louse *Lepeophtheirus salmonis* (Krøyer 1837) to the antiparasitic compound emamectin benzoate (EMB). Two laboratory-maintained *L. salmonis* strains that differ with respect EMB susceptibility and show stable susceptibility profiles over multiple generations were to be used as a model for these investigations. The current literature regarding avermectin (AVM) resistance in insects and nematodes would suggest that reduced EMB susceptibility in *L. salmonis* may involve target site modifications or changes in the molecular mechanisms responsible for drug detoxification. Additionally, some mechanisms involved in reduced EMB susceptibility may require transcriptional changes in response to EMB exposure, although other mechanisms may not include such changes. A global transcriptomic approach was selected for the analysis of EMB susceptibility in *L. salmonis* as such a strategy will detect transcriptional changes without bias, requiring no detailed knowledge of the biological systems being studied and facilitating the interrogation of multiple molecular mechanisms. With only limited sequence resources being available for *L. salmonis* at the beginning of this study, particularly in the absence of an annotated genome, a significant amount of the present work was concerned with generating new sequence to facilitate the analysis of EMB susceptibility in *L. salmonis* through the design of custom *L. salmonis* oligonucleotide (oligo) microarrays (Chapter 2) for the analysis of transcriptomic responses. These custom microarrays were used for the analysis of both constitutive differences in gene expression (Chapter 3) and EMB-induced transcriptomic responses (Chapter 4) in *L. salmonis* from drug susceptible (S) and EMB-resistant (PT) laboratory-maintained strains. Experiments included in this study were also aimed at the discovery of novel sequence in *L. salmonis* by generating a transcriptome through *de novo* sequencing a

pooled RNA library representing key stages of the salmon louse life cycle, using *L. salmonis* strain S (Chapter 2). Analysis of this transcriptome identified numerous candidates that may be suitable for further research to establish an association with reduced EMB susceptibility, including drug targets and drug detoxification mechanisms. Finally, complementary to the transcriptomic studies, a preliminary study used a genetic approach known as Restriction-site associated DNA sequencing (RAD-seq) to generate a collection of genetic markers in *L. salmonis* suitable for analysing genetic determinants of complex traits such as drug resistance. RAD-seq was successfully employed in the identification of Single Nucleotide Polymorphism (SNP) markers putatively associated EMB susceptibility and a robust sex-linked marker, using *L. salmonis* strains S and PT (Chapter 5).

6.1 The development of genomic resources for the study of salmon louse biology and reduced sensitivity to antiparasitics.

When this study was initiated, the genomic and transcriptomic resources available in *L. salmonis* were extremely limited, with no genome sequence assembly available. Therefore, to provide resources for the study of EMB susceptibility in *L. salmonis* a significant amount of sequence was generated during the current study through *de novo* sequencing. A major contribution to these resources stemmed from the generation of a transcriptome for *L. salmonis*, using representative samples from key stages of the life cycle. This transcriptome was produced using samples from the *L. salmonis* strain S, and therefore repeating this process with strain PT and/or other independent *L. salmonis* strains would generate a more comprehensive transcriptome resource for further research, although this was not achievable within the financial constraints of this project.

Two complementary methods were used for the assembly of these transcripts, where TopHat v2.0.4 (Kim *et al.*, 2013) used NCBI assembly ASM18125v2 of the *L. salmonis* genome as a scaffold for reference based assemblies and Trinity release 2012-06-08 (Grabherr *et al.*, 2011) was used to build a *de novo* assembly from the remaining sequence reads. A relatively stringent sequence assembly strategy was employed during this study to ensure the accuracy of assembled transcripts, which may explain conservative transcript numbers obtained compared to other species. A recent study (Yasuike *et al*., 2012) detailed sequence assembly using *L. salmonis* ESTs, which generated ~50% fewer putative transcripts in Atlantic and Pacific *L. salmonis* (14,466 and 16,108 respectively) compared to the current study. While several factors differed between this study and Yasuike *et al.*, the high power of next generation sequencing (NGS) approaches is likely to be the main reason for the higher number of putative transcripts obtained in the present work. This is supported by the fact that estimates of the number of putative transcripts generated for other copepods using NGS methods are at least in the range of that reported for *L. salmonis* here, *e.g.* ~40,000 for *Tigriopus californicus* (Barreto *et al.*, 2011) and 56, 809 for *Calinus sinicus* (Ning *et al.*, 2013). Transcriptomes have also been generated for a variety of crustaceans including *Parhyale hawaiensis* (Zeng *et al.*, 2011), *Macrobrachium rosenbergii* (Ventura *et al.*, 2013), *Macrobrachium nipponense* (Ma *et al.*, 2012) and *Euphausia superb* (Clark *et al.*, 2011) where putative transcript numbers varied from 22,177 (*E. superb*) to 81,411 (*M. nipponense*). A majority of these transcriptomes were generated using the alternative Roche 454 NGS platform (Barreto *et al.*, 2011; Clark *et al.*, 2011; Zeng *et al.*, 2011; Ma *et al.*, 2012; Ning *et al.*, 2013) and transcripts were assembled using a variety of software and strategies that differed from those used in the current study. It is also worth noting that differences in transcript assemblies have been demonstrated

between different versions of the Newbler software used to assemble Roche 454 sequence reads (Kumar and Blaxter, 2010). Given that *L. salmonis* has a relatively small genome size of 600 Mbp (The Salmon Louse Genome Project, 2013); the identification of 33,693 putative transcripts during this study is in line with the results detailed above that were obtained for other crustaceans, although it may be a rather conservative estimation.

Annotation of the *L. salmonis* transcriptome generated during this study identified members of gene families potentially involved in the toxicology of EMB. In particular, the family of ligand-gated ion channel (LGIC) subunits that is assumed to contain the putative pharmacological targets of AVMs, in addition to ABC (ATP-binding cassette) transporters and the detoxification enzymes cytochrome P450s (CYPs), glutathione-s-transferases (GSTs) and esterases that are members of gene families involved in detoxification pathways. Members of these gene families have previously been associated with drug resistance in ecdysozoan invertebrates and are therefore of interest for the study of EMB resistance in *L. salmonis*. When comparing the number of candidates identified in the transcriptome with those found among the sequences assembled from existing *L. salmonis* ESTs, it is evident that there are considerably more candidates in the *L. salmonis* transcriptome, which accordingly provides a larger pool of genes for further analysis to establish potential associations with reduced EMB susceptibility in *L. salmonis*. However, these candidates were identified in the *L. salmonis* transcriptome based on their functional annotation, which is likely to provide an under estimation of the true transcript numbers in the salmon louse. A more accurate and efficient method for candidate gene identification would be to employ a bioinformatic pipeline to align conserved signature motifs to query transcript sequences and perform BLASTx annotation, similar to that used to identify candidate insecticide

targets in the oriental fruit fly (*Bactrocera dorsalis*) (Hsu *et al.*, 2012) and greenhouse whitefly (*Trialeurodes vaporariorum*) (Karatolos *et al.*, 2011) transcriptomes, however, this was not achievable within the time constraints of the current study but will be performed in future studies.

Current knowledge of the gene families commonly associated with ecdysozoan drug resistance is very limited in sea lice (Tribble *et al.*, 2007a; Tribble *et al.*, 2007b Heumann *et al.*, 2012; Igboeli *et al.*, 2012; Igboeli *et al.*, 2013). While this study probably doesn't provide a comprehensive list of members from these gene families in *L. salmonis*, a wide array of candidate genes have been identified with potential roles in reduced EMB susceptibility that can be explored in future studies of salmon lice. This may include RNA interference (RNAi) studies that is a promising approach for functional studies of candidate genes, which has successfully been employed in the characterisation of a yolk-associated protein (LsYAP) (Dalvin *et al.*, 2009) and analysis of developmental and tissue distribution of prostaglandin E_2 synthase (Campbell *et al.*, 2009) in *L. salmonis*.

6.2 Transcriptional profiles observed in the absence of drug exposure in two salmon louse strains with differing EMB susceptibilities.

The main objective of transcriptomic profiling *L. salmonis* in Chapter 3 was to determine if any constitutive differences in mRNA expression existed between strains S and PT, which could be due to genetic changes in strain PT and may be associated with reduced EMB susceptibility. A global transcriptomic analysis strategy was used in the current study to avoid the limitations associated with candidate gene studies and increase the possibility of discovering novel mechanisms associated with drug resistance that may involve multiple gene families. However, measurement of mRNA

levels does not necessarily reflect protein levels that can be influenced by post-transcriptional regulatory mechanisms, and transcriptomic profiling cannot be used to detect mutations in the coding regions of genes encoding drug target sites or detoxification enzymes unless these gene mutations also result in changes in gene expression.

A significant limitation of the experimental design used in this study was that only one drug susceptible (S) and one EMB-resistant (PT) strain were available as a model for the experiments, due to the prohibitively high cost of establishing and maintaining *L. salmonis* on the salmonid host. Current literature would suggest that there is limited genetic variation among Atlantic *L. salmonis* populations (Glover *et al*., 2011) which suggests that transcriptomic responses identified using these strains would represent true responses in field *L. salmonis* populations, although it is possible that genetic diversity could be reduced in laboratory strains S and PT. Molecular determinants of ivermectin (IVM) resistance were identified in other ecdysozoan pests using transcriptomic profiling approaches (James and Davey, 2009; Dicker *et al*., 2011; Bariami *et al.*, 2012) which supports the decision to select a similar experimental design when exploring reduced EMB susceptibility in the salmon louse.

The transcriptomic profiling of *L. salmonis* during this study identified reduced expression of a wide variety of transcripts in strain PT compared to strain S, which were enriched for functions such as calcium ion binding and chitin metabolism that could not be directly associated with reduced susceptibility to EMB in the PT strain, but may indicate general differences between the two strains. Stress responses in marine copepods have been shown to have high intra- and inter-species variability that can be associated with previous exposure to stressors (Lauritano *et al.*, 2012). Strains S and PT differ with respect to drug exposure history, which will influence general stress

responses of these salmon lice. Alternatively, these results may indicate that there were problems with the synchronisation of cohorts of the two strains used for transcriptomic analysis. In order to mitigate potential random effects, constitutive differential gene expression between strains S and PT was analysed in two independent experiments, although only six replicate pools were used for each strain in the two experiments. If these results are considered as a true representation of differential gene expression between strains S and PT, then reduced expression of genes involved in metabolic homeostasis in strain PT may be indicative of differences in metabolism and growth rate between the strains. Increased expression of detoxification mechanisms can often have a fitness cost for the parasite through the need to reallocate resources and energy to facilitate drug metabolism that may impact development and reproduction or general health and survival of the individuals (Kliot and Ghanim, 2012). It may be possible that salmon lice from strain PT have a reduced metabolic rate to allow for fitness costs associated with allocating metabolic resources to mechanisms required to reduce the toxicity from EMB exposure. It has also been demonstrated that insecticide resistance has been associated with cuticle thickening in terrestrial arthropod pests (Wood *et al.*, 2010; Lin *et al.*, 2012) which reduces penetration of the active ingredient when it is applied topically. This type of resistance mechanism is unlikely to directly apply in *L. salmonis* resistance to EMB as field EMB exposure is normally through ingestion with food material.

This study identified a constitutively reduced mRNA expression of neuronal acetylcholine receptor (nAChR) and γ-aminobutyric acid gated chloride channel (GABA-Cl) subunits in the PT strain compared to strain S. The molecular targets of AVMs in crustaceans are not currently known, although glutamate-gated chloride channels (GluCls) are thought to be the main pharmacological target in nematodes and

insects (Cully *et al.*, 1994; Cully *et al.*, 1996; Dent *et al.*, 2000; Kane *et al.*, 2000; Bloomquist, 2003), however GABA-Cls are also thought to be targeted by AVMs (Feng *et al.*, 2002) and the AVM compound IVM has been shown to influence the activity of nAChRs (Krause *et al.*, 1998). Target site mutations are not necessarily associated with changes in gene expression but some studies demonstrate the influence of point mutations on the control mechanisms responsible for gene activation and expression (Beech and Silvestre, 2010; Beech *et al.*, 2011). This study's finding of reduced mRNA levels of nAChR and GABA-Cl subunits in the PT strain suggests that these LGIC subunits may represent additional pharmacological target sites for EMB in salmon lice.

Somewhat surprising, this study found no evidence for differences between the strains analysed with regard to mRNA levels of genes encoding enzymes and transporters involved in detoxification pathways. While comparatively little is known in crustaceans about the nature and role in environmental adaptation of detoxification pathways, these mechanisms play major roles in drug resistance in terrestrial ecdysozoan invertebrate pest species (Bass and Field, 2011). As genes involved in detoxification pathways may show altered mRNA levels either constitutively or in response to toxicant exposure, the involvement of detoxification pathways in reduced EMB susceptibility of *L. salmonis* will be discussed in section 6.3.

6.3 Analysis of transcriptomic responses in two *L. salmonis* strains following exposure to EMB.

The study reported in Chapter 4 of this thesis investigated transcriptomic responses induced by short-term EMB exposure of salmon lice from strains S and PT, which revealed modest changes in expression of a complex array of transcripts in salmon lice from strain S and few responses to exposure of *L. salmonis* from strain PT. As the present study involved sequencing subtractive cDNA libraries between the two

L. salmonis strains being studied and the inclusion of these transcript sequences on the microarrays employed in expression studies, it would be expected that any genes showing dramatic changes in expression between the strains should have been detected. The observations of this study did not include any changes in drug transporters or enzymatic defence mechanisms that are commonly associated with drug resistance in ecdysozoans which may suggest that the selected EMB exposure period and sampling points did not capture the period of exposure that elicits toxic responses in strain S or induces resistance mechanisms in strain PT. Additionally, the EMB concentration selected for this study may not have induced response levels in either *L. salmonis* strain that could be measured within the detection limits of a microarray hybridisation approach. The differences in route of EMB exposure between oral field exposure and aqueous laboratory exposure may have a significant influence on the transcriptomic responses measured in these experiments. It may be necessary for future investigations of EMB-induced responses in the salmon louse to use experiments where the parasite is exposed to EMB through natural exposure routes, although these experiments would involve replicate tanks of host fish which would be considerably more expensive than using an aqueous exposure assay and was not achievable within the financial constraints of this study. A reduction in EMB concentration was measured in the exposure solution sampled after completion of the assays in this study, which may be attributed to EMB adsorption to the glass containers used for exposure assays (Helgesen and Horsberg, 2013b) and could have influenced the transcriptomic response levels measured during this study.

The modest responses observed from exposure of strain S lice may be due to general responses to low-level toxicity and/or stress responses to drug exposure, removal from their host and/or the effects of the bioassay procedure. Detoxification

enzymes have been shown to be involved in the stress response of marine copepods (Lauritano *et al.*, 2012). Therefore, general stress response mechanisms in both strains that involve these detoxification enzymes could obscure any additional associations the enzymes may have with reduced EMB susceptibility in *L. salmonis*. Laboratory strains S and PT do not share a common genetic background and therefore differences in gene expression could be related to differential stress responses to EMB exposure or may reflect strain differences that are unrelated to EMB susceptibility. The results of this study may also suggest that non-essential cellular processes are reduced in strain PT, as numerous genes were down regulated in this strain including cytoskeletal proteins and associated binding proteins.

The P-glycoprotein (P-gp) ABC transporters have been found to be upregulated upon AVM exposure of several AVM resistant nematodes (Prichard and Roulet, 2007; James and Davey, 2009; Lloberas *et al.*, 2013) and therefore P-gp association with reduced EMB susceptibility in *L. salmonis* was explored in the current study. The P-gp homologue (SL-PGY1) and transcripts for three MRP in *L. salmonis* were represented on the microarray used in the current study but were not found to be significantly differentially expressed upon EMB exposure of strains S and PT. The results from this study do not provide evidence to support the involvement of these ABC transporters in reductions of EMB susceptibility in *L. salmonis* strains S and PT, however, post-translational phosphorylation of ABC transporter proteins could be involved in the control of EMB induced transporter activation, but would not be detected in the current study (Stolarczyk *et al.*, 2011).

Drug resistance of some insect and mite species have been associated with gene expression changes for different CYP genes and isoforms (Schmidt *et al.*, 2010; Liu *et al.*, 2011; Tao *et al.*, 2012) with only some isoforms being inducible upon drug

exposure (Liu *et al.*, 2011). No consistent regulation of CYP gene expression in response to EMB exposure of *L. salmonis* was observed in the present study. This observation is difficult to interpret, as the roles of CYPs in crustaceans and their inducibility by xenobiotics are not completely understood (Baldwin *et al.,* 2009; Dam *et al.,* 2008; Koenig *et al.,* 2012). It cannot be excluded that smaller changes in CYP expression may have remained undetected in the present study, particularly as the number of biological replicates (n=3-6) and thus statistical power was restricted in microarray experiments, and in the absence of a fully annotated genome assembly, there is no certainty that all CYP isoforms of *L. salmonis* were represented on the microarray. This study also did not provide evidence for the role of GST and esterase enzymes in reduced EMB susceptibility of *L. salmonis*. These enzymes have often been associated with insecticide resistance (Wang *et al,* 1991; Raymond *et al.*, 1998; Ranson *et al*, 2001; Wei *et al*, 2001; Hawkes and Hemingway, 2002; Cui *et al.* 2006), however it was not possible to fully represent these gene families during the current study.

To further explore the existence of EMB-induced mechanisms in *L. salmonis* using laboratory-maintained strains there are a number of strategies that could be employed in future experiments. Initially, it would be necessary to perform larger experiments that include numerous different EMB concentrations and exposure times, which was not achievable within the financial and time constraints of the current study. Additionally, these experiments should include a higher number of replicates and use multiple independent *L. salmonis* strains where all the cohorts used in the analysis are carefully synchronised The EMB-resistant strain (PT) available for this study is only moderately (~7-fold) resistant and therefore it would be beneficial to use aqueous EMB exposure assays to select resistant salmon lice for subsequent breeding of a highly resistant strain after numerous rounds of selection. Transcriptomic analysis of a highly

resistant *L. salmonis* strain in comparison to strain S would hopefully illuminate putative EMB-resistance mechanisms more efficiently. Additionally, the modification of existing EMB aqueous exposure bioassays to allow the selection of salmon lice with different degrees of EMB susceptibility may help in elucidating resistance mechanisms by selecting highly susceptible and resistant individuals for transcriptomic profiling. The alternative global transcriptomic analysis strategies mentioned above could include the use of an improved oligo microarray, which was described during Chapter 2 of this thesis and includes oligo probes that represent the transcriptome sequence also generated during this study. However, the most superior strategy for exploring transcriptomic responses associated with EMB resistance in *L. salmonis* would be to use RNA-seq, a NGS strategy that can not only be used for differential gene expression analysis, but can also detect splice variant abundances or target site mutations that cannot be detected using a microarray hybridisation strategy (Wang *et al.*, 2009).

6.4 Use of genome-wide SNP discovery to explore complex traits including drug resistance in the salmon louse.

Reduced drug susceptibility in ecdysozoan invertebrates has previously been associated with point mutations in target subunits that may influence the affinity of target sites for the drug or influence LGIC conformation upon drug binding (Beech and Silvestre, 2010; Beech *et al.,* 2011). Additionally, genetic polymorphisms may exist in EMB resistant *L. salmonis* that influence the control mechanisms responsible for gene activation and expression, or prevent the expression of functional proteins (Beech and Silvestre, 2010; Beech *et al.,* 2011). The transcriptomic analysis method used in Chapters 3 and 4 of this thesis is an approach that does not detect such genetic mutations and therefore a complementary genetic method was employed to identify SNPs in *L. salmonis.* RAD-seq technology was used to search for SNPs in *L. salmonis* through comparison of RAD-tag sequences between male and female salmon lice from strains S and PT. The selection of this approach was supported by previous studies that used similar approaches to identify associations between genetic markers and drug resistance in the diamondback moth (*Plutella xylostella*) (Baxter *et al.*, 2011) and the two-spotted spider mite (*Tetranychus urticae*) (Van Leeuwen *et al.,* 2012). In this study, a RAD-seq strategy successfully identified 15 SNP markers that were identified as polymorphic when comparing strains S and PT and were therefore putatively associated with reduced EMB susceptibility. As laboratory-maintained strains S and PT do not have the same genetic background further analysis is required to confirm an association of RAD markers with EMB resistance as was discussed in Chapter 5 of this thesis. Additionally, generation of F2 crosses using strain S and a highly EMB resistant *L. salmonis* strain would allow Quantitative Trait Locus (QTL) analysis for the association of SNP markers with EMB susceptibility, which was not possible during the current

study due to financial constraints. The suggestion of such an approach is supported by successfully incorporation of RAD-seq in a QTL study of Infectious Pancreatic Necrosis (IPN) in Atlantic salmon (Houston *et al.*, 2012). The identification of a robust SNP marker for reduced EMB susceptibility does however provide a valuable genetic tool that could be used for rapid identification of less susceptible field populations and incorporated in pest control strategies.

The study reported in Chapter 5 further included the identification of SNP marker *Lsa*101901 in *L. salmonis* that showed complete association with sex in 96 genotyped individuals from three different strains. These results strongly suggest that sex determination in *L. salmonis* is genetic and provide evidence for a female heterogametic (ZW/ZZ) system. Sex ratios of wild and laboratory-maintained populations of mobile *L. salmonis* are influenced by environmental factors and farm management practices but are usually close to 1:1 (Johnson and Albright, 1991b; Bron *et al*., 1993b; Ritchie *et al*., 1996; Hamre *et al*., 2009) which is consistent with a genetic mechanism of sex determination as suggested in this study. Cytogenetic investigations and studies of sex-linked marker heritability have identified diverse systems of genetic sex determination in crustaceans with male (XX/XY) or female heterogamety (ZW/ZZ) being most common (Legrand *et al*., 1987). Male and female heterogametic sex determination systems have also been identified in copepoda (Legrand *et al*. 1987) which suggests that it is possible that a ZW/ZZ genetic sex determination exists in *L. salmonis* as suggested in this study. The sex-linked SNP marker identified in this study represents a synonymous polymorphism in a prohibitin-2 homologue; however, the functional relationship of prohibitin-2 to sex determination remains uncertain. It has previously been demonstrated that there is differential EMB susceptibility between *L. salmonis* gender, with females being more susceptible than males (Westcott *et al.*, 2008). It has

also been recognised that the chalimus phase of the *L. salmonis* life cycle comprises two stages (Hamre *et al.*, 2013), rather than the previous hypothesis that there was successive chalimus stages (Johnson and Albright, 1991b; Pike and Wadsworth, 1999). This sex-linked SNP marker may therefore contribute to better understanding of how EMB susceptibility could be influenced in *L. salmonis* populations and the study of chalimus stage *L. salmonis* sex-ratios on salmonid hosts, to better understand how *L. salmonis* reproduction could be manipulated in the development of non-chemical based control strategies.

Conclusions

The studies detailed in this thesis demonstrate the use of current genomic and transcriptomic approaches to improving our understanding of the molecular mechanisms potentially involved in reduced *L. salmonis* EMB susceptibility. As a fully annotated *L. salmonis* genome is yet to be released, this project involved supplementing existing *L. salmonis* sequence resources to enable the study of EMB resistance. These new sequence resources facilitated the design of oligo microarrays for the analysis of transcriptomic responses in *L. salmonis* and provided candidates putatively associated with EMB resistance. The *L. salmonis* microarrays developed in this study were used for the transcriptomic profiling of constitutive gene expression and EMB-induced responses in the *L. salmonis* laboratory strains S and PT, which differ in susceptibility to the anti-parasitic drug EMB. Constitutively lower mRNA levels of nAChR and GABA-Cl subunits were found in the EMB resistant strain PT when compared to the reference strain S. In contrast, very few EMB-induced transcriptomic responses were measured in strain PT. In addition, RAD-seq technology was employed to discover SNPs in *L. salmonis* and perform parallel genotyping of individuals from strains S and PT. Genetic analysis identified SNP markers that were putatively associated with EMB susceptibility, and further isolated a sex-linked SNP marker showing complete association with gender in three different *L. salmonis* laboratory-maintained strains. The identification of alternative molecular targets for EMB and strain-linked SNP markers putatively associated with reduced *L. salmonis* EMB susceptibility provides candidates for future studies to establish an association with EMB susceptibility in *L. salmonis*. Finally, a robust SNP marker for *L. salmonis* gender may help advance our understanding of sex determination in sea lice and the development of improved control strategies for this species.

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Publications and Conferences

PUBLICATIONS

Carmichael, S.N., Bekaert, M., Taggart J.B., Christie, H.R.L., Bassett, D.I., Bron J.E., Skuce, P.J., Gharbi, K., Skern-Mauritzen, R. and Sturm, A. (2013) Identification of a sex-linked SNP marker in the salmon louse (*Lepeophtheirus salmonis*) using RAD sequencing. *Plos One* **8** (10), e77832.

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CONFERENCE PRESENTATIONS

Carmichael, S.N., Tildesley, A., Burgess, S.T.G., Ireland, J.H., Taggart, J.B., Skuce, P.J., Bron, J.E. and Sturm, A. (2012) A study of gene expression in Atlantic *Lepeophtheirus salmonis* populations with differing susceptibilities to emamectin benzoate. *Institute of Aquaculture PhD Research Conference*, 24th October 2012, Stirling, UK. **Poster presentation**.

Carmichael, S.N., Tildesley, A., Burgess, S.T.G., Ireland, J.H., Taggart, J.B., Skuce, P.J., Bron, J.E. and Sturm, A. (2012) A study of gene expression in Atlantic *Lepeophtheirus salmonis* populations with differing susceptibilities to emamectin benzoate. *9th International sea lice conference 2012*, 21st May 2012, Bergen, Norway. **Poster presentation**.

Carmichael, S.N., Nisbet, A.J., Tildesley, A., Gharbi, K., Skuce, P.J., Bron, J.E. and Sturm, A. (2011) High-throughput analysis of genes constitutively expressed in two populations of *Lepeophtheirus salmonis* with differing susceptibilities to emamectin benzoate. *Marine Alliance for Science & Technology (MASTS) Annual Science Meeting*, 22nd August 2011, Edinburgh, UK. **Poster presentation**.

Carmichael, S.N., Nisbet, A.J., Tildesley, A., Gharbi, K., Skuce, P.J., Bron, J.E. and Sturm, A. (2011) High-throughput analysis of genes constitutively expressed in two populations of *Lepeophtheirus salmonis* with differing susceptibilities to emamectin benzoate. *Society for Experimental Biology (SEB) annual main meeting*, 1st July 2011, Glasgow, UK. **Poster presentation**.

Carmichael, S.N., Skuce, P.J., Bron, J.E. and Sturm, A. (2010) Transcriptomic responses of the salmon louse *Lepeophtheirus salmonis* to emamectin benzoate. *Institute of Aquaculture PhD Research Conference*, 28th June 2010, Stirling, UK. **Poster presentation**.

Appendix

Appendix 1 General buffers & solutions

RNA Stabilisation Solution
(4.54 M ammonium sulphate, 25 mM trisodium citrate, 20 mM EDTA, pH 5.4)

Constituent	Volume/ Weight
0.5 M EDTA (pH 8.0)	20 ml
1 M Sodium Citrate	12.5 ml
Ammonium sulphate	300g
Milli Q Water	467.5 ml

RNA Precipitation Solution (0.8M Sodium Citrate, 1.2M Sodium Chloride)

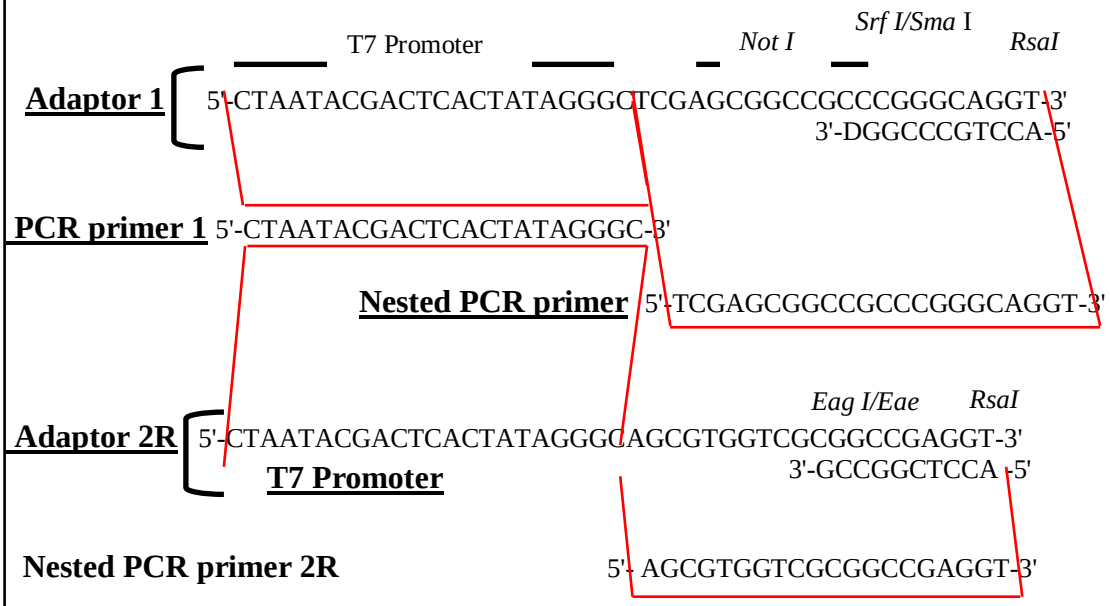
Constituent	Weight (g)
Sodium Citrate ($C_6H_6Na_2O_7 1.5H_2O$)	10.524
Sodium Chloride	3.506

Appendix 2 Salmon louse samples included in a total RNA pool for transcriptome sequencing.

Sample ID	Salmon louse stage
1	Egg strings (Light colouration)
2	Egg strings (Dark colouration)
3	Nauplius (24 hrs growth at 8 °C)
4	Nauplius (24 hrs growth at 10.5 °C)
5	Nauplius (48 hrs growth at 8 °C)
6	Nauplius (48 hrs growth at 10.5 °C)
7	Free-living Copepodid
8	Attached Copepod (24 hrs dpi)
9	Attached Copepod (48 hrs dpi)
10	Chalimus Ia (72 hrs dpi)
11	Chalimus Ib (96 hrs dpi)
12	Chalimus Ic
13	Chalimus 2a
14	Chalimus 2b
15	Preadult 1 Male
16	Preadult 2 Male
17	Adult Male
18	Preadult 1 Female
19	Preadult 2 Female
20	Adult virgin Female
21	Adult gravid female

Appendix 3 PCR-Select™ cDNA subtraction kit - Adaptor and primer sequences.

cDNA synthesis primer 5'-TTTTGTACAAGCTT$_{30}$N$_1$N-3'



Appendix 4 cDNA library details for the Nilsen Norway EST sequences.

Library ID	Library description	Number of Sequences
CC	Chalimus	120
FB	Adults (Colon/Intestine)	707
HA	Adults	680
L1T	T1 Adults	1,513
LF	Preadult I and II (with filament)	1,806
LNC	Copepodites	3,348
LNO	Preadult I and II (with filament)	16,079
LPA	Preadult Stages	89
LPU	Preadult Stages	1,340
ME	Adults (with blood)	4,031
NA	Nauplius	66
NLG	Egg string	3,541
PU	Preadult II	602
SB	Adult	142
	Total	34,064

The 35,577 EST sequences that originated from the 'Nilsen Norway' sequencing group were assessed to remove the short sequences (<100 bp). The remaining 34,064 sequences were grouped according to the sequenced cDNA library as detailed.

Appendix 5 *L. salmonis* oligonucleotide microarray designs

Microarray design	GAL file name
15K microarray design 1	033382_D_20110405.gal
15K microarray design 2	039612_D_20120319.gal
44K microarray design 1	048507_D_20130327.gal

Appendix 6 Reference gene primers for RT-qPCR analysis of relative gene expression (Chapters 3, 4 and 5).

Accession No.	Annotation	Primer sequence (5' - 3')	Ta	Fragment size (bp)
ACO14905.1	Hypoxanthine-guanine phosphoribosyltransferase (HGPRT)	GCAGCAAACATCGAATCTCA TCTTTGCACGAACAAACTGC	55	187
ACO15319.1	RMD5 homolog A	TCTCCTTATGCCCACTTGCT GAGTTCCGTCCTTTGCATTC	55	220
EF490880.1	Elongation factor 1α	CCAAATTAAGGAAAAGGTCGACAGACGTACTG TGCCGGCATCACCAGACTTGA	60	86
BT121430.1	40S ribosomal protein S20	AGTGTGGCCGGTGTTTAACAATCATCAA GGGCTTCGAGTCCTTGTATGCTGCTGCTACT	60	86
ACO10279.1	60s ribosomal protein L44	CCTAGCTGCAATCACCATGA CTCTTGCACTTGCTGCACTC	55	197

Appendix 7 Primers used for RT-qPCR analysis of relative gene expression between salmon louse strains (Chapter 3).

Accession No.	Annotation	Primer sequence (5' - 3')	Ta	Fragment size (bp)
ADD24187.1	Neuronal acetylcholine receptor subunit α3	GAATTTTGGTGAGGGGGAAT ACCATTGGACTTGACGATCC	55	208
EFN73916.1	GABA receptor subunit alpha	AATCTCACCGGATGGTCTTG ATGGTTGCTTTGGCAAGAGT	55	201
XP_003494528.1	Cytochrome p450 18a1	GAAAATTTGGCTCGAATGGA TTTTCACGTCCCGTGGTATT	55	163
AAS13464.1	Cytochrome p450 15a1	AAATGAGGCTCGCTTTACCA CCTTGCTGCTGGGATGTAAT	55	229
NP_001136104.1	Carboxylesterase	CAATTATTGGGCATGGCTCT CTTTCCCATTTTCCCACTCA	55	155
XP_797271.2	Maltase-glucoamylase	CAGGATCCAGGTCTCTTTGG GATTTCGGCAAACCATGTCT	55	180

Appendix 8 Primers used for RT-qPCR analysis of relative gene expression between on exposure to EMB (Chapter 4).

Accession No.	Annotation	Primer sequence (5' - 3')	Ta	Fragment size (bp)
AAS91796.1	Intestinal trypsin 5 precursor	GGAGTGGATCGCATTTTTGT CCAAAATGCAAACGTCATTG	55	226
XP_001850227	Metalloproteinase	GAAGATGCCTGCAAAATGGT ATCGCTTTTCGAATCAAGGA	55	163
ADD24462.1	Cerebellin-3	AAACGATGGGCGTATTTCAG GAGAATCCATGCGGTTTTGT	55	163
ADD38711.1	nAChR α3	TGGGCTGTCCATGAATGTAA CTCGGGACAGCACACATAAA	55	181
NP_001136346.1	Cuticular protein	GTGTCATCGGCTCATGTGTT CGAACTTTGTGTGGGTCCTT	55	225
ADD38289.1	Gamma-crystallin A	GGATTTATGGGAATCGAGCA ACTTGATCGGCAAAATGTCC	55	224
ACM68948.1	Selenium-dependent glutathione peroxidase	CGAACAGACATTGCTTGGAA GCAAATTCAGGCCATCCTAA	55	196
BAI79321.2	Duplex-specific nuclease	GCGGCTCCTCAATATCAAAG GTAACGAGGAGCGGGTATCA	55	188
ACO12859.1	Chloride intracellular channel exc-4	ACATTCCACCATCCCATCAT CAGGGCTTTCGGATGTATGT	55	190
ADD38201.1	TIP41-like protein	TCTCCCAAAGGTGGTTCATC AAAACCTCCACTGGACATGC	55	237

Appendix 9 Salmon louse immotility assessment time points

Observation	Time of exposure (hours)
1	0.3
2	0.7
3	1.0
4	1.5
5	2.0
6	2.5
7	3.0
8	4.0
9	5.0
10	6.0
11	8.0
12	10.0
13	12.0
14	15.0
15	18.0
16	21.0
17	24.0

Appendix 10 Illumina® adapter and primer sequences

P1 adapter sequences

P1 top:
5´AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTxx
xxxTGC*A -3´

P1 bottom:
5´PxxxxxAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTAT

CAT*T-3´

P2 paired end (PE) adapter sequences

P2 top:
5'-P-GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCAGAACAA-3'

P2 bottom:
5'CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGA

TC*T-3'

PE PCR Primer 1.0 5´-AATGATACGGCGACCACCGA-3'

PE PCR Primer2.0 5´-CAAGCAGAAGACGGCATACGA-3'

PE sequence primer - Read 1

5´-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

PE sequence primer - Read 2

5´-CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-3'

"P" denotes a phosphate group.

"x" refers to barcode nucleotides.

* denotes a phosphorothioate bond introduced to confer nuclease resistance to double stranded oligo.

Appendix 11 Multiplex barcode assignment and RAD-tag identification for individual samples from drug-sensitive (S) salmon lice.

ENA Sample ID	Barcode ID	Gender	Barcode Sequence	RAD Library	Filtered reads	Total RAD-tags
ERS225423	14	Female	CTCTT	2	2584676	130107
ERS225424	B05	Female	GAAGC	4	7065732	161378
ERS225425	18	Female	CCCCA	2	1923370	117510
ERS225426	B10	Female	GGGGA	4	5319672	157878
ERS225427	15	Female	CGTAT	1	2706698	134748
ERS225428	13	Female	CTTCC	3	3002946	131562
ERS225429	B11	Female	GTACA	4	6600830	158765
ERS225430	11	Female	AGTCA	3	2597410	125933
ERS225431	16	Female	CGCGC	1	2271838	128326
ERS225432	7	Female	GCTAA	1	2848806	134932
ERS225433	17	Female	CCTTG	2	2294860	124569
ERS225434	12	Female	AGCTG	3	2733504	128068
ERS225420	5	Male	GGTTC	3	3267350	137270
ERS225435	10	Male	GACTA	2	2855436	140236
ERS225421	9	Male	GATCG	3	3582112	145417
ERS225436	24	Male	TGCAA	4	2824930	148666
ERS225437	4	Male	GTCAC	1	2745824	138293
ERS225438	1	Male	ATTAG	1	2047938	128194
ERS225439	3	Male	GTTGT	2	3413014	151534
ERS225440	25	Male	TCTCT	4	897604	79038
ERS225441	8	Male	GCCGG	3	3454646	140592
ERS225442	2	Male	ATCGA	2	2706406	132272
ERS225443	B01	Male	CGATA	4	11697094	197636
ERS225436	6	Male	GGCCT	1	3590478	148027

Unique five base nucleotide barcodes were assigned to each salmon louse DNA sample. These samples were included in a multiplex RAD library and sequenced, which generated sequence reads that were quality filtered and used for the identification of paired-end RAD-tags in ≥ 75 % of the samples.

Appendix 12 Multiplex barcode assignment and RAD-tag identification for individual samples from EMB-resistant (PT) salmon lice.

ENA Sample ID	Barcode ID	Gender	Barcode Sequence	RAD Library	Filtered reads	Total RAD-tags
ERS226918	E5	Female	AGAGT	1	38702	1619
ERS226913	B04	Female	CTGAA	1	1446160	108344
ERS226908	D02	Female	TAGCA	1	10337800	158315
ERS226910	B08	Female	GCGCC	2	11648734	159572
ERS226912	D04	Female	TCGAG	2	9825154	157526
ERS226914	C5	Female	TGACC	2	22432	863
ERS226911	B06	Female	GAGAT	3	17341138	163812
ERS226915	B07	Female	GCATT	3	17928016	163311
ERS226907	27	Female	TATAC	3	2864762	127441
ERS226916	D09	Female	AACCC	4	5107894	148517
ERS226917	D11	Female	ACCAT	4	4780420	147905
ERS226909	D12	Female	ACTGC	4	4606692	144340
ERS226923	B03	Male	CTAGG	1	23818866	196804
ERS226930	26	Male	TCCTC	1	3034598	137401
ERS226927	23	Male	TGTGG	1	5571176	178547
ERS226922	B02	Male	CGGCG	2	17290824	188769
ERS226928	28	Male	TACGT	2	5386784	170448
ERS226920	21	Male	TTTTA	2	3244760	142556
ERS226924	20	Male	CACAG	3	3360070	146687
ERS226919	19	Male	CATGA	3	3127890	139270
ERS226925	22	Male	TTCCG	3	4342760	182092
ERS226926	B12	Male	GTGTG	4	10182526	176914
ERS226929	D01	Male	TAATG	4	8415250	210118
ERS226921	D03	Male	TCAGA	4	5587078	157408

For further details please see legend for Appendix 11.

Appendix 13 Primers used for RT-qPCR analysis of prohibitin-2 relative gene expression between male and female salmon lice (Chapter 5).

Accession No.	Annotation	Primer sequence (5' - 3')	Ta	Fragment size (bp)
BT121810.1	Prohibitin-2	GCGTATTCCCGACCAAAACT GAAGTTCTCAAGGGCGTTGT	55	167

Appendix 14 Additional file details

Additional file 2.1 Annotation of *L. salmonis* transcriptome sequences putatively associated with reduced EMB susceptibility.

Transcripts were identified as members of gene families putatively associated with reduced drug susceptibility in ecdysozoan invertebrates based on BLASTx annotation.

Additional file 2.2 GAL format file detailing the *L. salmonis* 15K oligo microarray design 1.

Description of oligo probe positions on the *L. salmonis* 15K microarray design 1.

Additional file 2.3 GAL format file detailing the *L. salmonis* 15K oligo microarray design 2.

Additional file 2.4 GAL format file of the *L. salmonis* 44K oligo microarray.

Additional file 4.1 Genes identified from the features grouped in network cluster 1.

Changes in expression of genes in cluster 1 (Chapter 4, Figure 4.6) observed following exposure of two salmon louse strains to EMB, expressed relative to gene expression in the matching solvent (SOL) control. Annotated genes (35%) are arranged by category of biological function. Features with identical annotation were removed prior to categorising biological function.

Additional file 4.2 Genes identified from the features grouped in network cluster 2.

Changes in expression of genes in cluster 2 (Figure 5) observed following exposure of two salmon louse strains to EMB, expressed relative to gene expression in the matching solvent (SOL) control. For further details, please see legend of Additional file 4.1.

Additional file 5.1 SNP alleles and RAD marker allele sequences for the *L. salmonis* strain-linked RAD markers.

Twenty seven strain-linked SNP alleles and RAD marker allele sequences were identified through the comparison of RAD-tag sequences between *L. salmonis* strains S and PT.