Molecular Characterisation of Immune Genes in Yellowtail Kingfish (*Seriola lalandi*)

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Abstract

The effective and efficient introduction of a new candidate species for aquaculture depends greatly on the understanding of the physiology of the organism and the ability to monitor a candidates' response to changes in their environment. For any farmed fish in an aquaculture setting, there will be a number of bottlenecks that restrict optimal growth and production. Being able to monitor and maintain health is one of these, with stress and pathogenic invasion from microbial organisms leading to a reduction in farming efficiency. Due to the very nature of aquaculture, stress and transmission of pathogens are very common, as fish are contained in close quarters. Thus, characterising and understanding the immune system of these fish will allow for better monitoring of fish health.

Yellowtail Kingfish (*Seriola lalandi*) is a strong candidate for introduction into the New Zealand aquaculture industry. The selection of this fish as a candidate for aquaculture is based on its economically beneficial traits that have potential in both domestic and export markets, particularly in the sashimi market in Japan. Because of this, it is important to begin investigations into the immune response of this species, so aspects of its health can be monitored during farming. The focus of this investigation was to identify immune genes in this species and to begin profiling their expression during the development of the fish.

Using bioinformatics approaches, the available gene databases and the implementation of a transcriptomic library prepared from the spleen tissue of *S. lalandi*, a number of immune genes were identified, which included, Immunoglobulin δ (IgD), Recombination Activation Gene 1 (RAG1) and Interleukin-1β (IL-1β). Primers were designed for each gene, to enable confirmation of the gene sequences, by PCR and RACE-PCR. Using this approach, no confirmed sequence was obtained for IL-1β, however sequence was obtained for part of the RAG1 gene and a large part of the IgD gene, including the 3'end.

The availability of these immune gene sequences, allowed methods to be developed for looking at gene expression. Primers were designed using the confirmed sequences for RAG1 and IgD, as well as the unconfirmed sequence for

IL-1β. These were used in qPCR to examine the expression of each gene during development, within larvae at hatch, 3 dph, 12 dph and 18 dph. Expression of each gene was found to increase by 12 dph, but then decrease at 18dph, with IL-1β showing the highest relative expression. However, no significant difference in expression was seen statistically between any of the time points.

Lastly, a protocol was developed for the histological sectioning of larval fish at different stages of development. A number of attempts were made to optimise an approach, with fish from 3 dph to 60 dph eventually being paraffin embedded, sectioned and stained with either toluidine blue or haematoxylin and eosin (H&E). Developing structures within the larvae, including immune tissues, were identified. Future work will use prepared sections to stain for the immune genes, to gain a better understanding of their role.

Discovery of immune genes from kingfish, such as the ones characterised in this investigation, will allow the development of invaluable tools. These tools can be used to monitor the health of fish during both the developmental and adult stages. This pave the way for informative studies that will benefit the future aquaculture of this species.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

°C	Degrees Celcius
AgR	Antigen Receptor
AMP	Antimicrobial Peptide
APC	Antigen Presenting Cell
BCR	B Cell Receptor
bp	Base Pairs
CART	Conserved Antigen Receptor Transmembrane Domain
CD	Cluster of Differentiation
cDNA	Complementary DNA
CDS	Coding DNA Sequence
CFU	Colony Forming Unit
CLR	C-type Lectin Receptor
Cq	Quantification Cycle
Ct	Threshold Cycle
CTL	Cytotoxic T Lymphocyte
CTLD	C-Type Lectin Domain
DAMP	Damage-associated molecular pattern
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
dpf	Days post fertilisation
dph	Days Post Hatch
DPX	Di-n-butyl Phthalate in Xylene
ErBr	3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide
EST	Expressed Sequence Tags
EtBr	Ethidium Bromide,
FAO	Food and Agriculture Organisation of the United Nations
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
H&E	Haematoxylin and Eosin
hpf	Hours Post Fertilisation
HSC	Haematopoietic Stem Cell
IFN	Interferon
Ig	Immunoglobulin
IgH	Immunoglobulin Heavy Chain
IgL	Immunoglobulin Light Chain
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1RAP	Interleukin-1 receptor accessory protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny Broth
LB+	Lysogeny Broth agar plates with ampicillin
lck	Lymphocyte-Specific Protein Tyrosine Kinase
LGP2	Laboratory of Genetics and Physiology 2
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeat
MAC	Membrane Attack Complex
MBL	Mannose-Binding-Lectin
MDA5	Melanoma Differentiation-Associated Gene 5
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA

NBR	Nonamer Binding Region
NCC	Nonspecific Cytotoxic Cell
NGS	Next Generation Sequencing
NK	Natural Killer Cell
NKT	Natural Killer T Cell
NLR	NOD-Like Receptor
NOD	Nucleotide-Binding Oligomerisation Domain
nt	Nucleotides
NVV	Nervous Necrosis Virus
OCT	Optimal Cutting Temperature Compound
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PRR	Pattern Recognition Receptor
p-xylene	Para-Xylene
qPCR	Quantitative PCR
RACE	Rapid Amplification of cDNA Ends
RAG-1	Recombination Activating Gene 1
RAG-2	Recombination Activating Gene 2
RIG-I	Retinoic Acid-Inducible Gene 1
RING	Really Interesting New Gene
RLR	RIG-I like Receptor
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNA-seq	RNA Sequencing
RSS	Recombination Signal Sequence
TAE	Tris-Acetate-EDTA buffer
Taq	*Thermus aquaticus* DNA Polymerase
TCR	T-Cell Receptor
TdT	Terminal Deoxynucleotidyl Transferase
Th	Helper T Cell
TIR	Toll-Interleukin-1 Receptor
TLR	Toll-like Receptor
T_m	Melting Temperature
TNF	Tumour Necrotic Factor
TRAIL	TNF-Related Apoptosis Inducing Ligand
Treg	Regulatory T Cell
Tris	(Hydroxymethyl) Aminomethane
Tween20	Polyoxyethylene 20 Sorbitan monolaurate
Uniprot	Universal Protein Resource
UTR	Untranslated Region
V(D)J	Variable, Diversity, Joining
VHSV	Viral Haemorrhagic Septicaemia Virus
VLR	Variable Lymphocyte Receptor
Wpf	Weeks post fertilisation
WPI	Weeks Post Immunisation
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZDD	Zinc-binding Dimerisation Domain
ZFA	Zinc Finger A
ZFB	Zinc Finger B

Chapter One: Introduction and Literature Review

1.1 Aquaculture

1.1.1 Global Aquaculture

Aquaculture is a technique by which species of fish, shellfish, and plants can be farmed (Naylor, Williams, & Strong, 2001). Aquaculture farms, which can be based in ponds, tanks or cages, have been established in freshwater and saltwater environments worldwide (Bostock et al., 2010). Nearly 50% of the total seafood produced around the world is farmed using aquaculture. This includes 13% of the protein produced from all animal meat industries (Bush et al., 2013). The aquaculture industry has grown over the past fifty years with reported yield of both animal and plant products reaching 68.3 million tonnes in 2008; the value of which was equal to US$106 billion (Bostock et al., 2010). In 2008, 310 different species were farmed in aquaculture, as recorded by the Food and Agriculture Organisation of the United Nations (FAO, 2010). However, global aquaculture production was dominated by only 20 species, representing 74% of the total aquaculture volume. These 20 species were mainly from the Cyprinidae family, and included a number of carp species such as the common carp (*Cyprinus carpio*), the silver carp (*Hypophthalmichthys molitrix*), and the grass carp (*Ctenopharyngodon idella*) (Bostock et al., 2010). The aquaculture industry is the fastest growing food sector with output almost doubling between 1998 and 2008. Aquaculture is also projected to surpass traditional capture fishing in popularity, as shown by the steady growth the industry is experiencing (Gjedrem, Robinson, & Rye, 2012; Naylor et al., 2001; Smith et al., 2010).

Seafood is the most traded food source internationally, so a successful aquaculture industry would offers benefits, both nutritionally and economically for fishing communities on a global scale (Smith et al., 2010). The nutritional value of seafood, especially the high levels of omega-3 and fatty acids, is an appealing detail which adds to the viability of aquaculture success(Smith et al., 2010). Currently, Asia is the largest source of aquaculture-based products with its output making up 89 per cent of total volume and 79 per cent of total value of the

world's aquaculture market. This can be attributed to population and economic growth in the region, as well as an increase in the export industry, and comparatively weaker regulatory policies (Bostock et al., 2010). An increase in industries, such as aquaculture, will be necessary to fulfil food requirements as the population of the world is projected to be over 9 billion by 2050(FAO, 2010; Gjedrem et al., 2012). To help aquaculture become more sustainable in the long term it will be important to improve farming practice and investigate alternative species.

1.1.2 Limitations of Aquaculture

There are still a number of sectors that require improvement to increase the efficacy of aquaculture. The primary areas are dietary limitations, monosex farming, and disease management. Fish may have herbivorous, carnivorous or omnivorous dietary requirements that must be accounted for when farming. Currently, feeding carnivorous fish is an inefficient practice. In order to produce 1 kg of farmed fish, more than 1 kg of fish feed is required (Taelman, De Meester, Roef, Michiels, & Dewulf, 2013). This inefficiency is caused by the protein requirement of these fish. It is understood that carnivorous fish require feed comprised of at least 400 g kg^{-1} of protein (Hardy, 1996). At present, research is focused on the possibility of replacing fish-based protein with other sources. This includes protein from poultry by-products and from soy (Watson, Buentello, & Place, 2014). Due to the presence of protein, lipids and carbohydrates microalgae is also a potential source for fish feed (Taelman et al., 2013).

Another aspect of aquaculture currently requiring optimisation is monosex farming. There is a sexual dimorphism in growth rates of some fish which is an attractive aspect for aquaculture. Monosex farming would benefit farming practices by focusing on those that have a faster growth rate. A commonly farmed species that exhibits this growth dimorphism is the *tilapia sp*. where males are found to have a higher growth rate than females (Singh, 2013). While growth rate is an important factor, there are other advantages of monosex farming. This includes prevention of breeding, which combats both overcrowding and potential introduction of invasive species into surrounding ecosystems (Singh, 2013).

The presence of diseases has a significant impact on aquaculture farms, where an entire farm's population can be diminished by an outbreak of disease

(Leung, Bates, & Dulvy, 2013). In order to effectively staunch the impact of disease throughout fish farming, the immune system of fish must be sufficiently understood. Adequate comprehension of immune systems leads to the efficient use of immunostimulants and an effective vaccination schedule (Scapigliati et al., 2002). Typically in aquaculture, vaccines can be delivered via immersion or injection. While injections are known to have a higher efficacy, it is a tedious process and only performed on valuable fish such as Atlantic salmon (*Salmo salar*). Therefore in most other farms immersion vaccination is performed instead (Rombout, Yang, & Kiron, 2014). Key immunological features related to the mucosal immune system possessed by mammals are absent in fish. Thus, a limitation arises whereby vaccine modes of action towards a specific antigen cannot be easily characterised (Bowden, Cook, & Rombout, 2005; Scapigliati et al., 2002). This explains the necessity to fully understand the processes behind the teleost immune system in fish.

1.1.3 New Zealand Aquaculture

In New Zealand, three major aquatic species are farmed; oysters, mussels and king salmon. Commercial aquaculture started in the north of the North Island during the 1960's, beginning with the farming of native rock oyster (*Saccostrea commercialis*) (Jeffs, 2005). However, with the unintentional introduction of the Pacific oyster (*Crassotrea gigas*), the native oyster species has become the less preferred option for farming. This is due to the superior growth rate of the newly introduced Pacific species. The Pacific oyster has now spread throughout the North Island and is mostly sourced from Northland (Jeffs, 2005; Ren, Ross, & Schiel, 2000). In 2011, 1,804 tonnes of Pacific oysters were exported accounting for NZ$16.6 million in New Zealand's total export revenue (Aquaculture New Zealand, 2012). Closely following the popularity of pacific oysters is the Greenshell mussel, *Perna canaliculus*. This species accounts for the largest proportion of aquaculture yield, with 101,311 tonnes produced in 2011 to a net value of NZ$218 million in export revenue (Aquaculture New Zealand, 2012). An estimated 80% of juvenile Greenshell mussels are sourced from Ninety Mile Beach in Northland, where they attach to debris, such as microalgae (Alfaro, McArdle, & Jeffs, 2010). The farmed juvenile Greenshell mussel species are transported from Ninety Mile Beach to other farms around New Zealand. The Marlborough region of the South Island is the largest producer of mature

Greenshell mussels with 69% of the total aquaculture yield (Alfaro et al., 2010; Aquaculture New Zealand, 2012). The farming of Chinook salmon (*Oncorhynchus tshawytscha*) began in the 1980s and has mostly been focused in Marlborough, with 64% of the total yield for New Zealand produced in the region (Aquaculture New Zealand, 2012; Jeffs, 2005). In 2011, the production of king salmon was at 14,037 tonnes which equated to NZD$63.4 million in export revenue (Jeffs, 2005). For aquaculture to expand, it is important to investigate new species to introduce to New Zealand's farms. There are a number of marine and freshwater species currently targeted as candidates (Alfaro, Jeffs, & King, 2014), including fish, such as eels (*Anguilla australis*), yellowtail kingfish (*Seriola lalandi*) and hapuku (*Polyprion oxygeneios*).

1.2 Yellowtail Kingfish

1.2.1 *Seriola lalandi*

The yellowtail kingfish (*Seriola lalandi*) belongs to the genus Seriola, and are commonly known as amberjacks. This species is a large, carnivorous finfish popularly known for the firm texture and rich flavour of their flesh. *S. lalandi* inhabit the Atlantic and Pacific oceans, but can also be found globally (Miller, Fitch, Gardner, Hutson, & Mair, 2011). Due to their fast growth and potentially large size, up to 2.5 meters long and up to 70kg in weight, they are excellent candidates for aquaculture (Miller et al., 2011). A large amount of research has been conducted in New Zealand to investigate the biological, technological, and economic feasibility of farming the species (Symonds et al., 2014). Although kingfish is not yet commercially cultured in New Zealand, there is now the capability to produce sufficient kingfish fingerlings to meet the needs of the early stages of an industry. *S. lalandi* culture has already begun within Australia where the industry is rapidly growing (Moran, Smith, Gara, & Poortenaar, 2007). At present, kingfish aquaculture industries are found in New South Wales and Western Australia, with the largest venture in South Australia (Miller et al., 2011). However, a number of obstacles exist in the kingfish sector of aquaculture, which greatly affects the economics of aquacultural farming. Currently, breeding stock has to be caught from the wild and maintained in large indoor broodstock tanks. This may have an adverse impact on wild *S. lalandi* populations. In addition, there are a number of other bottlenecks, including high mortality rates in young fish. Also to be considered is the continued presence of diseases and abnormalities in *S. lalandi* growth leading to deformities (Miller et al., 2011).

1.2.2 *Seriola quinqueradiata*

Another Seriola species currently being cultured is the Japanese Amberjack (*Seriola quinqueradiata*). It is farmed commercially in Japan, along with the Greater Amberjack (*S. dumerili*) and *S. lalandi*. These three species account for more than half of the fish species farmed in Japan (Kawanishi et al., 2005), giving around 156,000 tonnes in 2003 (Ohara et al., 2005). The Japanese Amberjack is most commonly found in the Sea of Japan, the East China Sea, and in the Pacific Ocean (Tian et al., 2012). The adult size of these fish are approximately 5 – 8 kg and may be up to 100 cm in length, though are often 70-80

cm (Ohara et al., 2005; Tian et al., 2012). The farming of these fish is achieved by using marine cages and net pens (Ohno, Kawano, & Hirazawa, 2008).Despite the success of cultured Japanese Amberjack, the impact of disease is a major limitation on the overall quality and production of the fish. Parasites cause considerable decline in the overall yield by either affecting the health of the fish, or lowering the quality of the final product (Burger, Barnes, & Adlard, 2008). In the 1980s, a Japanese aquaculture farm that cultured *S. quinqueradiata* was plagued by a parasitic infestation. The parasite, *Kudoa amamiensis,* caused visible white cysts on the skeletal muscle of the infected fish. These cysts rendered any infected fish unmarketable and the aquaculture farm was shut down (Burger et al., 2008). High mortality rates have also occurred in Japanese farms after infection of the parasite *Neobenedenia girellae*. After maturing, this parasite occupies the skin of the fish where they feed on the epithelial cells and the mucus lining of the fish's skin (Ohno et al., 2008). These cases demonstrate the necessity for research on the immune responses of any fish that is a candidate for aquaculture.

To date, limited research has been carried out in any *Seriola* species to understand their physiology at a genetic level, due to the lack of molecular tools that have been available. However, genetic techniques are being developed to help in our understanding of physiological aspects of farmed fish and shellfish species. These techniques allow insight into development, health, growth and reproduction, leading to the greater success for these species in aquaculture (Miller et al., 2011; Poortenaar, Hooker, & Sharp, 2001; Whatmore et al., 2013). Genes identified as being important in the physiology of an organism can be used as biomarkers to allow a greater understanding of the organism and its responses to changes in its environment (Whatmore et al., 2013). For currently farmed *Seriola* species, an understanding of their immune system, and the use of immune biomarkers to monitor a fish's health and responses to therapeutic approaches will be valuable for the aquaculture industry in New Zealand and worldwide.

1.3 Vertebrate Immunity

1.3.1 Vertebrate Immunology

The effective defence against invading pathogenic microbes is paramount to the survival of an organism. All multicellular organisms have some form of immune system to successfully protect against these microbes (Laird, De Tomaso, Cooper, & Weissman, 2000). Two classes of immune systems exist in nature, the innate immune system and the adaptive immune system. The innate immune system plays an integral part in immunity as a preliminary response, but is not specific for a particular pathogen. Instead the innate immune system targets highly conserved molecular patterns that only microbial pathogens and toxins possess (Chaplin, 2010). Vertebrates are the only phylum that is able to mount an additional, specific immune response: the adaptive immune system. The adaptive system adds another dimension of immune response by producing molecules that are able to target specific antigens, this includes the production of immunoglobulins (Igs) and T cell receptors (TCRs) (Chaplin, 2010; Janeway & Medzhitov, 2002). While vertebrates are the only phylum can mount an adaptive immune response, the adaptive system is not ubiquitous throughout all vertebrates (Figure 1).

Two distinct evolutionary groups can be seen in vertebrates, the jawless and the jawed vertebrates (Dong et al., 2005). Jawless vertebrates, or agnathans, appeared around 500 million years ago and are the most primitive form of vertebrates (Kasamatsu, 2013). Agnathans are known to lack the conventional adaptive immune response characterised by specific antigen receptors. These vertebrates are therefore crucial for the understanding of the evolutionary origin of the adaptive immune response (Herrin & Cooper, 2010). The most well studied agnathan species with regard to the immune system are the sea lamprey (*Petromyzon marinus*), followed by the hagfish (*Eptatretus burgeri*) (Herrin & Cooper, 2010; Suzuki, Shin, Kohara, & Kasahara, 2004). Although agnathans lack the conventional adaptive immune system, they have an alternative form of adaptive immunity. Antigen recognition is mediated by variable lymphocyte receptors (VLR) with specificity derived from a 24 amino acid, highly variable leucine-rich repeat (LRR) module (Boehm, McCurley, et al., 2012; Herrin & Cooper, 2010). The conventional adaptive immune system has only developed in

jawed vertebrates, or gnathostomes. It is understood that the adaptive immune system in gnathostomes evolved approximately 450 million years ago and began with the transposition of key immune genes into the vertebrate germ line (Hoffmann, 1999). Fish are the earliest organisms to possess the adaptive immune system. This makes them excellent candidates for comparative immunology studies to elucidate the evolutionary history of the adaptive immune system and, potentially, to better understand the human immune system (L. Y. Zhu, Nie, Zhu, Xiang, & Shao, 2013).

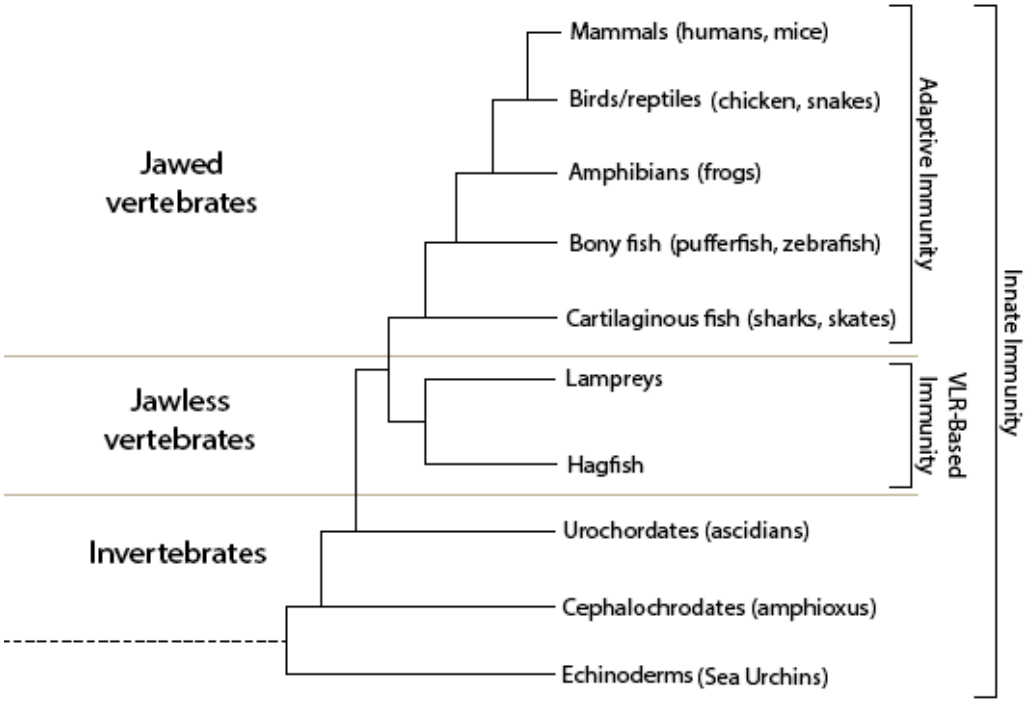

Figure 1: Evolutionary history of the immune system in animals. Innate immunity is shown to be present throughout the phylogenetic tree. VLR-based immunity is shown to be only found in jawless vertebrates, whereas adaptive immunity is shown to be exclusive to the jawed vertebrates. Image adapted from Kasamatsu, 2013.

1.3.2 Innate Immunity in Fish

Evolutionarily, the innate immune system is the most ancient and is conserved within both animals and plants. This suggests the innate immune system appeared before the split of these eukaryotic kingdoms (Jones & Dangl, 2006). The innate immune response provides an early defence against microbial infection. Primarily, non-specific defence is provided by anatomical traits possessed by the organism. This includes skin, mucus layered epithelial cells, epithelial cilia that remove the aforementioned mucus once contaminated, presence of secretory lysozymes, and hostile physiological environments, such as stomach acidity (Chaplin, 2010; Turvey & Broide, 2010). Pathogens are often able to bypass this rudimentary defence; thus creating the necessity for further,

innate systems. Once physical barriers have been evaded, further cellular and humoral components of the innate system advance the immune response. Cellular components can be divided into the non-specialised physical barriers and the specialised innate immune cells (Takashi Aoki, Takano, Santos, Kondo, & Hirono, 2008). All blood cells are developed using the process of haematopoiesis, whereby a haematopoietic stem cell (HSC) differentiates into one of the numerous blood types (Figure 2) (Granick, Simon, & Borjesson, 2012). This includes the white blood cells or leukocytes involved in both the innate and adaptive immune response. The principle cells involved in the innate immune response are the myeloid-derived neutrophils, eosinophils, basophils, mast cells, macrophages, dendritic cells (conventional and plasmacytoid), and the lymphoid-derived natural killer (NK) cells (Chaplin, 2010).

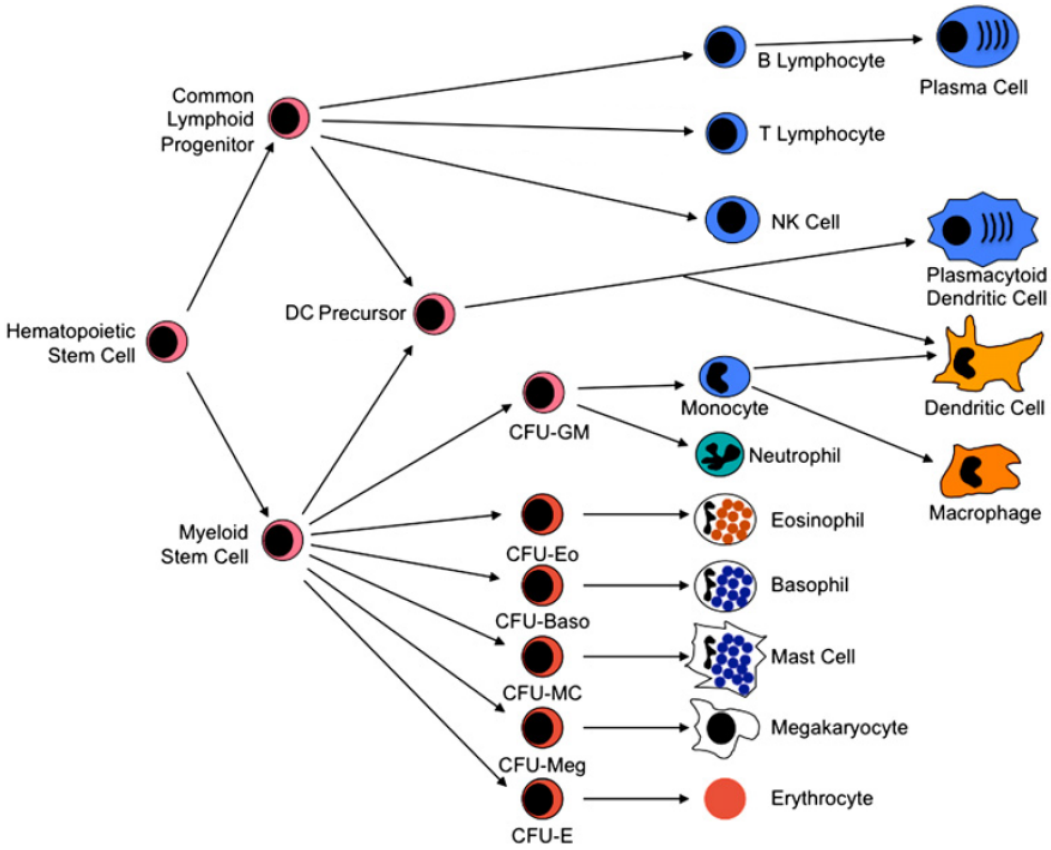

Figure 2: Potential blood cell lineages for a Haematopoietic Stem Cell. An HSC may differentiate into one of two cell types: a Myeloid Stem Cell or a Common Lymphoid Progenitor. Common Lymphoid Progenitors differentiate to produce B and T lymphocytes and the NK cells. Myeloid stem cells further produce colony forming units (CFUs) of their respective blood cell type. It is important to note the further differentiation of monocytes into macrophages and dendritic cells. Dendritic cells may also arise from a dendritic cell precursor, which itself may be derived from either myeloid stem cells or common lymphoid progenitors. Image adapted from Chaplin, 2010.

1.3.3 Cells of the Innate Immune System

The cell mediated response of the innate immune system is made up of a number of types of cells. Initial detection of pathogens is mediated via tissue resident macrophages with the aid of pattern recognition receptors (PRRs) (Grayfer, Hodgkinson, & Belosevic, 2014). In the absence of infection, macrophages are tasked with the clearing of debris such as dead erythrocytes and other cells (Keightley, Wang, Pazhakh, & Lieschke, 2014). One of the key features of macrophages is that they are antigen presenting cells (APCs). During a period of infection, macrophages are able to phagocytise invading pathogens, present the antigen on an MHC protein and release cytokines that further the immune response (Gordon, 2007). Similarly, the innate system also utilises dendritic cells which are important APCs. Dendritic cells are able to phagocytise pathogens, migrate to secondary lymphoid tissue and present antigens on upregulated MHC proteins (Shao et al., 2015). A second form of dendritic cell occurs in nature, named the plasmacytoid dendritic cell. Plasmacytoid cells are particularly important for defence against viruses, which is shown by the ability of these cells to release interferon type I (see section 1.3.11) (Lewis, Del Cid, & Traver, 2014). Macrophage and dendritic cells are part of the mononuclear phagocyte family and are both antigen presenting cells. These cells are both derived from monocytes; cells that circulate throughout the organism and are able to differentiate into macrophages or dendritic cells (Lewis et al., 2014).

Another group of immune cells is the granulocytes which include neutrophils, eosinophils, basophils and mast cells. Neutrophils are the first immune cells to be recruited in the case of pathogenic infection (Keightley et al., 2014). These cells have a number of functions to combat pathogens which includes phagocytosis and degranulation. The latter of which involves releasing antimicrobial peptides such as lactoferrin, and defensins (Kolaczkowska & Kubes, 2013). In mammals, eosinophils have been characterised as having the function of defence against helminths (Jacobsen, Helmers, Lee, & Lee, 2012). However, it has proven difficult to determine the exact function of eosinophils in teleosts. It is thought that teleost eosinophils have ribonuclease and antibacterial properties which are released upon degranulation (Balla et al., 2010). It is also likely that lysozyme is also released with the degranulation of eosinophils (Uribe, Folch, Enriquez, & Moran, 2011). Mammalian basophils are also utilised in defence

against helminth infection. They are involved in the allergic response and are able to produce histamine upon degranulation (Sullivan et al., 2011). Teleost basophils have proven difficult to characterise due to the small number of cells they contain. Doubt had been cast as to the occurrence of basophils in teleosts because of this small number (Tavares-Dias, 2006b). However, despite this difficulty basophils have now been identified in a number of freshwater and marine species (Tavares-Dias, 2006a). Mast cells play a role in allergic reactions, and are situated under epithelial membranes within proximity to blood vessels (Ola B. Reite, 1998). Mast cells are similar to basophils with one major difference, being their localisation to tissue, compared with basophil bloodstream motility (Kawakami & Galli, 2002). Much like basophils, the presence of mast cells in teleost is under debate. Histochemical analysis has led to the discovery of an 'eosinophilic granule cell' that resembles the mammalian mast cell, but has red granules after staining with haematoxylin and eosin (O. B. Reite & Evensen, 2006). In a study of a salmoniform species, *Esox Lucius*, it was found that these cells are homologous to the mast cells found in mammals (Sfacteria, Brines, & Blank, 2015).

While NK cells are granular, they are classed as large granular lymphocytes (Chaplin, 2010). Mammalian NK cells have non-specific cytotoxic properties and are able to induce apoptosis in cells that are not presenting a 'self' signal, i.e. from an MHC class I (Fischer, Koppang, & Nakanishi, 2013). Teleosts are thought to possess a precursor cell type in lieu of NK cells. Nonspecific cytotoxic cells (NCC), first characterised in channel catfish (*Ictalurus punctatus*), are smaller cells and agranular. These cells localise in lymphoid tissue and are able to target invading parasitic organisms (Shen et al., 2002).

1.3.4 Pattern Recognition Receptors

Crucial to the non-specific detection of pathogens are the pattern recognition receptors (PRRs). These germline encoded receptors are able to recognise highly conserved features possessed by pathogens; the pathogen-associated molecular patterns (PAMPs) (T. Aoki, Hikima, Hwang, & Jung, 2013). There are four families of PRRs in the immune system of vertebrates: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) (A. Y. Chen & Chen, 2013).

TLRs are the most widely studied PRR and were first characterised in the fruit fly, *Drosophila melanogaster* (Rebl, Goldammer, & Seyfert, 2010). In humans, ten TLRs have been characterised, which is less than mice which have 12 (Eastmond, Schuler, & Rupa, 1995). Within avian, amphibian and teleost species, 26 TLRs have been characterised, including the teleost exclusive TLR 22 (Pietretti et al., 2014; Rauta, Samanta, Dash, Nayak, & Das, 2014). TLRs have a range of activating ligands including lipopolysaccharide, and both double and single stranded nucleic acid molecules (Rauta et al., 2014). TLRs utilize a LRR motif to identify pathogens which induces expression of pro-inflammatory genes or Interferon (INF) type I. The cytokines expressed are determined by which TLR is activated and is aided by the intracellular toll/interleukin-1 receptor domains (Matsushima et al., 2007; Y. Xu et al., 2000).

In response to intracellular viral nucleic acids, particularly double stranded RNA (dsRNA), RLRs are able to induce expression of IFN type I (Eastmond et al., 1995). In mammals and teleosts, there are three RLR members: retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (T. Aoki et al., 2013). Viral recognition in RLRs is mediated by the functional RNA helicase domain that can bind to viral RNA (H. X. Xie et al., 2006). Functionally, RIG-I is able to recognise DNA and RNA viruses and may have the capacity to recognise bacterial pathogens. MDA5 has been found to recognise long strands of dsRNA, provided the RNA has assumed a structure that is related to viral infection (H. X. Xie et al., 2006). Finally, LGP2 has the role of a negative regulator by disrupting the binding of viral RNA to RIG-I and MDA5 (Eastmond et al., 1995). It is also thought LGP2 may be a positive regulator of RIG-I and MDA5 and increases the production of IFN (H. X. Xie et al., 2006).

Nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) are known to detect cytosolic PAMPs following phagocytosis (Livak & Schmittgen, 2001). Primarily, NLRs are activated upon recognition of bacterial pathogens (J. Xie, Hodgkinson, Katzenback, Kovacevic, & Belosevic, 2013). Peptidoglycan of gram positive and gram negative bacteria has been shown to initiate interleukin-1β production and lead to phagocyte recruitment through the NLR pathway (Livak & Schmittgen, 2001). NLRs have been conserved over

evolution, demonstrated by the presence of a plant homolog that is involved in responding to plant viruses (Kanneganti, Lamkanfi, & Nunez, 2007).

C-type lectins are used in the innate system in the recognition of carbohydrate PAMPs. This is mediated via the carbohydrate recognition domain of the CLR, the C-type lectin domain (CTLD) (A. Y. Chen & Chen, 2013). Most CTLDs are known to have dependence on Ca^{2+} ions for recognition function and are able to recognise a wide range of carbohydrate PAMPs (Vasta et al., 2011). CTLs have been found in a number of teleost species that are important to aquaculture, including rainbow trout (*Oncorhynchus mykiss*), pufferfish (*Takifugu rubripes*) and Atlantic salmon (*Slamo salar*) (Genten, Terwinghe, & Danguy, 2009; McInerney, Adams, & Hadi, 2014).

1.3.5 Antimicrobial Peptides

One of the most ancient components of the innate immune system is antimicrobial peptides (AMP). They have been found in microorganisms, plants, invertebrates, and vertebrates (Rieger & Barreda, 2011). AMPs are classified as oligopeptides, which have a wide range of microbial targets, including viruses, fungi, and both gram positive and negative bacteria (Izadpanah & Gallo, 2005). The mode of action AMPs varies but generally they disrupt various components of a pathogen. The most common target for AMPs is the cell membrane, but they also target DNA/RNA synthesis, proteins and other intracellular features of a pathogenic cell (Bahar & Ren, 2013). Cell membrane disruption is driven by the electrostatic attraction of the AMP to the cell membrane. Following this interaction, the AMP infiltrates the cell membrane and causes lysis in numerous ways, such as pore-formation and solubilisation of the membrane (Izadpanah & Gallo, 2005). Chronologically, the first mammalian AMPs discovered were defensin in rabbits, followed by bombinin and lactoferrin in epithelia and cow milk, respectively (Bahar & Ren, 2013).

Due to the colder environment and higher exposure to waterborne pathogens, the teleost adaptive system is less effective than mammals' and other vertebrates'. Thus, AMPs and other innate components are more relied upon in teleosts (Tessera, Guida, Juretic, & Tossi, 2012). In teleosts, most AMPs have antibacterial/bacteriostatic properties, with a few having antiviral effects (Rajanbabu & Chen, 2011). This includes β-defensin which has been

characterised in rainbow trout (*Oncorhynchus mykiss*) during the infection by haemorrhagic septicaemia virus (Rajanbabu & Chen, 2011). AMPs in teleosts are produced in immunologically active tissues, including the spleen and kidney, and often populate the skin mucosa (Rieger & Barreda, 2011).

1.3.6 Complement Molecules

The complement system is made up of over 35 plasma proteins and is involved with both the innate and adaptive immune responses (Boshra, Li, & Sunyer, 2006). The functions of these complement molecules are divided into three pathways: the classical, alternative, and the lectin (Gasque, 2004). The common feature of each pathway is the lysing of cells by formation of pores in the membranes of pathogens (Holland & Lambris, 2002). Complement molecules are also able to opsonise pathogens, recruit phagocytes and promote their activation, thus increasing the efficiency of pathogen removal (L. Y. Zhu et al., 2013). The classical pathway involves the C1 protein binding to an immunoglobulin that has bound to its cognate antigen, i.e. an Ab-Ag complex (S. Zhang & Cui, 2014). C1 is made up of two C1r and C1s subunits, and one C1q subunit. The C1q binds to the Ab-Ag complex and activates the serine protease properties of the C1s which acts on the molecule C4 (Holland & Lambris, 2002). This molecule is cleaved to form C4a and C4b, the latter binds to the membrane of the pathogen and then to the complement molecule, C2. The C2 is cleaved by C1s into C2a and C2b which results in the formation of the C4bC2a complex, called C3 convertase (Holland & Lambris, 2002). The lectin pathway follows a similar process to the classical pathway; however initiation of this pathway does not depend on the presence of an Ag-Ab complex. Instead, the lectin pathway begins with the binding of a mannose-binding-lectin (MBL) to a carbohydrate pattern on the surface of a pathogen (Boshra et al., 2006). The steps that follow mirror the classical pathway which results in the formation of the C3 convertase.

Crucial to the success of each pathway is the C3 protein. Cleavage of C3 can occur spontaneously, as part of the alternative system, or by C3 convertase. It can be cleaved into C3a, which is involved with pro-inflammatory responses, and C3b, which opsonises to pathogenic cells (Cresci, Allende, McMullen, & Nagy, 2015). C3b can be further cleaved to produce an inactive form referred to as iC3b, which can be recognised by phagocytic leukocytes (Nakao, Tsujikura, Ichiki, Vo,

& Somamoto, 2011). C3b binds to the C3 convertase to form C4bC2aC3b, the C5 convertase. As previously mentioned, the spontaneous cleavage of C3 initiates the alternative pathway which also involves factors B, D, and P (Cresci et al., 2015). C3b opsonises to the pathogenic cell which is followed by the binding of factor B to produce C3bB. Factor D is then recruited to the C3bB complex and cleaves factor B. This produces C3bBb, the alternative pathway equivalent of C3 convertase (Turner & Moake, 2013). C5 convertase in the alternative pathway is referred to as C3bBbC3b, whereas C5 convertase in the classical and lectin pathways is referred to as C4bC2aC3b (Turner & Moake, 2013). C5 convertase cleaves C5 into C5a and C5b, the latter forms a complex with C6, C7, C8, and C9 to form the membrane attack complex (MAC) (Gasque, 2004). The MAC lyses the cell by forming a pore in the membrane. Currently, evidence shows the presence of the classical and alternative pathway in teleosts. While evidence is lacking to support the presence of the lectin pathway in teleosts, there have been successful characterisations of MBL in teleosts (L. Y. Zhu et al., 2013).

1.3.7 Adaptive Immunity in Fish

In higher vertebrates (reptiles, birds and mammals) and lower vertebrates (bony and cartilaginous fish) the adaptive or acquired immune response provides a specific defence against pathogens (Rauta, Nayak, & Das, 2012). This response is influenced by the initial innate immune response, where TLR interactions with the microbe have been shown to help initiate the appropriate activation of the adaptive immune system (Ward & Rosenthal, 2014). Lymphoid tissues are an important component of the adaptive immune response in vertebrates (Boehm, Hess, & Swann, 2012). They can consist of both immature haematopoietic cells, which give rise to functional immune cells of the innate and adaptive immune responses, and stromal cells. These stromal cells are able to produce signals for the complete maturation of immune cells, such as lymphocytes (Boehm, Hess, et al., 2012).

Lymphoid organs are divided into two classes, either primary or secondary. This division is based on attributes such as function or ontogeny of the tissue (Ángeles Esteban, 2012). Primary lymphoid tissues generate effector lymphocytic cells, whereas secondary lymphoid tissues provide a location for lymphocytes to produce an immune response towards an antigen (Boehm, Hess, et

al., 2012). A number of lymphoid tissues characterised in mammals are not found in fish. This includes bone marrow for B lymphocyte generation, lymph nodes, and Peyer's patches (Morrison & Nowak, 2002; Press & Evensen, 1999). However, lymphoid organs such as the thymus, kidney and spleen do exist in both fish and mammals (Rauta et al., 2012). In fish, the primary lymphoid tissues that have the function of lymphocyte production are the thymus and the head kidney (Austbo et al., 2014; Boehm, Hess, et al., 2012). The most evolutionarily ancient vertebrate primary lymphoid organ is the thymus, which is involved in the adaptive immune response with the role of producing T cells (Boehm, Hess, et al., 2012; Bowden et al., 2005). The role of the thymus in T cell production is shown by locating T cell specific markers, such as T cell receptor-α (TCR-α), or lymphocyte-specific protein tyrosine kinase (lck) (Trede, Zapata, & Zon, 2001). The thymus in teleost species is situated in the pharynx towards the anterior of the fish and may be visible in fish from 24 hours post fertilisation (hpf), as seen in *Tilapia* species, or 60 hours post fertilisation, such as in zebrafish (Press & Evensen, 1999; Zapata, Diez, Cejalvo, Gutierrez-de Frias, & Cortes, 2006).

The function of the head or anterior kidney is comparable to bone marrow in mammals. This is due to the haematopoietic capacity of the kidney, particularly in producing B cells, but also granulocytes, and macrophages (Kondera, 2011; Uribe et al., 2011). It is understood that the head kidney is also involved in the processing of antigens, phagocytosis and IgM production (Reyes-Cerpa et al., 2012). Current findings suggests that the head kidney is involved in the development of B cells, with the presence of related lymphocytic maturation markers (Bromage, Kaattari, Zwollo, & Kaattari, 2004). This includes factors involved in V(D)J recombination, such as terminal deoxynucleotidyl transferase (TdT) and the recombination activating genes RAG-1 and RAG-2. Also present is Ikaros, a transcription factor related to B cell development (Bromage et al., 2004; Kirstetter, Thomas, Dierich, Kastner, & Chan, 2002; Litman, Rast, & Fugmann, 2010).

The head kidney is also thought to possess secondary lymphatic properties, however the key secondary lymphoid tissue in fish is the spleen (Koppang et al., 2010). The most evolutionarily ancient organ in the lymphoid system is the spleen which has the primary function of filtering blood (Boehm, Hess, et al., 2012; Reyes-Cerpa et al., 2012). In an immunological setting, the

spleen is understood to be a location for the activation of B cells and the formation and differentiation to plasma cells. Furthermore, blood filtering enables the spleen to trap antigens, which can then be detected by lymphocytes and an immune response can be mounted (Salinas, Zhang, & Sunyer, 2011).

1.3.8 B Cells and Immunoglobulins

The cell mediated aspect of the adaptive immune system is comprised of two groups of cells: B cells and T cells. While each group of cells have different functions, they are related in that they target specific antigens (Laing & Hansen, 2011). The fundamental purpose of B cells is the production of immunoglobulins which may take on one of two roles. The primary niche immunoglobulins fill is bound to the membrane of B cells, and are known as B cell receptors (BCRs). Secondary to this role is secreted immunoglobulins (Litman et al., 2010). Naïve B cells are derived from HSCs with the intermediary cell stages pro-A and pre-B cells preceding the naïve B cell (Shapiro-Shelef & Calame, 2005). Naïve B cells detect their cognate antigen via the BCR, at which point the antigen is engulfed by the B cell. The antigen is then processed within the B cell and presented extracellularly as part of an MHC class II complex (Yuseff, Pierobon, Reversat, & Lennon-Dumenil, 2013). Once a corresponding $CD4^+$ helper T cell (T_h cell) identifies the presented antigen (see section 1.3.9), it releases effector cytokines that leads to proliferation and differentiation of the B cell (Laing & Hansen, 2011). Differentiation of a naïve B cell may result in one of two potential cell types following differentiation, memory B cells or Plasma cells (Odaka et al., 2011).

Immunoglobulins, also referred to as antibodies, are small proteins made up of two heavy chains and two light chains which are linked with disulphide bonds. Immunoglobulins are typically a Y-shaped molecule that has an antigen-binding variable region and a constant region (Georgiou et al., 2014). Mammals have five isotypes of immunoglobulins: IgM, IgD, IgE, IgG, and IgA. The isotype is determined by the constant region of the immunoglobulin heavy chain (IgH), of the following: μ, δ, ε, γ, α (Hikima, Jung, & Aoki, 2011). Mammals are also known to have two light chain (IgL) isotypes referred to as kappa, Igκ and lambda, Igλ (Hsu & Criscitiello, 2006).

In contrast, studies have ascertained that three immunoglobulin isotypes are present in teleosts: IgM, IgM and IgT/Z (Sunyer, 2013). The most highly secreted immunoglobulin isotype is IgM, which is commonly expressed as a tetrameric complex that contains eight antigen binding sites (Solem & Stenvik, 2006). While the exact function of IgD has not been discovered, it has been found that Teleost IgD varies in molecular mass between fish species, but is thought to be a monomeric immunoglobulin (Edholm, Bengten, & Wilson, 2011). IgT, occasionally referred to as IgZ, is considered to be the final immunoglobulin to be discovered in vertebrates. Again, its complete function has not been determined; however it is known that IgT is monomeric in serum and polymeric in the gut mucus (Y. A. Zhang et al., 2010).

1.3.9 T Cells and the Major Histocompatibility Complex

T cells form the other major class of adaptive lymphocytic immunity. Two groups of T cells exist in the immune system which are categorised by the chains that make up the T cell receptors (TCR). As previously mentioned, T cells are produced in the thymus as part of the adaptive immune system (Bowden et al., 2005). T cells can be divided into two groups based on the chains that make up the TCR. Most T cells possess TCRs comprised of a TCR-α and TCR-β chain which have similar diversity to immunoglobulins. However, the γδ T cells are made up of a TCR-γ and TCR-δ chain (Castro et al., 2011). Briefly, γδ T cells have limited diversity in antigen detection abilities and are thought to detect lipid and phosphorylated metabolites from microbes, using the TCR-γδ as a pattern recognition receptor (F. Buonocore et al., 2012). The most common TCR is made up of the TCR-α and TCR-β chains. These can further be divided based on functional aspects and key co-stimulatory molecules they possess. The two major T cell classes are cytotoxic T lymphocytes (CTL) and helper T cells (Th). Key co-stimulatory factors are the CD4 and CD8 molecules found in Th cells and CTLs, respectively (Laing & Hansen, 2011). Immature T cells begin as double positive for CD4 and CD8, denoted as $CD4^{+}CD8^{+}$ T cells until a differentiation event occurs that produce $CD4^{+}CD8^{-}$ Th or $CD8^{+}CD4^{-}$ CTLs (Germain, 2002).

Critical to Th and CTL modes of action are the major histocompatibility complex molecules (MHC), class I and class II. The common feature of these molecules is the presentation of antigenic peptides on a host cell surface to T cells

which leads to an immune response (Dixon & Stet, 2001). The primary functional difference between the MHC class I and class II is the source of the presented antigen. Class I proteins are expressed in nearly all cell types in the possession of a host, and are formed from two non-covalently bound peptides: an α heavy chain and a β-2-microglobulin (L. Y. Zhu et al., 2013). MHC class I molecules are involved with presenting intracellularly processed peptides which includes peptides normally expressed by the host cell. A cancerous cell or a cell that has been commandeered for viral production produces abnormal proteins. These peptides are also presented via the MHC class I system (Dirscherl, McConnell, Yoder, & de Jong, 2014). CTLs recognise the MHC class I-antigen complex with the TCR, specificity increases with the CD8 co-receptor interaction with the MHC class I molecules (Laing & Hansen, 2011). Once a CTL recognises an abnormal peptide, it may induce apoptosis by using one of two methods: a secretory or a non-secretory pathway. Secretory apoptosis relies on the release of cytoplasmic granular toxins that induce apoptosis, whereas the non-secretory pathway involves activating cell death receptors with their specific ligand e.g. Fas activation by Fas ligand (FasL) (Nakanishi, Toda, Shibasaki, & Somamoto, 2011).

MHC class II molecules are used to present extracellular antigens to T cells. The class II molecules are formed from the non-covalent bond between two chains, α and β (Dixon & Stet, 2001). Where MHC class I is expressed in nearly all cell types, class II is only expressed in the antigen presenting cells (APCs) of the immune system (L. Y. Zhu et al., 2013). Once phagocytosis of a microbe occurs, a peptide fragment is loaded onto the MHC class II to be detected by a T cell (Cuesta, Angeles Esteban, & Meseguer, 2006). The TCR of Th cells is able to recognise the MHC class II-antigen complex, much like the CTLs. However, it is the CD4 co-receptor that interacts with the class II molecule to allow specific recognition of the antigen and class II MHC (Laing & Hansen, 2011). In mammals, this activation leads to the differentiation of the Th cell to one of the following: Th1, Th2, Th17 or Treg. Current research has not confirmed the presence of Th2, Th17 or Treg cells in teleost species, however functional assays support the notion of teleost Th1 cells (Castro et al., 2011).

1.3.10 V(D)J Recombination

The key mechanism that drives the adaptive immunity is the specificity of BCRs and TCRs. These specific cell receptors are able to target particular antigens due to a highly variable region, referred to as the antigen receptor (AgR) (Figure 3) (Corcoran, 2010). Driving this specificity is V(D)J recombination, a process that takes a selection of gene segments to form a BCR or a TCR (Market & Papavasiliou, 2003).

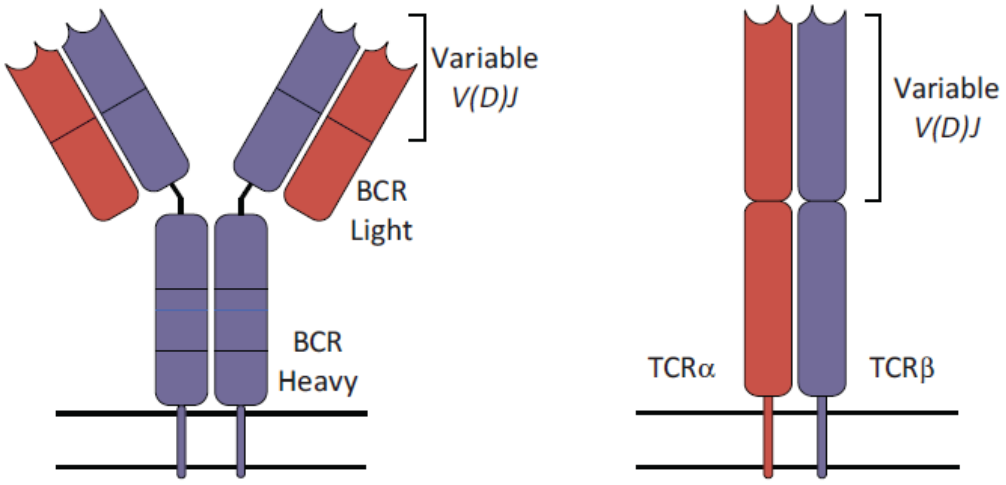

Figure 3: Structures of the BCR and TCR. BCR shows heavy chain (purple) and light chain (red) with the variable region denoted on the upper portion of the structure. TCR is shows the alpha (red) and beta (purple) chains, with the variable region again in the upper portion. Image reprinted from Calis &Rosenberg, 2014.

Three gene segments are involved in producing the variable region of BCRs and TCRs, they are named the Variable (V), the Diversity (D), and Joining (J) (Dong et al., 2005). The V, D, and J gene segments are all used to produce IgH, TCR-β and TCR-δ, however only the V and J gene segments are used in Igκ, Igλ, TCR-α, and TCR-γ (Jung & Alt, 2004). These gene segments are associated with a constant region, which forms the non-antigen binding region of the BCR or TCR (Market & Papavasiliou, 2003). The critical principle of V(D)J recombination is the essentially random selection of gene segments to produce a variable region, mediated by the recombination activating genes, RAG1 and RAG2. Each of the VDJ gene segments are flanked by a recombination signal sequence (RSS) which enables the recognition of a gene sequence by the recombination machinery (Patrick C. Swanson, 2004). The RSS is composed of a heptamer and a nonamer of conserved nucleotides that are separated by a spacer sequence made up of either 12 or 23 unconserved nucleotides (Figure 4) (Market & Papavasiliou, 2003; Patrick C. Swanson, 2004).

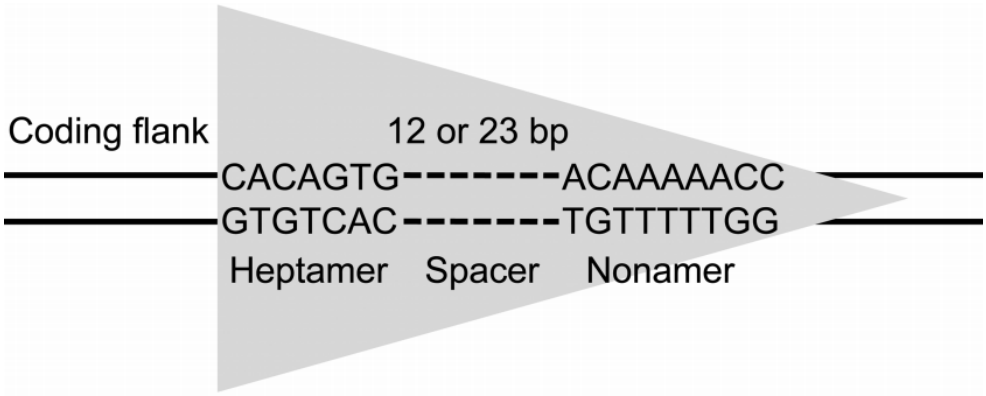

Figure 4: Diagram of a Recombination Signal Sequence. The heptamer and nonamer have conserved sequences and a separated by a spacer sequence of 12 or 23 unconserved nucleotides. Image reprinted from Yin et al, 2009.

RAG1 binds to both the 12 and 23 bp RSS motifs and the recruits RAG2 which leads to the formation of a protein complex that is referred to as RAG (Market & Papavasiliou, 2003). The fundamental mechanism of RAG is to perform a cleavage reaction that brings together the V, D and J gene segments (Figure 5) (Jung & Alt, 2004). Two steps are involved in this reaction, first a nick is introduced in the DNA upstream of the heptamer which produces a 3' hydroxyl group (Yin et al., 2009). Second, transesterification occurs where the 3' hydroxyl group covalently binds to the phosphate on the adjacent DNA strand (P. C. Swanson, 2001).

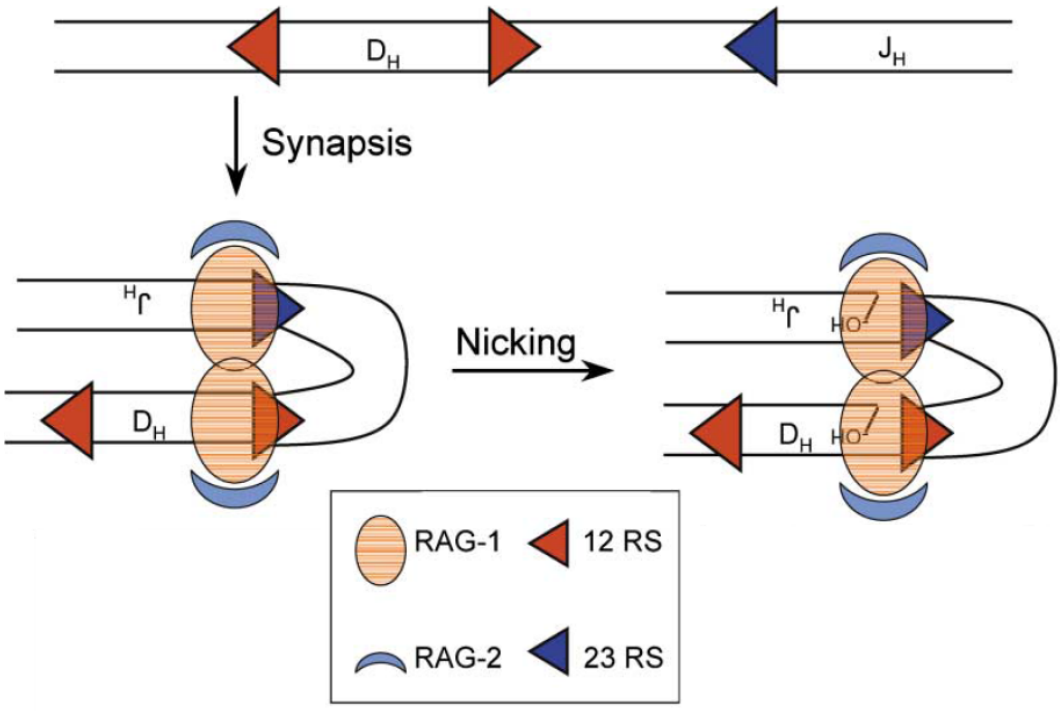

Figure 5: Recombination event mediated by RAG. RAG first creates a nick and then cleaves at the 5' end of the RSS. This produces a hairpin structure at the coding region which is then joined to bring the D and J segments together. Image adapted from Jung & Alt, 2004.

1.3.11 Regulation of Immunity in Fish

Both innate and adaptive immune responses utilise regulatory proteins called cytokines, which are groups of proteins used for cell signalling and modulation of an individual's immunity. They are used in response to bacterial, viral, or parasitic pathogens (Barrett, 1996; L. Y. Zhu et al., 2013). Cells in the immune system secrete cytokines that bind to specific cellular receptors through autocrine or paracrine mechanisms. There are over 100 separate genes coding for cytokine-like activities, many with overlapping functions and many still unexplored. Today, the term "cytokine" encompasses interferons (IFNs), the interleukins (ILs), the tumour necrosis factor family (TNFs), the chemokine family, the mesenchymal growth factors and the adipokines (L. Y. Zhu et al., 2013).

In response to viral pathogens, IFNs act by altering the regulation of mRNA transcription to produce an antiviral state in cells (Robertsen, 2006; Whyte, 2007). INF's are divided into three groups: Type I, Type II, and Type III and each have specific members (L. Y. Zhu et al., 2013). In mammals, there are about 20 members of Type I IFN, which includes IFN-α and IFN- β proteins. However, only one member has been found for Type II and Type III IFNs, which are INF-γ and IFN-λ, respectively (L. Y. Zhu et al., 2013; J. Zou & Secombes, 2011). Each type of INF cause the expression of selected IFN stimulated genes to occur, such as the antiviral protein, Mx, which produces an antiviral state in the targeted cells. However, INF-γ is also found to be involved in pro-inflammatory cytokine induction, which includes IL-1β, IL-6, IL-12 and TNF (J. Zou & Secombes, 2011). Each type of IFN has been discovered within bony fish and appears to have very similar activity to that seen in mammals (J. Zou & Secombes, 2011).

TNFs are implicated in a number of immune responses, including inflammation, autoimmunity, cellular apoptosis and host defence (L. Y. Zhu et al., 2013). Although up to 19 mammalian TNF members have been identified, few have been identified in teleosts and of the ones identified most appear to be similar to mammalian TNF-α (L. Y. Zhu et al., 2013). In response to bacterial lipopolysaccharide (LPS), TNF-α is thought to elicit macrophage activity and have a particular role in the neural immunoendocrine functions and induction of

IL-1β and IL-8 (Rauta et al., 2012; Uribe et al., 2011; Whyte, 2007). Another TNF of significance that has a homologue in bony fish is the TNF related apoptosis inducing ligand (TRAIL) that this is involved in inducing apoptosis in mammals (L. Y. Zhu et al., 2013).

Interleukins (ILs) are a sub-category of cytokines involved with intercellular signalling, particularly, but not exclusively to the immune system (Secombes, Wang, & Bird, 2011). There are over 37 different ILs that have been found in mammals, each characterised and grouped by function, similarities of receptors, or genetic homology (Akdis et al., 2011). The first and most commonly described of the IL families in mammals is the IL-1 family, which has 11 members, and is involved with pro-inflammatory responses (Lu, Chen, He, & Shi, 2013). The most commonly studied members of the mammalian IL-1 family are IL-1α, IL-1β, and the receptor antagonist IL-1RA. Another well studied IL-1 member is IL-18, which is able to induce production of INF-γ, stimulate cell differentiation of T_H1 cells, and improve NK cells cytotoxicity (Huising, Stet, Savelkoul, & Verburg-van Kemenade, 2004). In bony fish, the most studied of the IL-1 family is IL-1β, which was also the first IL to be characterised (Ogryzko, Renshaw, & Wilson, 2014). IL-1β was the earliest IL-1 member to be found in bony and cartilaginous fish, and was readily identified due the high levels of expression after contact with bacteria (Huising et al., 2004; Secombes et al., 2011).

There are a number of other interleukins present in the cell signalling network with varying effects on the immune system. IL-2 has primary signalling implications with T-cell maturation and subsequent apoptosis after repeated signalling (Akdis et al., 2011). It is understood that IL-2 is crucial for Treg development (Akdis et al., 2011). IL-2 also has effector roles in other cells including B cells, neutrophils, NK cells, monocytes and macrophage. Teleost IL-2 was first characterised in fugu, followed by rainbow trout (S. Bird et al., 2005; Y. A. Zhang et al., 2009). IL-6 has been found in mammals to have a range of functions in both the innate and the adaptive system. In the innate system, IL-6 can promote pro or anti-inflammatory effects, with the latter achieved by suppressing other cytokines, such as IL-1, IL-10 and TNF-α (H. H. Chen, Lin, Foung, & Han-You Lin, 2012). Innate cells including mast cells, dendritic cells and macrophages are all capable of producing IL-6. Non-immune cells, such as

epithelial and endothelial cells, are also able to produce an IL-6 response to a stimulus (Dienz et al., 2012). Another important interleukin found in teleosts is IL-10. The characteristic role that IL-10 plays in the immune system is an anti-inflammatory cytokine (Akdis et al., 2011). Activated macrophages and Th2 cells are responsible for the production of IL-10 which inhibits inflammatory signals such as IL-1, TNFα, and the expression of MHC class II proteins (J. Zou, Clark, & Secombes, 2003). The fugu IL-10 was the first teleost IL-10 to be characterised and was found using the fugu genome (J. Zou et al., 2003) and has since been found in a number of other teleosts (Savan & Sakai, 2006).

1.4 Immune Gene Discovery in Fish

The transcriptome is the collection of all RNA species in a cell or collection of cells (McGettigan, 2013). Transcriptomics, the study of the transcriptome, focuses on a number of aspects. This includes: quantification of each transcript, 3' and 5' ends, start sites, and any post-transcriptional modifications to the RNA (Z. Wang, Gerstein, & Snyder, 2009). Early transcriptomic work relied on microarray analysis. Microarrays are a surface on which a number of oligonucleotides are bound. These are designed to complement target RNA derived cDNA sequences (Malone & Oliver, 2011). Microarrays provide a comparison platform whereby RNA is extracted for a reference tissue and a comparison tissue. The two sets of RNA are independently reverse transcribed to cDNA and dyed with different fluorescent dyes (Valdés, Ibáñez, Simó, & García-Cañas, 2013). The cDNA is applied to the microarray and hybridised with its cognate probe, this is followed by a wash step that removes residual cDNA that has not hybridised. The microarray is then analysed by laser and computer systems with different dye intensities relating to the expression of a target gene (Karakach, Flight, Douglas, & Wentzell, 2010; Quesada-Garcia et al., 2014).

With the advent and increasing accessibility of next generation sequencing, RNA-seq is becoming a popular option for transcriptomic library formation. This method of sequencing involves the reverse transcription of sample RNA to cDNA which is then sequenced to produce a library of short reads (Garber, Grabherr, Guttman, & Trapnell, 2011). Transcriptomic analysis is normally paired with genome mapping; however this is limited to species with a reference genome (Zhao et al., 2011). In species without a reference genome, RNA-seq provides a practical solution in *de novo* assembly where short sequence reads can be assembled without a reference sequence (Robertson et al., 2010). Assembly is usually performed by genome-independent reconstruction algorithm software. This produces consensus transcripts that can be used to search gene and protein databases (Garber et al., 2011). The pivotal element of the work carried out for this research was transcriptomic libraries derived from RNA-seq.

Initial immune-related gene identification in fish has used methods such as homology cloning (Jun Zou, Grabowski, Cunningham, & Secombes, 1999),

subtractive hybridisation (Tsoi et al., 2004), and expressed sequence tags (Altmann, Mellon, Distel, & Kim, 2003). However, with the beginning of the next generation sequencing (NGS) era these techniques have fallen out of favour to methods like genome and RNA sequencing. A number of teleost species have a completed and annotated genome: such as *Takifugu rubripes* (Aparicio et al., 2002), *Danio rerio* (Howe et al., 2013) and *Xiphophorus maculatus* (Schartl et al., 2013) which has benefited the identification of immune genes in these teleosts. Transcriptomics for teleost species without a completed genome can allow similar gene discovery to occur. This is due to the comparative element introduced when looking at tissue specific transcripts (Z. Wang et al., 2009). In *Seriola quinqueradiata*, tissue specific immune gene profiling has been achieved using microarray analysis (Darawiroj, Kondo, Hirono, & Aoki, 2008). However, other research in *S. lalandi* relied on physiological analysis such as studying mucus and serum (Leef & Lee, 2008), or the concentration of blood lactate and plasma osmolality (Mansell, Powell, Ernst, & Nowak, 2005). A greater understanding is required for the immune system of *Seriola sp.* and NGS technology provides the data needed for comprehensive study of these species.

1.5 Aims

Understanding the Yellowtail Kingfish's immune system will benefit the aquaculture industry, by allowing better monitoring of fish health and the development of more efficient therapeutic approaches. However, for the successful introduction of any new species into aquaculture, it is important to characterise genes involved in innate and adaptive immune responses, so that informative studies can be completed, and responses to changes in the environment can be determined. This study will:

1. Use bioinformatics and transcriptomics to identify candidate genes related to the immune system of *S. lalandi*.

2. Characterise the complete mRNA sequence of the candidate genes using molecular techniques.

3. Determine the expression profile of the candidate genes in juvenile fish from 3 dph to 60 dph.

Chapter Two: Methods

2. Methods

All molecular work was carried out in C.2.03 Molecular Biology Laboratory at the University of Waikato. Work was carried out on a designated laboratory bench cleaned with 70% ethanol, unless otherwise stated. All waste was collected in a container filled with Virkon® disinfectant solution and then autoclaved on a waste cycle. Initial preparation of tissue for histology was also carried out in the C.2.03 Molecular Biology Laboratory. Further preparation for paraffin embedding was carried out in R.2.43. The sectioning of paraffin embedded samples was carried out in R.2.43a. Samples for cryostat sectioning were initially prepared in the C.2.03 Molecular Biology Laboratory. Further preparation and cryostat sectioning was carried out in E.2.14 Physiology and Biochemistry Laboratory.

2.1 Yellowtail Kingfish Samples

Larvae and juvenile yellowtail kingfish were collected at NIWA Aquaculture Park. Sample collections were taken at regular intervals from hatching until 60 days post-hatch. Up to 50 larvae were preserved in RNA*later*® (Life Technologies). Each stage had four replicates and twenty larvae fixed in either buffered glutaraldehyde or RNase-free buffered paraformaldehyde, and stored in methanol. For the adult samples, wild yellowtail kingfish, S. lalandi, (one male, one female) were caught near Gannet Island, off the coast of Kawhia, New Zealand. Samples were taken from various organ types: anterior kidney, brain, gill, liver, muscle, pituitary gland, spleen, ovaries and testes and transferred to tubes containing RNAlater® (Life Technologies). All samples were stored at -20°C until required for further use.

Molecular Techniques

2.2 Database Searching and Primer Design

Geneious v.7 (Biomatters Ltd.) software was used to identify potential immune genes from the previously constructed *S. lalandi* spleen transcriptome Ion

Torrent RNA-Seq library, provided by Dr. Steve Bird. A reference protein sequence for the gene of interest from a closely related species (in the order Perciformes) was retrieved from the NCBI protein database, and the RNA-Seq library was searched using tBLASTn. Results from this search that were inclusive of as much of the gene of interest as possible were downloaded and aligned to the reference protein sequence. The nucleotide sequence for each of these was obtained and used to construct a consensus sequence. Appropriate regions of the consensus sequence with high quality data were then used to design primers using Primer3 (http://primer3.sourceforge.net/) for subsequent amplification of the initial gene sequences and 5'- and 3'-RACE PCR. Primers were designed (Table 1) with the following ideal specifications in mind; Melting temperature (Tm): 60-62°C, Length: ~20nt, GC content: 40-60%, 3'end GC clamp (1-2 G or C at the 3' end promotes better binding). However, due to sequence quality obtained from the library, specifications were not always able to be met for all primers designed.

Table 1: Primers designed for use in this investigation. Also included are the M13 forward and reverse primers and the T7 primer for sequencing.

Primer	Gene Target	Primer Sequence (5' – 3')	Tm (°C)
slRAG-F1	*Rag1*	CTGAGAGAGAGCGGGATG	58.8
slRAG-F2		ATGGGCGATGTCAGCGAGAAG	59.7
slRAG-R1		AGAGTCTTGTGCAGGTAATTG	54.9
slRAG-R2		GCAAAGTGCTGCGAGTAAAAC	57.5
slIGD-F1	*IgD* (Heavy Chain)	TCTCCAGAGGAATTGAATGTG	52.1
slIGD-F2		TGTGGACGGAGACAATAAAAC	52.8
slIGD-F3		ATCACTAGACGCTTCACTGTCC	56.4
slIGD-F4		GAAGAGTCAGGACCACAGAG	54.4
slIGD-F5		GAGCCATTAGTGAGTCACAG	52.7
slIGD-F6		GTAACGGCACAGAGACTTTGC	56.4
slIGD-R1		GATGTCATCAAAGAAGTCACAG	51.4
slIGD-R2		AGTGCTCCAATGTGCCTG	55.6
slIGD-R3		CAGGGATAGGGTGGTGTTTAC	54.9
slIL1B-F1	*IL1β*	ATGGAATCCGAGATGAAATGC	53.1
slIL1B-F2		TTGAGCGAGATGTGGAGCTC	57.0
slIL1B-F3		GGACTGGACCTGGAGATCTC	56.4
slIL1B-F4		TGATGACGGTGAACAGGATG	54.7
slIL1B-R1		GGCATATTTAGTAACAACGGAC	51.5
slIL1B-R2		TTTCACATCATTTTGCCTCAGG	53.8
slIL1B-R3		CTGACGTTGAAGGAGGTGAC	55.4
slBACTIN-rtF	*Actb*	CACAGACTACCTCATGAACATC	52.3
slBACTIN-rtR		CCGATCCAGACAGAGTATTTAC	52.3
M13 Forward	*lacZ*	GTAAAACGACGGCCAGT	50.7
M13 Reverse		AGTGTGTCCTTTGTCGATACTG	47.0
T7 Promoter	T7	TAATACGACTCACTATAGGG	47.5

2.3 RNA Extraction

Due to availability, two different approaches were used for RNA extraction, and are detailed below:

2.3.1 TRIzol Reagent

Total RNA was extracted from juvenile or adult yellowtail kingfish tissue using the TRIzol® reagent protocol. First, the tissue was removed from the RNA*later*® and added to 1mL of TRIzol® reagent in a DNase/RNase free 2ml screw cap tube. The tissue was homogenised using 0.1mm silica/zirconia and 0.5mm glass beads (dnature) and shaken at 4800 Oscillations/minute for 10sec with a mini-beadbeater (Alphatech). Samples were checked for incomplete homogenisation and shaking repeated until a complete homogenised sample was obtained. After a 5 minute incubation period at room temperature, 0.2mL of chloroform was added to the homogenate. The sample was then shaken by hand for 15 seconds and left to incubate for 2-3 minutes at room temperature. After incubation, the sample was centrifuged (Eppendorf) for 15 minutes at 12,000 x g at 4°C. This resulted in proteins and DNA localising in the lower, red organic phase and white interphase and RNA exclusively remaining in the colourless aqueous phase. The aqueous phase was transferred to a new DNase/RNase free 1.5ml tube with care taken to not disturb the interphase. A disturbance could lead to phenol contamination which can disrupt later enzymatic reactions, such as cDNA synthesis. Following RNA collection, 0.5mL of 100% Isopropanol (Sigma) was added and the tube incubated for 10 minutes at room temperature. The sample was centrifuged for 10 minutes at 12,000 x g at 4°C and a pellet of RNA was formed. Frequently, the RNA pellet was invisible and the location of the pellet was noted by consistent positioning of the tube in the centrifuge, with the hinge facing up. The supernatant was removed by decanting and 1mL of 75% ethanol (Sigma) was added to the tube containing the RNA pellet. The sample was shaken with a vortex and centrifuged for 5 minutes at 7500 x g at 4°C, ensuring the hinge of each tube was facing up. The supernatant was removed using a pipette, taking care not to disturb the invisible RNA pellet and the remaining supernatant was left to air dry for 5-10 minutes.

2.3.2 R&A-BLUE Total RNA Extraction Kit

Tissue used for RNA extraction was added to a DNase/RNase free 2ml screw cap tube with 1mL of R&A-BLUE™ solution (iNtRON Biotechnology). Also added to the tube were 0.1mm silica/zirconia and 0.5mm glass beads (dnature). The tissue was homogenised by shaking at 4800 Oscillations/minute for 10sec using a mini-beadbeater (Alphatech) and each tube was visually checked for complete homogenisation. Upon completion of this step, 200µL of chloroform (Sigma) was added to each tube. This was followed by a 15 second vortex. The tubes were then centrifuged (Eppendorf) at 12,000 x g for 10 minutes at 4°C. This produced three layers within each tube with the RNA situated in the top aqueous layer. This aqueous layer was carefully removed using a pipette and transferred to a new DNase/RNase free 1.5ml tube. As an added precaution, a portion of the aqueous layer was left. This was to prevent disruption of the mid-layer which can lead to contamination from genomic DNA, proteins, or other organic compounds. Then, to precipitate the RNA, 400µL of isopropanol (Sigma) was added to the new tube containing the aqueous layer and then mixed by inverting the tube seven times. The tube was then centrifuged at 12,000 x g for 10 minutes at 4°C, which resulted in a pellet of RNA at the bottom of the tube. The supernatant was carefully removed using a pipette, to not disturb the pellet. The pellet was then washed by adding 1mL of 75% ethanol (Sigma) and the tube centrifuged at 13,000 x g for one minute at room temperature. Again, the supernatant was removed using a pipette, being careful not to disturb the pellet. The tube was then left for five minutes to allow the RNA pellet to dry.

In each extraction, the RNA was dissolved in 20µL of DEPC (Sigma) water and the sample placed on a heating block (Eppendorf) at 55-60°C for 10-15 minutes. The final quality and concentration of RNA was determined using the NanoDrop^{TM} 2000 (ThermoScientific). The NanoDrop 2000 uses a wide spectral range (190-840nm), allowing the concentration of DNA & RNA (260nm), proteins (280nm) and other contaminants (230nm) to be determined. First, 2µl of DEPC water was used to blank the instrument. Then 2µL of each extracted RNA solution was used to determine the quantity of RNA at 260nm and the purity was determined by the 260/280nm absorbance ratio where a ratio of ≥1.8 showed sufficient purity.

2.4 cDNA Synthesis

Total RNA was immediately used, once extracted to synthesis cDNA, due to the higher stability of cDNA over RNA. Two different approaches were used for cDNA synthesis, dependent on the intended use of the cDNA, and are detailed below:

2.4.1 qScript cDNA Synthesis

The qScript™ Flex cDNA Synthesis (Quanta Biosciences) kit included; qScript Flex 5x Reaction Mix that is made up of 5X buffer, magnesium, dNTPs and stabilizers, 10X Oligo$(dT)_{20}$ primer, 10X random hexamer primer (both primers are in a solution containing gene-specific priming (GSP) enhancer), qScript reverse transcriptase and nuclease-free water. An extra 10X GSP was supplied in the kit, but was not used. A reaction protocol using both primers was made up in an RNase/DNase free 0.2 mL PCR tube. To each tube, 2 µL of both the Oligo dT and random primers were added, followed by x µL of RNA, to give at least a concentration of 1ug. Finally, nuclease-free water was added to bring the final volume to 15 µL. The tubes were briefly mixed by vortex and pulse spun in a centrifuge (Total Lab Systems Ltd) for 5 seconds to ensure all the contents were at the bottom of the tube. The tubes were then incubated at 65°C for 5 minutes in a thermocycler (MJ Research) and immediately snap chilled in ice after the incubation period. Once the mixture had been chilled, 4 µL of 5x qScript Flex 5x Reaction Mix and 1 µL of qScript reverse transcriptase was added to each tube to bring the final volume to 20 µL. The contents of the tubes were briefly mixed by vortex and pulse spun in a centrifuge (Total Lab Systems Ltd) for 5 seconds before being placed in a thermocycler (MJ Research) with the following program:

Table 2: qScript cDNA thermocycler program to allow for primer annealing, reverse transcription and termination of the reaction.

	Temperature	Time
Random Primer Annealing	25°C	10min
Reverse Transcription	42°C	90min
Reaction Termination	85°C	5min
Holding Temperature	4°C	5min

2.4.2 Tetro cDNA Synthesis

A reaction protocol using both primers was made up in an RNase/DNase free 0.2 mL PCR tubes. To each tube, 1 µL of the Oligo $(dT)_{18}$ primer was added, followed by x µL of RNA, to give at least a concentration of 1ug. Next, 1µL of 10mM dNTPs were added, followed by 4 µL of 5x RT buffer. RNase inhibitor was also added at 1 µL to prevent digestion of RNA, followed by 1 µL of 200 u/µL reverse transcriptase. Finally, nuclease-free water was added to bring the final volume to 12 µL. Then the tubes were briefly mixed by vortex and pulse spun in a centrifuge (Total Lab Systems Ltd) for 5 seconds to ensure all the contents were at the bottom of the tube. The tubes were then placed in a thermocycler with the following program:

Table 3: Tetro cDNA kit thermocycler program for reverse transcription and termination of reaction.

	Temperature	Time
Reverse Transcription	45°C	30min
Reaction Termination	85°C	5min

The cDNA produced by both methods was kept in the freezer until use within polymerase chain reaction (PCR).

2.5 cDNA Amplification

Once the cDNA synthesis has been completed, a PCR was used to amplify specific genes from the cDNA. HOT FIREPol® DNA polymerase (Solis BioDyne) was used for the PCR reaction mix. This was a chemically altered DNA polymerase which is only activated by a 15 minute incubation period at 95°C. Thus, the polymerase is inactive at room temperature, lowering the chance of non-specific gene amplification. A PCR master mix was made in a RNase/DNase free 1.5 ml tube and included HOT FIREPol® polymerase, a Mg^{2+} free HOT FIREPol® 10X buffer B2 which contained Tris-HCl, $(NH_4)_2SO_4$ and detergent, $MgCl_2$ at 25 mM, 20 mM dNTP mix and DEPC water. The final 22 µl volume included 2.5 µL of both 10X buffer and $MgCl_2$, 0.5 µL of dNTPs, 0.25 µL of polymerase enzyme, and 16.25 µL of DEPC water.

Each reagent was multiplied depending on the number of reactions that were being run plus an additional reaction mix to ensure enough master mix for

each tube. To each RNase/DNase free 0.2 mL PCR tube 1 µL of forward and 1 µL of reverse primer was pipetted to the side of the tube, followed by 1 µL of the cDNA template. Finally, 22 µL of master mix was added to each tube to make a final volume of 25 µL. The contents of the tubes were briefly mixed by vortex and pulse spun in a centrifuge (Total Lab Systems Ltd) for 5 seconds before being placed in a thermocycler (MJ Research) with the following program:

Table 4: Thermocycler program for the amplification of cDNA using a designed primer to target a specific gene

	Temperature	Time	Cycles
Initial Denaturation	95°C	15 min	1
Denaturation	95°C	30 sec	35-40
Annealing	50-60°C	30 sec	
Extension	72°C	30sec-3min	
Final Extension	72°C	10 min	1

The extension time, annealing temperature and cycle number varied depending on the primers, length of the gene targeted for amplification and the intensity of the band obtained. Generally, annealing temperature was determined by the Tm of the primers and extension time was determined based on predicted size with the polymerase enzyme able to amplify at 1kb per second.

2.6 Gel Electrophoresis

The amplified PCR products were separated via gel electrophoresis. A 1.5% TAE gel was made by dissolving 0.525g of agarose powder (dnature) in 35 mL of TAE buffer (Sigma). The mixture was then microwaved and mixed until the agarose was completely dissolved and no crystals were visible. The solution was left to cool and 1µL of ethidium bromide (10mg/ml) was added. The cooled solution was then carefully poured into a gel mould to avoid the introduction of bubbles, and a 12 well comb was placed into the mould. The gel was left to set for approximately 20 minutes and the gel combs were carefully removed, so as to not collapse the wells. The gel was then placed in an electrophoresis mini gel system tank (VWR) with 1x TAE buffer covering the gel. Matching the number of PCR samples, 3µL drops of 6x gel loading buffer (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue) were placed on Parafilm® (Sigma) and the gel was loaded

with 5µL of 100bp ladder (GenScript). Loading the gel involved adding 10µL of PCR product to one of the 3µL loading buffer drops and mixing by pipetting up and down. Then 10µL of PCR product and loading buffer mix was pipetted into one of the wells. Once the gel had been loaded, the cover and electrodes were connected to the gel tank. The voltage was set to 90V with a run time of 25 minutes. At the completion of the run, the gel was taken from the tank and visualised using a UV trans-illuminator (Life Technologies). A photograph of the gel was taken with a High Performance CCD Camera (Cohu) and edited using Scion Image v0.4 (Scion Corporation).

2.7 Nested PCR

A nested PCR was used to overcome non-specific amplification obtained during the first round of PCR. An extra set of primers for each gene were designed and nested inside the first set of primers. This resulted in two forward and two reverse primers for each gene that was to be amplified. The first primer set, denoted as F_1 and R_1 were the original primers designed for PCR and were used in the first round of nested PCR. These primers were designed to anneal as close to the 3' and 5' end as possible. The second set of primers denoted F_2 and R_2, were designed to anneal slightly further into the sequence than the first primer set, sometimes with slight sequence overlap occurring between the primers. Nested PCR was run in two rounds. The first round PCR used the protocol set out in the aforementioned cDNA Amplification (see section 2.5) and used the F_1R_1 primer set to amplify from the cDNA template. The first step included a positive β-actin control with the same template DNA, but with β-actin primers, to determine that each PCR run was working correctly. The PCR products were run on a 1.5% agarose gel (see section 2.6) to confirm presence of the amplified gene of the right length. For the second round, template DNA derived from the previous first round PCR and three PCR tubes were set up with a different combination of primer in each (see section 2.5). There were three possible combinations of primers that were used: F_1R_2, F_2R_1, and F_2R_2, however, F_2R_2 was usually the preferred combination. Following amplification, the products were run on a 1.5% agarose gel (see section 2.6).

2.8 PCR Product Purification

To maximise the efficacy of further downstream applications, such as ligations, PCR product purification was used. This method used the DNA Clean & Concentrator™-5 (Zymo Research) kit. The PCR tubes each had a volume of 15µL due to the 10µL used for gel electrophoresis. To each tube, 75µL of DNA Binding Buffer was added to give 90µL total. A labelled Zymo-Spin column was placed into a collection tube and the 90µL of DNA sample was loaded into the column. The column was then centrifuged (Total Lab Systems Ltd) at 13,000 x g for 30 sec at room temperature. Once the centrifuge had finished the flow-through was discarded and 200µL of DNA Wash Buffer was added to the column. The column was then centrifuged at 13,000 x g for 30 sec at room temperature and the flow-through was discarded. The wash step was then repeated, again using 200µL of DNA Wash Buffer and centrifuging at 13,000 x g for 30 sec at room temperature and discarding the flow-through. The column was removed from the collection tube and placed into a named and labelled RNase/DNase free 1.5mL tube. To the column matrix, 10µL of DEPC water was added and the columns were centrifuged at 13,000 x g for 30 sec at room temperature to collect the purified PCR product were immediately used for cloning of the PCR product.

2.9 Cloning of PCR Products

2.9.1 Ligation of PCR Product into a TA Cloning Vector

PCR products that were good candidates were ligated into a vector for subsequent cloning in bacteria. The kit used was the pLUG-Prime® TA-Cloning Vector Kit (iNtRON Biotechnology, Inc.) and reactions were set up for each PCR product that was deemed a suitable size from the gel electrophoresis. Each tube contained the following:

Table 5: Reaction mix for ligation of PCR product into the TA cloning vector.

Reagent	Volume
10X Ligation Buffer A	1 µL
10X Ligation Buffer B	1 µL
TA-Cloning Vector	2 µL
T4 DNA Ligase	2 µL
PCR Product	4 µL
Total	10 µL

The contents of the tubes were briefly mixed by vortex and pulse spun in a centrifuge (Total Lab Systems Ltd) for 5 sec. The tubes were then refrigerated overnight to maximise the efficiency of the T4 ligase enzyme, before they were used to transform bacteria.

2.9.2 Lysogeny Broth (LB)+ Agar Plates

In order to grow successfully transformed bacteria, LB agar plates that contained ampicillin were made. The plates were made using agar (Coast Biologicals Limited), LB (USB Corporation) and ampicillin (Calbiochem). LB agar was made up in 500mL glass laboratory bottles (Duran), containing 7.5g of agar, and 12.5g of Lysogeny broth, made up to 500mL using double deionised water. The solution was mixed and the bottle was then autoclaved (Astell). During the autoclave cycle, a water bath (Life Technologies) was set to 60°C. Once the autoclave had completed, the bottle was placed in the water bath and left for 1 hour. This allowed the agar to cool enough for ampicillin to be added, but kept the agar warm enough to prevent solidification. To the agar, 5 µL of 100µg/mL ampicillin was then added and mixed by gently inverting the bottle. A sterile

environment was established by cleaning the bench surface with 70% ethanol and having a blue Bunsen flame in close proximity. Petri dishes (Global Science) were arranged on the work bench with the lid slightly ajar. The agar bottle was taken from the water bath and each plate was filled with agar until an even layer of agar was produced at 0.5 cm deep. Once the plates were poured, they were left to cool to 4°C and set. The LB+ plates were then closed, stacked and wrapped in aluminium foil.

2.9.3 Transforming Chemically Competent Bacteria

Two types of chemically competent cells were used for bacterial transformation, *E. cloni*® 10G (Lucigen) and Mix and Go *E. coli* (Zymo Research) due to availability. Before the transformation step, the tubes containing the ligation mix were initially placed in a thermocycler (MJ Research) at 70°C for 15 minutes. This was to heat inactivate the T4 ligase enzyme and without this enzyme inactivation the success of the transformation could be compromised. In addition, before bacterial cells were added to the LB+ agar plates, 40µL of IPTG (200mg/ml; Biotech) and 2% Xgal (Progen) was spread onto them using a glass hockey stick spreader over a blue Bunsen flame. To ensure the spreader was sterile, it was first dipped into 100% ethanol and flamed with a Bunsen burner. The spreader was cooled by touching the edge of the agar, and was then used to spread the solutions evenly over the plate. Upon completion, the plates were left to dry within a 37°C incubator (Precision Scientific) for 1 hour.

E. cloni 10G Chemically Competent Cells

Tubes containing 40 µL of *E. cloni*® subcloning grade cells (Lucigen) were removed from storage in the -80°C freezer and placed into ice to thaw for 10 minutes. Once the cells had thawed, 4µL of ligation mix was added to the tubes containing *E. cloni*® and gently mixed. The tubes were then returned to ice for a 30 minute incubation. Following the incubation, the cells were heat shocked in a water bath (Life Technologies) at 42°C for 50 seconds and then immediately placed back on ice for 2 minutes. To each tube of *E. cloni*® cells 960µL of Recovery Medium (Lucigen) was added which was followed by incubation in a shaking incubator (Bioline) for 1 hour at 37°C at 200 rpm. In preparation for plating one LB+ plate for each tube of cells was taken from the media only fridge. At this point the previously prepared, pre-warmed LB+ plates containing IPTG

and Xgal were named and labelled with the date and the name of the ligated gene. Under a blue Bunsen flame, 200µL of the transformed cells were applied and spread with a hockey stick. The hockey stick had previously been prepared by dipping into 100% ethanol, flaming with a Bunsen burner and then cooled by touching the edge of the agar. This application method was repeated for each plate to prevent both cross-contaminations by killing the cells with the high temperature of the spreader. The plates were then returned to the 37°C incubator (Precision Scientific) and left overnight.

Mix and Go *E. coli* Transformation Kit and Buffer Set

This kit involved the preparation of bacterial competent cells using the Mix and Go *E. coli* Transformation Kit and Buffer Set (Zymo Research). A colony of DH5α *E. coli* cells was taken from a previously grown plate to inoculate 100 ml of LB media. The colony was incubated in a shaking incubator (Bioline) overnight at 37°C at 200 rpm. The next day a 500mL culture flask was set up with 50mL of ZymoBroth™ which was inoculated with 0.5mL of the overnight DH5α culture. The flask was plugged with cotton wool, capped with aluminium foil and then placed in a 37°C shaking incubator (Bioline) at 200rpm. After one hour the optical density of the culture was determined by NanoDrop™ 2000 (ThermoScientific) using ZymoBroth™ as the blank. The optical density required was between 0.4 and 0.6 at 600nm. If the optical density was lower than 0.4, the flask was returned to the shaking incubator for another hour. This was repeated until the optical density reached 0.4, where the culture was then transferred to a 50mL Falcon Tube (Thermofisher) and put on ice for 10 minutes. The culture was then centrifuged (Eppendorf) at 4°C for 10 minutes at 2,500 x g. The 2x Wash Buffer and 2x Competent Buffer were diluted to 1x by adding 2.5mL of Wash, or Competent Buffer, to 2.5mL of Dilution Buffer, and remained on ice until required. After centrifugation of the bacterial cells, the supernatant was removed and 5mL of 1x Wash Buffer was used to resuspend the pellet. The cells were again centrifuged at 4°C for 10 minutes at 2,500 x g. Then the Wash Buffer was completely removed and replaced with 5mL of Competent Buffer. The cells were gently resuspended in the buffer and 0.2 mL of the cells were then gently divided into DNase/RNase free 2ml screw cap tubes by pipetting. The divided cells were stored in the -80°C freezer until required. The transformation procedure began by

thawing an aliquot of cells in ice for 10 minutes, after which the procedure for transforming and plating them out was the same as for the *E. cloni*® cells.

2.9.4 Screening of Transformed Bacteria

The TA-cloning vector allowed for two levels of screening of the transformed bacterial cells. The inclusion of the vector into the cells gives ampicillin resistance, which allowed for the growth of the bacteria on LB+ plates, provided by the inclusion of the β-lactamase gene in the vector. The second level of confirmation involved blue-white colony screening which determined if the PCR product has been successfully inserted into the vector. The site of product insertion interrupts the LacZ gene in the vector, which produced the enzyme β-galactosidase that is able to hydrolyse Xgal. This hydrolysis resulted in an end product with a distinct blue colour. Interruption of the LacZ gene and the inability for the transformed cell to produce β-galactosidase prevented hydrolysis of the Xgal, which is characterised by the presence of white colonies. This screening is a good preliminary analysis of the success of the ligation. To further validate this, white colonies were selected and a PCR was run which targets the region of the ligated PCR product. On the underside of each plate 10 isolated white colonies were marked with a pen and labelled from one to ten. Inoculation of ten DNase/RNase free 0.2mL PCR tubes containing 2µL of DEPC water was carried out using one of the ten marked colonies. The tubes were inoculated by touching a sterilised 10µL pipette tip to one of the colonies and transferring a small portion of the colony to the DEPC water. Care was taken not to contaminate the tube with agar as this can inhibit the PCR. M13 forward (5'- GTAAAACGACGGCCAGT-3') and M13 Reverse (5'-AGTGTGTCCTTTGTCGATACTG-3') primers were used to specifically target the ligated DNA. Each tube containing the bacterial template was inserted with 1µL of each primer to give a total volume of 4µL in every tube. A master mix was made up (see section 2.5) that contained 1 µL less water to account for the extra water to which the colonies were transferred. Master mix measuring 21 µL was added to the tubes containing 2 µL of inoculated water, which was followed by a 5 second centrifuge (Total Lab Systems Ltd). The tubes were then added to a thermocycler (MJ Research) to allow for amplification of the region between the M13 primers. The PCR products were run on a 1.5% agarose gel (See section2.6) to confirm presence of the ligated PCR product. Successful insertion of the product was shown by the presence of

one band. However an extra 172bp was added to the expected product size as the M13 primers amplified part of the vector, producing a slightly larger band.

2.9.5 Culturing Successfully Transformed Bacteria

Colonies containing the PCR product were grown in LB media overnight. LB media was made up in a 500mL glass laboratory bottles (Duran) by dissolving 12.5 g of LB (USB Corporation) in 500 mL of double deionised water. For each selected colony, a 14 mL sterile culture tube (Greiner Bio One) with 5mL of LB broth and 5µL of 100ng/mL ampicillin (Calbiochem) was set up. A sterile 10µL pipette tip was used to transfer the colony containing the correct PCR insert from the agar plate to the culture tube. Once a colony had been picked, the tip was dropped into the culture tube. The tubes were then left in a shaking incubator (Bioline) at 37°C at 200rpm overnight.

2.9.6 Extraction of Bacterial Plasmid

The Presto™ Mini Plasmid Kit (Geneaid) was used for plasmid isolation from previously cultured bacterial cells. Initial preparation of the kit included adding the supplied RNase A to the PD1 buffer and storing the buffer at 4°C and adding 100mL of absolute ethanol (Sigma) to the supplied wash buffer. This kit included the TrueBlue lysis buffer which acted as an indicator during the cell lysis and neutralisation steps. Each centrifugation (Total Lab Systems Ltd) performed in this plasmid extraction was at 13,000 x g, and room temperature. To RNase/DNase free 1.5mL microcentrifuge tubes, 1.5mL of cultured bacteria was transferred and centrifuged for 1 minute. The supernatant was removed and the pellet completely resuspended in 200µL of PD1 buffer and 2µL of TrueBlue lysis buffer. Also added to the resuspended cells was 200µL of PD2 buffer and each tube was gently inverted at least 10 times. The tubes were then left to incubate at room temperature for 2 minutes to allow for homogenisation of the lysate. At this stage, the entire solution had become blue, indicating complete lysis. Incomplete lysis was indicated by regions of brown cells or colourless areas within the solution. After incubation, 300µL of PD3 buffer was added to the suspension and each tube was immediately inverted at least 10 times. Successful mixing was indicated by the solution becoming colourless. Tubes that still showed blue areas were inverted until the entire solution became colourless. Tubes were centrifuged for 3 minutes and the supernatant was transferred to a PD column that was placed

within a 2mL collection tube. The columns were centrifuged for 30 sec and the flow-through obtained in each collection tube was discarded. For plasmids to be sequenced a preliminary wash step was carried out, 400µL of W1 buffer was added to each column and each tube was centrifuged for 30 sec and the flow-through was discarded. Wash buffer measuring 600µL was added to each column, and then the mixture was centrifuged for 30 seconds. Then the flow-through was discarded and the columns were dried in the centrifuge for a further 3 minutes. A new RNase/DNase free 1.5mL microcentrifuge was named and labelled with the name of the ligated PCR product, the colony that was used to produce the cultured cells, and the date. The lid hinge of each tube was removed using scissors and the dried columns were extracted from the 2mL collection tube and placed into the prepared tubes. DEPC water measuring 30µL was added directly to the centre of each column and allowed to stand for 2 minutes for complete absorption into the column matrix. The columns were then centrifuged for 2 min to collect the plasmids in the tube. The concentration of each plasmid was determined using the NanoDrop^{TM} 2000 (ThermoScientific). First, 2µl of DEPC water was used to blank the instrument. Then 2µL of each extracted plasmid solution was used to determine the quantity of DNA at 260nm. The purity of the DNA was determined by the 260/280nm absorbance ratio where a ratio of ≥1.8 showed sufficient purity. The required plasmid concentrations for sequencing were 150ng/µL, thus plasmids at this concentration or higher were used for downstream applications. Plasmid concentrations lower than 150ng/µL were kept and pooled after repetition of the plasmid extraction. Pooling the plasmids was achieved by using the originally obtained 30µL of plasmid at the elution step of the repeated plasmid extraction.

2.9.7 Restriction Digest of the Vector

Before sending the plasmid for sequencing, a final confirmation that the isolated plasmid contained the correct PCR product was required. The conformation was achieved through restriction digest of the plasmids. Two restriction sites found within the pLUG plasmid map were exploited to cut the ligated DNA from the plasmid. A restriction digest was set up in RNase/DNase free 0.7µL PCR tubes as follows:

Table 6: Reagents used for Restriction digest of the plasmid and the volume

Reagent	Volume
Plasmid DNA	1.00 µL
Buffer H	2.50 µL
***Eco*RI**	2.00 µL
***Pst*I**	2.00 µL
DEPC Water	17.5 µL
Total	25.0 µL

The contents of the tubes were briefly mixed by vortex and pulse spun in a centrifuge (Total Lab Systems Ltd) for 5 seconds to collect the reagents at the bottom of the tube. The tubes were then left overnight in the 37°C incubator (Precision Scientific). After incubation, the samples were run on a 1.5% agarose (see section 2.6). A successful digest was indicated with two distinct bands. The first band was greater than 1,500bp and represented the plasmid. A smaller band was also seen that was slightly larger than the ligated PCR product size that had been cut out of the vector by the restriction enzymes. The presence of this band at the correct size confirmed the success of the ligation.

2.9.8 Sequencing

Upon confirmation of successful ligation by the restriction digest, the plasmids were prepared for sequencing. The required volume of the plasmid was 12µL at a concentration of 150ng/µL. The formula $C_1V_1 = C_2V_2$ was used to determine the volume of plasmid required to make up a 12µL aliquot. The concentration of the plasmid is represented by 'C_1', and 'V_1' was the symbol used for the required volume of the plasmid. The final concentration is 'C_2', and 'V_2' was the final volume. Thus, to work out V_1 the equation must be rearranged to $V_1 = \frac{C_2V_2}{C_1}$:

$$V_1 = \frac{(150\text{ng/µL} \times 12\text{µL})}{\text{Plasmid Concentration}}$$

The volume, V_1, was then added to a named and labelled RNase/DNase free 0.7mL PCR tube, and DEPC water was added to make the final volume 12µL. The tubes were sent to the Waikato DNA Sequencing Facility where the plasmid

DNA was sequenced using BigDye® Terminator v3.1 Cycle Sequencing kit (Life Technologies). The sequence was run on a 3130xl Genetic Analyser System (Life Technologies). Plasmids were sequenced in one direction using the M13 Forward primer (5'-GTAAAACGACGGCCAGT-3'), or both directions using the M13 Reverse primer (5'-AGTGTGTCCTTTGTCGATACTG-3').

2.9.9 Sequence Analysis

Sequences were translated using the ExPASy translate tool (http://web.expasy.org/translate/), and alignments were ClustalX 2.1 (http://www.clustal.org/clustal2//). Phylogenetic trees were constructed using Figtree (http://tree.bio.ed.ac.uk/software/figtree/) software. Sequences were first aligned by ClustalX using the BLOSUM cost matrix, and trees constructed using the Figtree. Pairwise amino acid identity was determined using SIAS (http://imed.med.ucm.es/Tools/sias.html).

2.10 RACE PCR

2.10.1 3' RACE PCR

The FirstChoice® RLM-RACE kit (Life Technologies) was used for the rapid amplification of cDNA ends (RACE). This method allowed for the amplification of the 3' end of the gene of interest. Using the confirmed sequence, gene specific primers were designed (Table 7) to amplify the 3' end of the genes including the poly-A tail. Forward primers (F_1 and F_2) were designed near the end of the confirmed sequence. RNA was extracted from kidney, liver and spleen (see section 2.3) and reverse transcribed using the Tetro cDNA kit (see section 2.4.2). However, instead of Oligo $(dT)_{18}$ primers, the same volume of 3' RACE adapter was added. This added a known sequence to the terminal end of the RNA strands. Following a cDNA synthesis, first round PCR was performed (see section 2.5) using the supplied R_1 primer that anneals to the 3' adaptor and run on a gel (see section 2.6). A nested PCR, using F_2 and the supplied R_2 primers, was then performed (see section 2.7) to reduce the number of bands from non-specific amplification. The band was gel purified (see section x) and then cloned, sequenced, and added to the existing confirmed sequence (see section 2.9).

Table 7: Primers designed for the 3' RACE PCR used in this investigation. Also included is the 3' RACE adaptor and the primers that target this adaptor.

Primer	Gene Target	Primer Sequence (5' – 3')	Tm (°C)
3'RACE Adaptor	-	GCGAGCACAGAATTAATACGACTCACTATAGGT12VN	61.2-62.6
3'RACE Outer		GCGAGCACAGAATTAATACGACT	55.1
3'RACE Inner		CGCGGATCCGAATTAATACGACTCACTATAGG	60.7
slIgD-3'F1	*IgD*	ACACCTTCAGCCACCTTG	55.2
slIgD-3'F2		GGACCAAAGGAATTGTTGG	51.5
slRAG-3'F1	*Rag1*	AGGTGGTGTGTGAACTGGTGC	60.1
slRAG-3'F2		AGGAAAGGAGAGAGGCCCTGAG	60.2
slIL1B-3'F1	*IL-1β*	GGACTGGACCTGGAGATCTC	56.3
slIL1B-3'R1		TGATGACGGTGAACAGGATG	54.7

2.10.2 Gel Purification

Gel purification was used to isolate a single band on an agarose gel after a second round PCR for RACE. Often, despite using nested PCR for RACE, multiple bands remained, though non-specific bands were fainter. The Zymoclean™ Gel DNA Recovery kit (Zymo Research) was used to purify a single band from an agarose gel. While the gel is under UV light, a scalpel was used to cut around the band. The section of gel was placed into a 1.7 mL DNase/RNase free tube and weighed. Agarose dissolving buffer was added to the tube at three times the mass of the gel section, and incubated for 10 minutes at 50°C. After observing the tube to ensure the gel had completely dissolved, the solution was transferred to a Zymo-Spin column and centrifuged for 30 seconds at 13,000 rpm. The resulting flow-through was discarded and 200 µL of DNA wash buffer was added. The column was centrifuged for 30 seconds at 13,000 rpm, and again the flow through was discarded. This step was repeated and then followed by a drying step which entailed an extra 13,000 rpm centrifugation for 30 seconds. A new 1.7 mL DNase/RNase free tube was labelled and the lid was cut off. The spin column was placed into the new tube with 10 µL of DEPC H_2O and centrifuged at 13,000 rpm for 30 seconds.

2.11 Quantitative PCR

2.11.1 Primer Design

Using the online program Primer3 (http://primer3.sourceforge.net/) forward and reverse primers were designed for housekeeping genes and the genes of interest (Table 8). The following characteristics were adhered to as much as possible when designing primers. Primers had a 40-60% GC content to ensure stability of the product and the amplicon length was between 50 and 150 bp as longer products could lead to poor amplification efficiency. Primers also ended with a G or C residue as T and A residues can bind non-specifically to DNA more easily. The melting temperature of the primers was between 54 and 60°C with the forward and reverse primers being within 1°C of each other. The number of possible interactions within a primer and between primers was to prevent hairpin loops or primer dimers, respectively. Lastly, the primers and amplicons were BLASTed against both the public database and the transcriptome to help confirm that the primers targeted the correct gene of interest.

Table 8: Primers designed for qPCR for genes of interest and housekeeping genes.

Primer	Gene Target	Primer Sequence (5' – 3')	Tm (°C)
slRAG-RTF1	*Rag1*	GATGTTTGTGGATGAGTCAGA	53.5
slRAG-RTR1		TCTCTTTCATTGCATTACGTC	53.3
slRAG-RTF2		CTCGGAGCTATCCTGTAAGC	54.6
slRAG-RTR2		TTCATTGCTAAGCCTCTGC	53.8
slIgD-RTF1	*IgD* (Heavy Chain)	ATGGATGCAGATGTGAACGA	54.2
slIgD-RTR1		GTCAAGACACAATAGATGAAAGAG	53.1
slIgD-RTF2		GGTGATGCATGTAAACACCA	53.3
slIgD-RTR2		CCATGTCTTCGTTCACATCTG	53.6
slIL1B-RTF1	IL-1β	GACGAAACGGTTGTGAAGAC	54.0
slIL1B-RTR2		GATGATGTCTTTCTTCTGGGTG	53.3
slIL1B-RTF2		AAGTACATCTCCTGTGGTGC	54.5
slIL1B-RTR2		CATTTCTCCAGATGCAGCTC	53.6
slbactin-RTF1	β-Actin	TACAACGAGCTGAGAGTTGC	54.8
slbactin-RTR1		GTTGAAGGTCTCGAACATGAT	53.5
slgapdh-RTF1	GAPDH	GCTCATCTCTTGGTATGACAATG	53.5
slgapdh-RTR1		TGCATGTACATCAGCAGG	55.0

2.11.2 RNA Extraction and cDNA Synthesis

Total RNA was prepared from a number of different larvae and tissues. Spleen and kidney tissues were targeted for use in determining the specificity of each primer set designed and for testing their efficiencies. Preliminary examination of the expression of the genes of interest during development was

achieved using four replicates of 50 larvae that had their RNA extracted from day 0, 3, 12 and 18.

Preparation of high purity RNA, with no genomic DNA contamination was crucial for achieving the best results from qPCR and helping to prevent the amplification of non-specific products. To achieve this, the Quick-RNA™ MiniPrep kit (Zymo) was used, which was able to reliably and rapidly isolate DNA-free RNA from the tissue samples. The RNA isolation consisted of three steps: sample lysis, sample clearing and gDNA removal, and RNA purification. First, Kingfish tissues or pooled larvae from each time point were added to 2 ml polypropylene RNase/DNase free microcentrifuge tubes containing 600 µL RNA Lysis Buffer. Approximately 50 µL of 0.1mm and 0.5mm diameter glass beads were added and the tubes were placed into an Alphatech Mini-beadbeater™. The tissues were homogenised at 4800 oscillations per minute for 10 seconds. This was repeated if the tissue did not break down completely. Homogenisation caused the lysis buffer to foam, which was cleared by centrifuge at 13,000 x g for 1 minute. The supernatant was then transferred into a Spin-Away™ Filter (yellow), which was placed into a Collection Tube. The sample was centrifuged at ≥10,000 x g for 1 minute to remove the majority of gDNA, which remained in the filter. The flow-through in the Collection Tube was then used for RNA Purification. To the flow-through, which contains the sample in RNA Lysis Buffer, 1 volume of ethanol (95-100%) was added (1:1) and mixed well. This mixture was transferred to a Zymo-Spin™ IIICG Column (green) in a Collection Tube and centrifuged 13,000 x g for 30 seconds. The flow-through was discarded, leaving the total RNA within the column. Next, to ensure complete removal of any trace genomic DNA, the column underwent DNase I Treatment. The column was prewashed with 400 µl RNA Wash Buffer, centrifuged 13,000 x g for 30 seconds and the flow-through discarded. For each sample to be treated, a DNase I Reaction Mix was prepared in a 2 mL polypropylene RNase/DNase free microcentrifuge, which contained 5 µL DNase I DNA and 75 µL Digestion Buffer. This 80 µL DNase I Reaction Mix was added directly to the column matrix and incubated at room temperature (20-30 ºC) for 15 minutes. The column was then centrifuged 13,000 x g for 30 seconds and 400 µL RNA Prep Buffer was added to the column. Again the column was centrifuged 13,000 x g for 30 seconds and the flow-through discarded to ensure complete removal of the DNase I Reaction Mix. To wash the

RNA, 700 µl RNA Wash Buffer was added to the column and centrifuged 13,000 x g for 30 seconds and the flow-through discarded. Another 400 µl RNA Wash Buffer was added, but this time centrifuged 13,000 x g for 2 minutes to ensure complete removal of the wash buffer. Finally, the column was transferred to a 1.5 mL polypropylene RNase/DNase free microcentrifuge, with the hinge removed and 30 µL of DNase/RNase-Free water was directly added to the column matrix and centrifuged 13,000 x g for 30 seconds. The eluted RNA was measured using the Nanodrop 2000 (Thermo Scientific) and the 260/280 absorbance ratio measured to ensure purity was approximately ≥1.8. The RNA was immediately used for cDNA synthesis to minimise RNA degradation.

To synthesise cDNA the HiSenScript™ RH(-) cDNA Synthesis Kit (iNtRON) was used which has been designed for the sensitive and reproducible detection and analysis of full-length cDNA copies from a total RNA sample. For each sample, into a 0.2 mL DNase/RNase free PCR tube: 1 µg of total RNA, 10 µL of 2X RT Reaction Solution, 1 µL of Enzyme mix Solution and DNase/RNase Free Water up to a total volume of 20 µL. Each tube was then mixed well by vortexing and then the mixture was spun down using centrifugation. The tubes were placed into a Biorad T100 thermal cycler and tubes were incubated at 42°C for 1 hour to allow reverse transcription, followed by an inactivation step of 85°C for 5 min. Each cDNA obtained from the spleen and kidney was stored at 4°C until being used in q PCR for testing primer efficiencies. The cDNA produced from pooled larvae was immediately diluted into 300 µl of DNase/RNase free water and subsequently stored at 4°C until they were used for measuring the expression of the genes of interest in qPCR.

2.11.3 Quantitative PCR

Quantitative PCR was performed using the Rotor-Gene 6000 (Corbett). The RealMOD^{TM} GH Green q PCR Master Mix Kit (iNtRON) was used following the manufacturer's instructions. Briefly, 10 µL of 2x RealMOD^{TM} GH Green qPCR Master Mix, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM) and 8 µL of previously synthesised cDNA was added into a 0.2 mL RNase/DNase free PCR tube (Axygen). Amplification was carried out using the following program: Initial denature at 94°C for 5 minutes, followed by 40-50 cycles of denaturation at 94°C for 20 seconds and annealing/extension at 60°C for

30 seconds. Fluorescence outputs were measured and recorded at 80°C and a melt curve for each sample was performed between 72 and 94°C to ensure that only a single product had been amplified. Using the graphs generated, a threshold line was set on the amplification curves to generate the threshold cycle (Ct) value for each reaction, which is where a detectable amount of amplicon product had been generated during the early exponential phase of the reaction.

2.11.4 Primer Testing and Efficiencies

Prior to determining the expression of the genes of interest, the primer efficiencies were tested to determine the amplification efficiencies. The previously prepared cDNA from the spleen and kidney tissues underwent a 4-fold serial dilution. This produced cDNA that was undiluted or diluted 1:4, 1:16 or 1:64. For each cDNA dilution, each pair of primers was run in duplicate as well as a negative control. This control contained DNase/RNase free water in lieu of cDNA template to check for contamination. The determination of primer efficiencies was run in duplicate to ensure accurate results. At each dilution, Ct values were obtained from each amplification curve. The Ct values were plotted against the initial amounts of template on a semi-$\log_{10}$ plot. A line of best fit was applied to the points and the gradient of the line and the R^2 value was calculated. This showed how close the points were to line of best fit. Efficiencies (E) of the primers was calculated using the equation $\mathbf{E= 10^{(-1/s)}-1}$, where "s" is the slope of the line. The qPCR products were also run on a 1.5% agarose gel, using the same protocol that was previously described for PCR, to confirm amplification of a single product (see section 2.6).

2.11.5 Measuring Gene Expression Over Development

The housekeeping genes were used to normalise the expression in each pool of larvae and expression analysis was achieved using the geometric means of these genes (Vandesompele et al., 2002). Using the comparative ΔCq method (Silver, Best, Jiang, & Thein, 2006), the relative expression levels was determined. This method was a variation of the Livak method (Livak & Schmittgen, 2001) which is used to evaluate gene expression, where $\Delta Cq = 2^{Cq(ref)-Cq(target)}$. Statistical significance was determined using ANOVA and an independent Student's t-test, where values $P<0.05$ were considered significant.

Histological Techniques

2.12 Gelatine Slide Preparation

Prior to beginning the sectioning, 26x76mm slides (Fronine) were prepared. Coating the microscope slides in gelatine ensured sections remained in place and attached to the slide. This was essential for the following wash and dye steps. First, one litre of gelatine solution was made up in a 1000mL beaker (Kimax). This was achieved by dissolving 5 g of gelatine (Sigma) and 0.5 g of $CrK(SO_4)_2$ (Ajax Chemicals) in 1 L of double distilled water. A stir bar was placed in the beaker and then onto a magnetic stirrer/hot plate (Barnstead Thermolyne) that was set to 45°C. The solution was left until the gelatine and $CrK(SO_4)_2$ were completely dissolved. This was determined visually by checking for any suspended particles. Upon completion, the solution was left to cool at room temperature. During this cooling stage, a 10 cc/mL syringe (Terumo) and 0.45 μm syringe filter (Sartorius Stedim) were prepared. Once the gelatine had sufficiently cooled, the solution was filter sterilised and transferred to a 1000 mL glass laboratory bottle (Duran). Slides to be gelatine coated were placed in a metal slide rack and then the dish was filled with gelatine solution. Enough gelatine solution was poured into the dish to ensure the slides were completely submerged during the next step. The slide rack was then dipped into the gelatine solution five times each for five seconds. The slides were then stored at room temperature for 48 hours to ensure the slides were completely dried.

2.13 Preparation of Juvenile Kingfish for Paraffin Embedding

Before the fish could be embedded in paraffin, a number of wash steps to dehydrate the fish were performed. First, the fish were placed in a 50mL falcon tube and submerged in 1x phosphate buffered saline (PBS) for five minutes in the 4°C cold room. After the 5 minute incubation, the PBS was removed and replaced with fresh 1x PBS. The PBS wash steps were repeated a total of three times. The following steps required three different concentration ethanol solutions a 5%, 25% and 75% which were diluted using 1XPBS. These steps were used to dehydrate the sample and were achieved by submerging the fish in increasing ethanol/1XPBS solutions for five minutes at room temperature. Under a fume hood, the sample was then transferred to 100% ethanol for 30 minutes at room

temperature. This step was repeated twice more, each step again incubated for 30 minutes at room temperature. This was followed by three, 30 minute xylene washes at room temperature, again under a fume hood. This xylene was a mixture of p-, o-, and m-xylene isomers. After the final xylene wash, the samples were taken to R.2.43 where an equal volume of liquid paraffin wax was added. This 1:1 xylene and paraffin mixture wash incubated at 60°C (Contherm) for 45 minutes. Finally, three 60°C paraffin washes were performed by submerging the samples in paraffin and leaving for at least 20 minutes. The samples were then ready for paraffin embedding.

2.13.1 Alternative Preparation Protocol

A second protocol was trialled as a possible way in which section quality could be increased. In lieu of the PBS washes, the samples were transferred to 100% ethanol for five minutes. The ethanol was then replaced with fresh 100% ethanol and left to incubate overnight. The ethanol was removed the next day and replaced with p-xylene. This differs from the previously in that it is a single isomer of xylene. The samples were washed for 30 minutes with xylene, and then repeated twice more. An equal volume of liquid paraffin was added to the samples and incubated at 60°C for at least 30 minutes. Once the paraffin had liquefied, the tubes were inverted three times to mix the 1:1 xylene paraffin solution. Three paraffin washes were then performed where paraffin was added to each tube and incubated at 60°C for 45 minutes to enable maximum wax infiltration.

2.14 Paraffin Embedding

Embedding of the juvenile kingfish samples was completed using a HistoStar™ Wax Embedding Station (Thermo Scientific). The embedding station was set up the day prior to use, by turning on the machine and adding Surgipath® Paraplast® paraffin to the wax tank. The tank was heated to 60°C and left for 24 hours to allow the paraffin to become liquid. On the day of embedding, the 60°C hot spot and cold module were turned on and left to reach the desired temperature. The mould and specimen tanks were also turned on and the heat set to 60°C. A mould for each specimen was placed in the mould tank to heat up to 60°C. This completed the set up stage of the embedding station. Orientation of the fish in the mould was important for later microscopy work. The fish was required to be sectioned through the transverse plane i.e. from head to tail. A mould was taken

from the mould tank and placed on the hot spot underneath the wax spout. A small amount of wax was added to the bottom of the mould, enough to create a small layer. The fish was transferred from the final paraffin wash step and placed head first into the wax using tweezers. The mould was then placed on the cold spot to allow the wax to slightly solidify and hold the fish in place. It was important that the wax not be allowed to completely solidify, as adding more wax after this would produce two distinct layers. This layering causes weakness in the structure of the final paraffin block. Once the initial wax had cooled enough, it was put back on the hot spot and the fish was covered in wax. When the fish was entirely covered, a Tissue-Loc cassette (Thermo Scientific) was placed on top of the mould and filled with wax. The mould was transferred to the cold module and left for at least 20 minutes to ensure the wax was completely set. Once cooled, the paraffin block was carefully removed from the mould.

2.15 Sectioning with the Wax Microtome

The paraffin block required trimming before sectioning took place. Each side of the block was trimmed using a razor blade at a 45° angle to form a trapezoidal prism. This was to produce smaller sections with the fish in the middle of the block. Next, named and labelled gelatine slides were placed on an RNase-free heating block (Leica) at 37°C, and 0.2% ethanol was dropped onto each slide. The paraffin block was secured into the wax microtome (Leica) and a blade was carefully placed in the blade holder. The microtome was adjusted to position the block so that it was parallel to the blade. This was to ensure an even section was produced. The orientation of the block relative to the blade was predetermined through trial and error while cutting the extra paraffin prior to the fish blocks. Position of the block was controlled from a control panel that could bring the block forwards or move it back. This control panel could also be used to position the blade; most blade movements were controlled by a hand wheel. Once tissue was at the surface of the wax, the microtome was set to cut 6 µm thick sections. Once a section was produced, a fine bristle brush was used to transfer the section to the 0.2% ethanol on a gelatine slide. This was repeated until ten sections were produced. Once ten sections had been produced, the microtome was set to 20 µm. This was followed by five rotations of the hand wheel. These steps were repeated until the fish had been sectioned to a point of interest.

2.16 Preparation of Juvenile Kingfish for Cryostat

Before preparing the specimens for cryostat sectioning, sucrose was made up at 30%. First, 150 g of sucrose was measured out and added to 50 mL of 10X PBS in a 500mL beaker. The volume of the solution was then increased to 500 mL by adding DEPC H_2O. The solution was mixed and sterilised using a syringe and filter, then was transferred to a 500 mL laboratory bottle (Duran). The fish were first placed in a tissue well and submerged in 1X PBS as a quick wash. The wash was immediately removed and replaced with fresh 1X PBS and incubated for 10 minutes. Two more wash steps were performed, followed by a quick wash of 30% sucrose. After this quick wash, two 10 minute wash steps were performed with 30% sucrose. Next the fish were submerged in 30% sucrose and incubated overnight at 4°C while being gently rocked by orbital shaker. After overnight incubation, two-thirds of the 30% sucrose was removed and replaced with optimal cutting temperature compound (OCT) (Frozen Section Compound, Surgipath). They were then incubated for one hour at 4°C while rocking gently. The fish were then taken to the E.2.14 physiology lab for further cryostat preparation. A cryomold® (Tissue-Tek) was filled with OCT and a fish was transferred to the mould using sterile tweezers. The fish was positioned vertically along the mould and a note was taken of the orientation of the fish. The mould was then sprayed with Frostbite® (Surgipath) to set the OCT. The block was removed from the mould and stored for later use. The frozen block was wrapped in tin foil and stored at -80°C.

2.17 Sectioning with the Cryostat

The cryostat (Leica) was prepared before sectioning took place. It was checked for any debris from previous sectioning and cleared if not already clean. The cryostat was then set to -25°C and left to cool. The frozen fish blocks were required to be mounted to a specimen disc before sectioning could occur. The specimen disc was covered in a layer of OCT and the block containing the sample was placed on the specimen disc. The block was orientated so the head of the fish was pointed out head first. Once the block was correctly orientated they were sprayed with Frostbite® to set it in place. The block was then placed on the quick freeze shelf in the cryochamber and the heat extraction cylinder was positioned over the block. The Peltier element was activated and the block was left to

solidify for 10 minutes. Following this cooling step the specimen disc was loaded and secured into the specimen head. The anti-roll plate was taken off the knife holder base. A knife was then carefully placed in the knife holder. The specimen block was then moved towards the blade using the control panel. The microtome was set to cut 10 μm sections. Gelatine slides were named and labelled before sectioning took place. The hand wheel was used to cut away at the preliminary OCT prior to the cutting of the fish. Once tissue was exposed, the anti-roll plate was placed on the knife holder base and a section was taken. The anti-roll plate prevented the section from curling and folding over on itself. The section was transferred onto a slide by removing the anti-roll plate and pressing the slide onto the section. This was repeated until at least six sections were taken, and was followed by cutting 100 μm into the fish. These steps were followed until each fish was completely sectioned.

2.18 Paraffin Staining

Two staining protocols were used to stain the sections of fish, toluidine blue and haematoxylin and eosin. These were used to visualise the sections under microscope and distinguish various tissue types. Two types of staining protocols were used during this experiment, toluidine blue and haematoxylin and eosin (H&E). Prior to staining, slides were placed into a slide rack and washed. The wash protocol went as follows: two xylene washes for each slide, for 10 minutes. Then a 100% ethanol wash for five minutes, a 95% ethanol wash for two minutes. A wash of 70% ethanol for two minutes, 35% ethanol for one minute, and then washed in tap water for at least 30 minutes. The initial xylene wash was performed to dissolve the paraffin wax, and was followed by gradual rehydration of the tissue by decreasing ethanol concentrations. This rehydration allowed the successful penetration of the stain throughout the tissue. Between each solution the slides were allowed to dry for approximately 30 seconds. Following the 30 minute wash in tap water, the slides were air dried for ten minutes.

2.18.1 Toluidine Blue Stain for Paraffin Sections

Slides were placed on a dry paper towel and a small drop of 1% toluidine blue solution was added to each tissue sample using a P1000 pipette. Once the toluidine had been applied, the slides incubated at room temperature for two

minutes. This was followed by a tap water wash step. The slides were held under a gently running tap until any residual solution had been removed.

2.18.2 Haematoxylin and Eosin (H&E) Stain for Paraffin Sections

The H&E stain was also used for staining paraffin sections. Following the wash step, the slides were placed on a paper towel and a small drop of haematoxylin was added to each tissue sample. Once the haematoxylin was applied, the slides were left to incubate at room temperature for five minutes. Following this incubation, the slides were washed under a gently running tap until residual haematoxylin was removed. This step was followed by returning the slides to the rack and submerging the slides in tap water for ten minutes. The slides were removed from the tap water and air dried for at least 30 seconds. The slides were then dipped in 0.1% HCl three times, followed by dipping the slides in 0.1% NH_4OH three times, and finally dipping the slides in tap water three times. The slides were left to dry on a paper towel in preparation for the eosin counterstaining protocol. As with the haematoxylin, small drops of eosin solution were added to each section. The slides were then left to incubate at room temperature for three minutes. They were then placed in the rack and a dipping procedure was followed where the slides were dipped five times for five seconds in each solution. First, the slides were dipped in 100% ethanol + 0.1% acetic acid, followed by dipping the slides in 100% ethanol which was repeated. The next two steps involved dipping the slides in acetone, again five times for five seconds. Finally, the slides were dipped in xylene five times for five seconds; this xylene dipping step was then repeated.

2.18.3 Preliminary Microscopy and Cover Slips

Once the slides had dried, the quality of the sections and the staining was determined. An Axiostar Plus compound microscope (zeiss) was used to visualise the tissues, noting successful sections for later microscopy. Slides that were stained using toluidine blue required dehydration before a slide cover could be applied. This was completed by washing the slides in 35% ethanol for 20 seconds, 70% ethanol for 20 seconds, 95% for 20 seconds, two 100% ethanol washes for 20 seconds and two five minute xylene washes. The H&E stained slides did not require this step. Application of DPX mounting media (Sigma) to each successful

section was performed. This was followed by placing a 22x40mm cover slip (Menzel-Glaser) on each slide and leaving the slides to set overnight.

2.19 Cryostat Toluidine Blue Staining

Slides that were prepared via cryostat required a different staining method for toluidine blue. First, the slides were placed in a PBS bath for 20 minutes in order to dissolve the OCT. An attempt was made to stain the slides using the 1% toluidine blue previously used for paraffin sections. However, this did not work and a new toluidine stock was made up. A new stock of toluidine blue was made up that contained 100% ethanol and urea (Appendix II). A small drop of the toluidine blue solution was added to each tissue sample using P1000 pipette. Once the toluidine had been applied, the slides were left to incubate at room temperature for two minutes. This was followed by a tap water wash step where the slides were held under a gently running tap until any residual solution had been removed. Once the slides were dried, the Axiostar Plus compound microscope was used to visualise the tissues, noting successful sections for later microscopy. Application of Shandon Immu-mount™ (Thermo Scientific)) to each successful section was performed. This was followed by placing a 22x40mm cover slip on each slide and leaving the slides to set overnight.

2.20 Microscopy

High quality pictures were obtained by using a DMRE microscope (Leica) equipped with a DP70 camera attachment (Olympus). Fitted on the microscope was HC Plan 25mm 10x eyepieces and HC Fluotar objectives. Photos of the section in this work used 5x, 10x and 20x objectives. The upper and lower filter prisms were set to bright field and brightness was not raised above 6 to maximise the life of the bulb. Before using the microscope the motorised focus upper threshold was calibrated to prevent the slide and the objective coming into contact. A slide was placed on the stage under the 10x objective and was brought into focus using the manual focusing wheel. Calibration was completed upon pressing the upper threshold calibration button once to activate set mode and once more to set the current focus position. Slides were positioned using the X-Y controls and sections were focused. On a computer attached to the camera, DP imaging software (Olympus) was used to capture the desired images of the sections. Depending on the thickness of the section and the objective used, the

exposure was adjusted and the white balance and level adjustment tools were used to produce high quality images.

Chapter Three: Results

3. Molecular Results

3.1 RNA Extraction and β-Actin

The initial sequence confirmation of immune genes was achieved using RNA from an adult in *S. lalandi*. Four tissue types were used to isolate total RNA: kidney, spleen, liver and gill. These were selected due to the immunological activity each tissue type possesses within fish. Total RNA was extracted and quantified using the NanoDrop 2000 (Table 9). This was used to determine the purity of the RNA sample and its concentration, which was used to determine the volume of RNA necessary for cDNA synthesis.

Table 9: NanoDrop analysis of extracted RNA from kidney, spleen, liver and gill.

Tissue Type	RNA Concentration	260/280	260/230
Kidney	1143.9 ng/µL	2.00	1.59
Spleen	3183.3 ng/µL	2.07	1.94
Liver	7452.6 ng/µL	2.07	1.98
Gill	1839.5 ng/µL	2.05	1.97

Once cDNA had been synthesised from the total RNA, a PCR was run using β-actin primers (Figure 6). This was a positive control and was used to show successful cDNA synthesis. The presence of bands at 476 bp were expected and showed the cDNA was successful synthesised. The cDNA could then be used for further work. Most work was carried out on spleen, kidney and liver tissue with gill being used in case of failure in the other tissue.

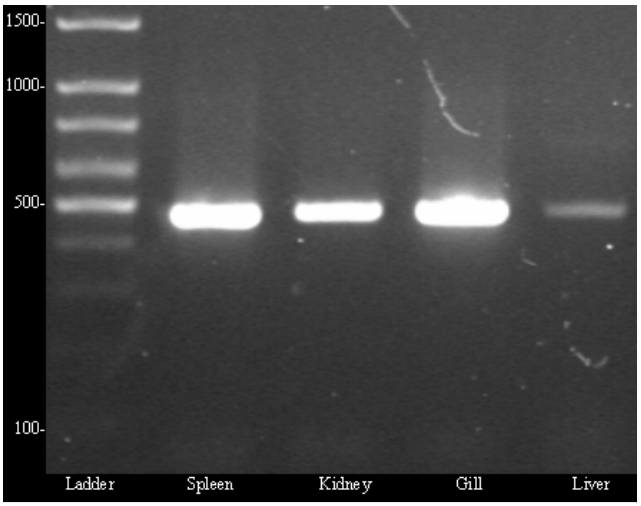

Figure 6: Amplification of β-actin from cDNA of different tissue. This gel image shows that cDNA from spleen, kidney, gill and liver tissue was successfully synthesised from RNA and could be used for further analysis.

3.2 Immunoglobulin Delta

The *Siniperca chuatsi* (ACO88906.1) amino acid sequence was found in the NCBI database and used to search the transcriptome library for IgD. The Geneious software was used to perform the transcriptome search. Nucleotide sequences were obtained from the tblastn search and were assembled, to produce a consensus sequence of *S. lalandi* IgD. The consensus sequence was translated using ExPASy and aligned to the *S. chuatsi* IgD amino acid sequence which gave a 28.39% identity (Figure 7).

```
S_lalandi       ------XXXGQGDTSHCXXXXKVNSGPHQSDSSAPLVASLQRN-----------------
S_chuatsi       MFSVALLLLLAAGCVKCEQLTQPASVTVQPGQRLTITCQVSYSVSSYRTAWIRQPAGKGL
                      ..  :*    :  * . *...  .:...:. .
S_lalandi       MWSGNRKXNLYILYQX---KESCXSVDGDNKTFSLIS-EIQPN---------------MT
S_chuatsi       EWIGIRRVGYTTYYKDSLKNKFSIDLDSSSNTVTLNGQNVQPEDTAVYYCAREILYWAFD
                 * * *: .    *:    :: . .:*...:*.:* . ::**:                :
S_lalandi       EWKRGLSFTCKAIHDKKNLXX------PRRGRSLLVNCLTAL-------MKLSARTSACV
S_chuatsi       YWGKGTTVTVTSATSTGPTVFPLMQCGSGTGDTVTLGCLATGFTPSSLTFTWSKNGAALT
                 * :* :.* .:  ..           .  * :: :.**::        :. * . :* .
S_lalandi       QFILALLLQFTWRFPASRLXXX-----RESDVIATCSVLTGFDVKMTWLMDGLNRTXERR
S_chuatsi       DFIQYPPVQKGDVYTGISQIHVRRQDWDARESFQCAVTHPAGNVQTNFIKPSQRVDSPNI
                :**    :*    :..             : :  . . .. :*: .::  . .    .
S_lalandi       CKHNS-NQSCDSSLKRLETTQDDNM-S-P-VFSHPTGQ-MSQGLQLRFRQWK--------
S_chuatsi       TLHPVWEGEFGASPVRLICTLSGFFPDKLNVTWQRDNRALDGQIQRKLQSVEGVEKTFSL
                  *   : . .:*  **  * .. : .   *  :  .: :.  :* :::. :
S_lalandi       ----SRDLSQIC-RGNLCSVSXXAKAQIC-RGNLCSSVTSHNSPQVTSTSPFRPTMLXX-
S_chuatsi       SSEIEPNMTEWADGSSFTCMSIHNNSEFRKTISICQIHSRAAPSIHVEIPSFKTVMMSAS
                    . :::: .  ..: .:*   ::::    .:*.  :   ..  .. ..*:..*:
S_lalandi       ------------------------------------XXESMLIFLKPWAFIQSLDASL
S_chuatsi       EVKATCSLRTVFDAKVTWLMDGKLPSRDQVNKVTNTTHLISTLTVSLNQWKQLKLLNCKA
                                                    .:: : *: *  :: *:..
S_lalandi       SLKVTSKKARIS--PAKSIKASPAASSQTQQAIFLG-------------THLWNFFWSX
S_chuatsi       EHRCFSSAEETTNVSRPAVTAPSVEIRRSLPDLLKGNSAVLECDITQLSSSDLYVTFQAN
                . :  *.  . :  .  ::.*...   ::   :: *             :.*:  * :
S_lalandi       --------------------------------XAKA-THLWNFFWSPVKSQDHRDSCAL
S_chuatsi       SVDISDKQYVDLPEDPGLHSVSRRFTVPPSHWKKDTSFTCKVNQGFSSNFESNSTGKIFV
                                                .: *   *  :*.  ..:  ..  :
S_lalandi       DGASILKLN---------------GFLTLIKDLIQTMSSPXXXXDVWQ-PX-------NX
S_chuatsi       DPSVELLLVPSKESGPQRLVCSGWGFDPQIKWFSESQQRSPSTNDISMGADGRVAVTSQL
                * :  * *                ** . ** : :: . .    *:   .        :
S_lalandi       XXLKKSGKXGRSXLVXCLTALXMXTVSKNISLCSVTPASSQVVGVYVQGPPLQESSEXXX
S_chuatsi       HIPQTEWKTGKGFTCKVSDKSLNKIVEKEISLCSVTPASSQIVGVYVQGPPLQELQSQGQ
                   :.. * *:.            *.*:************:************ ..
S_lalandi       XXXXXXXXAKAGRPSS--FLHHL-SRWGHTAPKFLY-AISESQ-RHRDFAELLQYVSRGL
S_chuatsi       VTVTCLLVGPSLNDFS--------ITWKVGGIKYSLNVHTEPPVSHSNGTESLRSFLN--
                      . :  .  *          *   . *:   . :*.   * : :* *: . .
S_lalandi       ARI-KSVL-SKAPVLXPRXQTPYKQKQRSTNS--EDNATNSL-ALYVRRPHTNLPSFWIF
S_chuatsi       ---------VSAEDWHAYKQVSCEGKHRCSNQGYEDHISKSR-DLYPPTVKIIQPTASEL
                         .*    .  *.. : *:*.:*.  **: ::*   **    :   *:   :
S_lalandi       SIQHHSVLGRX--GQGASNIIVYWEXXGXRLQPNSLHQQXLHGNTQGAAXYSMSSRLNVS
S_chuatsi       STSDVLTLICLVSGFFPSNIIVYWEENGQRLPSTRYTNS-PAWKYTGSSTYSMSSRLNAS
                * ..  .*     *  .******** * ** ..   :.    :  *:: ********.*
S_lalandi       KTEDKNSTYSCAVRHESSERPFESYINNVFATVPXXAKAXXXXATLLQGTNELVCLVFGF
S_chuatsi       KTEDKQSTYSCVVRHESSETLLESTIKDVFATEPYSEPS----ATLLQGSGELVCLVFGF
                *****:*****.*******  :** *::**** *    :    ******:.*********
S_lalandi       SPASINITWYDGPKELLDYNTSEPHRGXERKVQYPKPPSSVPGQLVTSVYGKVITCRVMH
S_chuatsi       SPASINITWFDDTKELLDYNTSEPHRGPNGKFSIQS---HLRLSQVNWLPGAVLTCRVTH
                *********:*..************** : *..  .    :  . *. : * *:**** *
S_lalandi       VNTTLSLNISKPGTLEHCDFFDDIMDAD--------------------------------
S_chuatsi       ANTTQSLKIAKPDILEDCLFLDDIMHADVSQDIDVESWYMACIFLLFFLISIIYGVLATV
                .*** **:*:**. **.* *:****.**
S_lalandi       ----
S_chuatsi       IKTK
```

Figure 7: Amino acid alignment for Immunoglobulin Delta from the reference species and the *Seriola* transcriptome. A pairwise alignment was performed using ClustalX 2.1 using *S. chuatsi* IgD sequence (Accession no. ACO88906.1) and IgD translated from the *S. lalandi* transcriptome. An asterisk represents complete identity, a colon represents conservative substitutions and a dot represents a semiconservative substitution.

3.2.1 Amplification and Cloning of IgD

Various primers for the amplification of the coding DNA sequence (CDS) region of IgD were designed and used in spleen cDNA. The first primer combinations used were F1R1, F2R2 and F3R2 which were predicted to produce products of 2,302 bp, 2,201 bp and 1,466 bp (Figure 8). These primer combinations did not result in any bands of expected size.

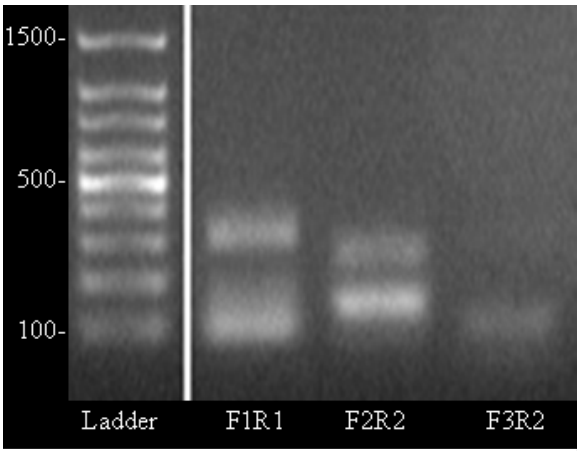

Figure 8: Gel image of the unsuccessful amplification of IgD. Primers were designed to amplify larger products than shown which were 2,302 bp, 2,201 bp and 1,466 bp. However the largest product was approximately ~350 bp when a larger product was expected.

Additional primers were subsequently designed to amplify smaller products and were trialled. These included F4R3, F5R3 and F6R3. While F4R3 did not produce the expected 1,237 bp product (not shown), the combinations F5R3 and F6R3 produced bands at around 800 bp and 781 bp, respectively. These bands were approximately similar to the size predicted (Figure 9). The gel analysis showed a strong, singular band in each lane, except in Kidney F5R3 which also had a faint band at approximately 300 bp. A nested PCR was not required due to band strength and number. Two products were selected for further ligation which was Spleen F5R3 and Kidney F6R3.

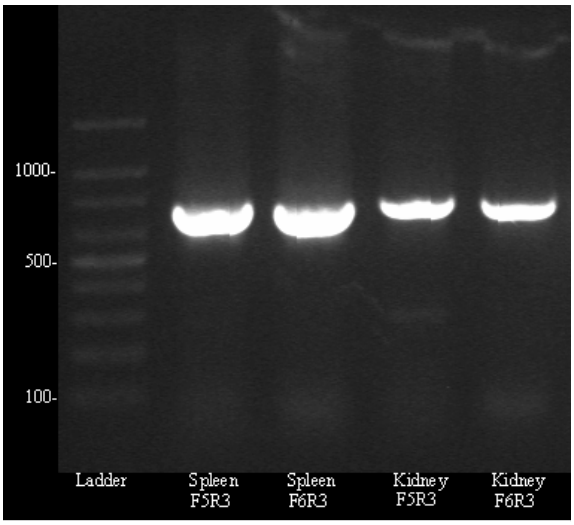

Figure 9: Gel analysis of IgD PCR products. Primer combinations F5R3 and F6R3 were using to amplify IgD in spleen and kidney tissue. The gel showed strong bands in each lane of approximately the correct size. A second faint band was seen within the Kidney F5R3 lane. Spleen F5R3 and Kidney F6R3 were ligated into vectors.

3.2.2 Colony PCR for IgD

Upon successful ligation and transformation of bacterial cells, two colony PCRs were performed. One for the spleen insert and one for the kidney insert. Six colonies from each plate were screened for colony PCR and subsequently run on a gel. As M13 forward and reverse primers were used to screen the colonies, the expected product sizes were the number of bases of the insert with 172 bases added from the vector. A band of 972 bp was expected from colonies with the spleen IgD insert (Figure 10). A band of 953 bp was expected from colonies with the kidney insert (Figure 11).

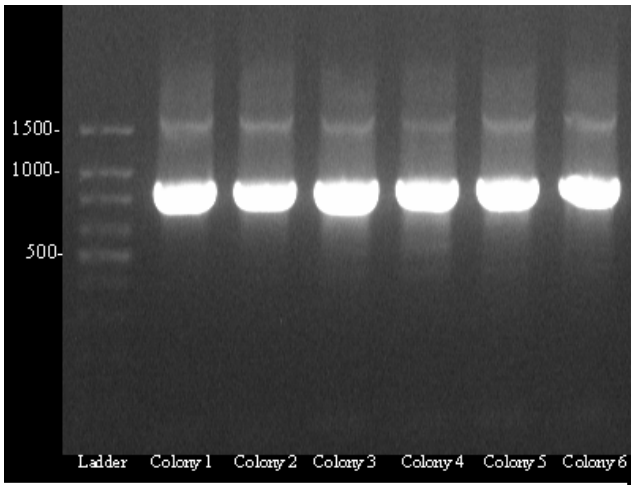

Figure 10: Colony PCR for Spleen IgD insert. Gel shows very strong bands at the expected size of 972 bp which indicated all colonies possessed the insert. Bands larger than 1,500 bp were also seen which could be due to non-specific amplification of the plasmid. Some smaller products were also seen, however due to the strength of these bands compared to the ladder, they were difficult to see once the image brightness was adjusted.

All colonies from the spleen plate and two colonies from the kidney plate produced bands that were approximately the size that was predicted. Colonies two, four and five from the spleen plate and colonies one and three from the kidney plate were grown up overnight for plasmid extraction.

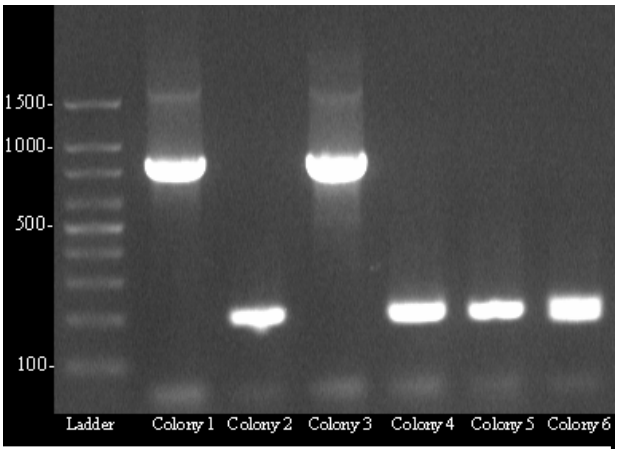

Figure 11: Colony PCR for Kidney IgD insert. Gel shows very strong bands at the expected size of 953 bp in only two of the colonies. Bands larger than 1,500 bp were also seen which could be due to non-specific amplification of the plasmid. Amplified products within the other lanes, represent the plasmid amplified by the primers, which contain no insert

3.2.3 Restriction Digest of Plasmid with IgD Insert

Plasmids were extracted from the cultured bacterial cells that contained the insert. Then a restriction digest was set up and incubated overnight. A restriction digest was used to ensure the insert was still present in the vector (Figure 12). Two bands were expected to be seen on a gel, a band at approximately 800 bp and a >1,500 band which represented the empty plasmid. Instead, three bands were seen which included the >1,500 bp plasmid and two smaller products. Although these bands were not of the predicted size they were still sent for sequencing, as the bands were predicted to represent the ligated product that had also been cut by the restriction enzyme.

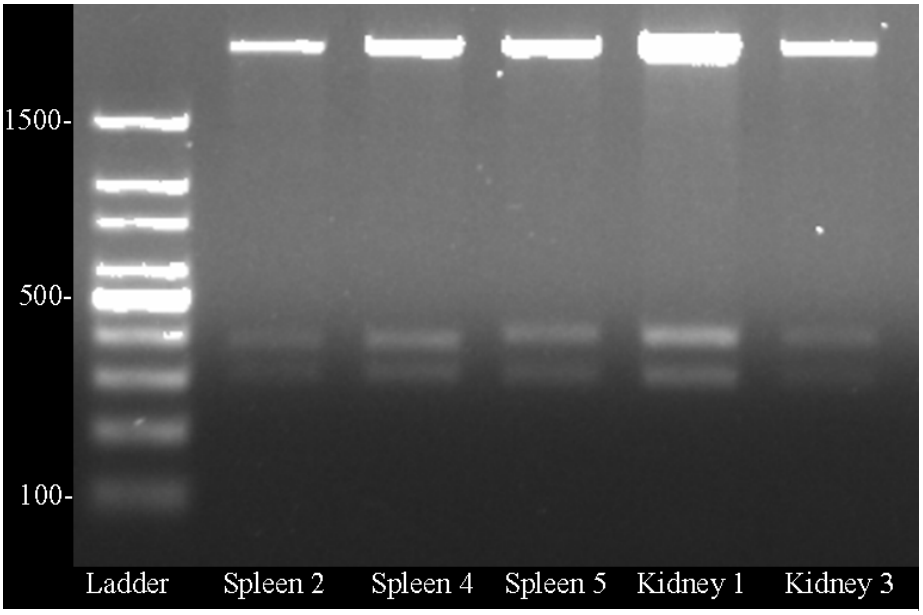

Figure 12: Gel analysis of the IgD restriction digest. Three bands were seen after running the gel. The largest band was expected as it represents the empty plasmid that the insert was digested from. The other two bands were not expected due to their smaller size, however the addition of the sizes of the bands together, does give the expected size, indicating the insert was also digested.

3.2.4 Sequencing results

Plasmids were analysed using the Nanodrop 2000 to determine sample concentration and purity (Table 10).

Table 10: Nanodrop results for plasmids containing IgD insert. Concentration and purity of the plasmids were found and used to determine the quantities needed for sequencing.

Sample	Concentration	260/280	260/230
Spleen Colony 2	105.2 ng/µL	1.91	2.02
Spleen Colony 4	224.2 ng/µL	1.86	1.65
Spleen Colony 5	142.9 ng/µL	1.86	1.78
Kidney Colony 1	92.3 ng/µL	1.84	2.13
Kidney Colony 3	223.3 ng/µL	1.88	2.19

All plasmids were prepared and sent for sequencing. Once sequencing data were returned, the forward and reverse primer sequences used in the initial amplification were searched for in the nucleotide sequence. Any remaining sequence before the forward primer and after the reverse primer was omitted, as this represented vector sequence. Sequencing results from spleen colony 5 were of poor quality and were not included in the rest of the analysis. The remaining sequences were aligned and a consensus sequence was determined (Figure 13), where at least three sequences matched. The consensus sequence (Figure 14) was then aligned with the transcriptome-derived consensus sequence and used to replace part of the transcriptomic sequence with the confirmed sequence. This confirmed sequence was subsequently used to design primers for RACE PCR and qPCR.

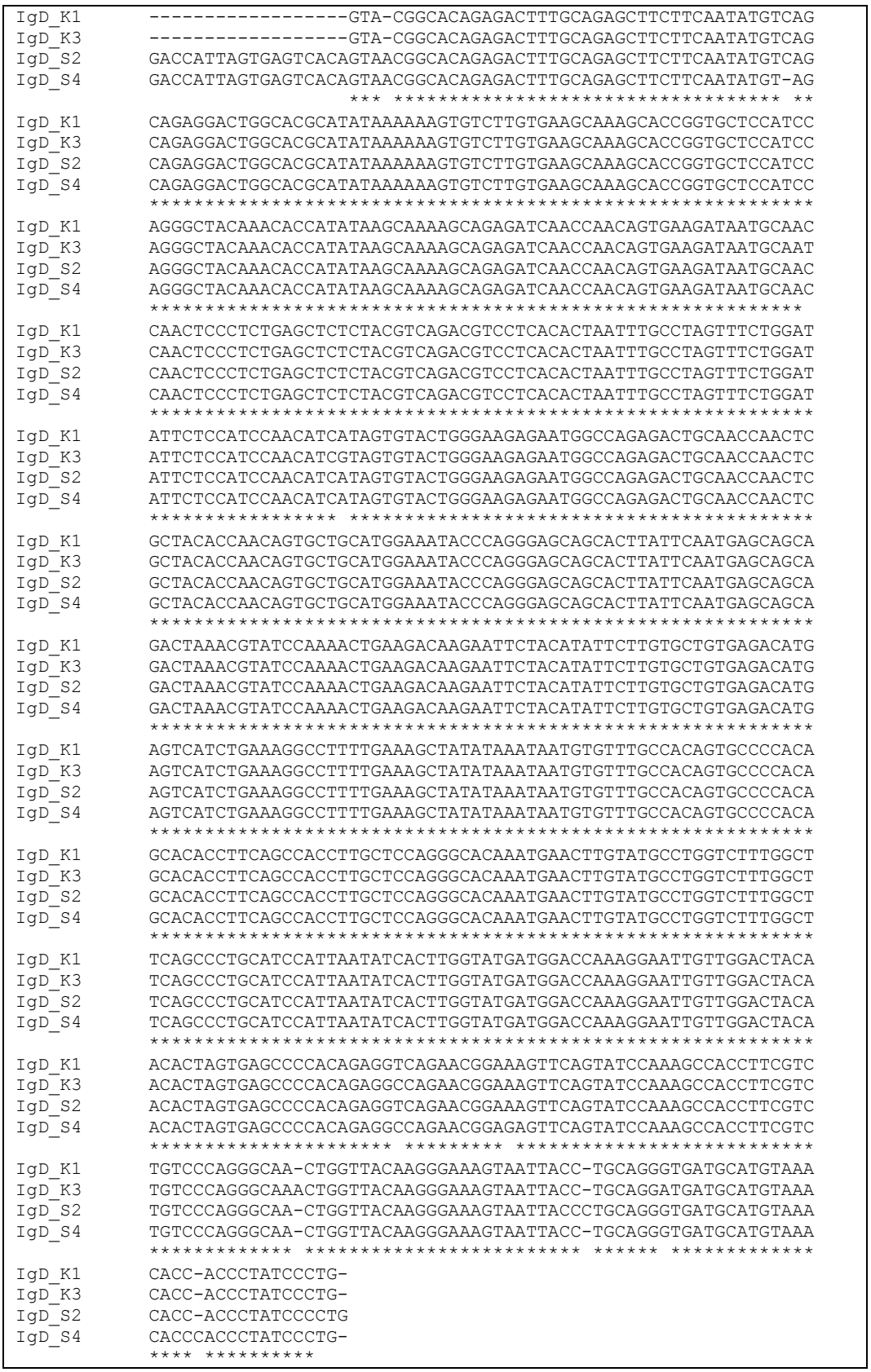

Figure 13: Alignment of IgD sequencing results. The alignment showed high similarity between all sequencing results. A consensus sequence based on these nucleotides was formed with differences being resolved by taking the most common nucleotide that was supported by at least three sequences. An asterisk represents complete identity.

NNNNNNNNNNNGGCCAAGGCGACACATCACATTGTASSCYRWSKSGGAAGGTGAATTCGGGGCC
TCACCAGTCAGACTCATCTGCACCCTTAGTGGCTTCTCTCCAGAGGAATTGAATGTGGAGTGGC
AACAGGAAARCAAATCTCTACATACTGTACCAANTCAAAGAAAGCTGCNAGAGTGTGGACGGAG
ACAATAAAACCTTCAGTCTAATCAGTGAGATTCAGCCAAATATGACAGAGTGGAAAAGAGGCTT
GAGTTTTACATGCAAGGCCATTCATGACAAAAAGAATTTGANNNGGCCAAGGCGGGGAAGGTCT
TTACTTGTGAACTGTCTGACAGCTCTCTGAATGAAACTGTCAGCAAGAACATCAGCCTGTGTTC
AGTTTATTCTAGCGCTGCTCCTTCAATTCACGTGGAGATTCCCAGCTTCCAGACTGTRRYSAMR
GCGAGAATCTGATGTGATTGCAACATGTTCTGTCCTCACTGGTTTTGATGTCAAAATGACTTGG
CTGATGGATGGACTGAATCGCACAGGNGAAAGACGATGCAAACACAACTCATAAAATCAGTCAT
GTGACAGTTCCCTTAAGAGACTGGAAACAACTCAAGATGATAACATGTAAAGCTGACCATAAGT
GTTTTCTCATCCAACGGGACAGTAAATGTCGCAGGGGCTGCAGTTACGGTTCCGTCAGTGGAAA
TCAAGAGATCTTTCCCAGATTTGCTGAAGGGGGAACTTGTGCTCAGTGTCACANNNGGCCAAGG
CGCAGATTTGCTGAAGGGGGAACTTGTGCTCGAGTGTCACATCACACAACTCTCCTCAAGTGAC
CTCTACGTCACCTTTCAGGCCAACAATGTTGASRYCWMKGMSAGAGAGTATGTTGATCTTCCTG
AAGCCGTGGGCCTTCATTCAATCACTAGACGCTTCACTGTCCCTAAAAGTCACTTCAAAAAAGG
CACGAATTTCACCTGCAAAGTCAATCAAGGCTTCTCCAGCAGCTTCGAGTCAAACACAACAGGC
GATATTTTTGGGGACCCATCTATGGAACTTCTTTTGGTCCCCNNNGGCCAAGGCGTGAACCCAT
CTATGGAACTTCTTTTGGTCCCCAGTGAAGAGTCAGGACCACAGAGACTCTTGTGCTCTGGATG
GGGCTTCAATCCTCAAATTAAATGGCTTTCTGACTCTCATCAAGGACCTTATTCAAACCATGAG
TTCACCATGRGYSMAGRYGGACGTGTGGCAGTGACCAGTRAACTTMMWATCMCTCAAAAAGAGT
GGAAAACMGGGAAGGTCTTYACTTGTGAASTGTCTGACAGCTCTCTGWATGNAAACTGTCAGCA
AGAACATCAGCCTGTGTTCAGTAACTCCAGCATCATCTCAGGTAGTTGGTGTATATGTTCAGGG
ACCACCACTCCAGGAGAGTTCAGAACANNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGCCAAG
GCGGGTCGGCCCTCATCTTAATGATTTCTCCATCACCTGTAAAGTAGATGGGGACATACAGCTC
CAAAATTTCTATATTGAGCCATTAGACCATTAGTGAGTCACAGTAACGGCACAGAGACTTTGCA
GAGCTTCTTCAATATGTCAGCAGAGGACTGGCACGCATATAAAAAAGTGTCTTGTGAAGCAAAG
CACCGGTGCTCCATCCAGGGCTACAAACACCATATAAGCAAAAGCAGAGATCAACCAACAGTGA
AGATAATGCAACCAACTCCCTCTGAGCTCTCTACGTCAGACGTCCTCACACTAATTTGCCTAGT
TTCTGGATATTCTCCATCCAACATCATAGTGTACTGGGAAGAGAATGGCCAGAGACTGCAACCA
ACTCGCTACACCAACAGTGCTGCATGGAAATACCCAGGGAGCAGCACTTATTCAATGAGCAGCA
GACTAAACGTATCCAAAACTGAAGACAAGAATTCTACATATTCTTGTGCTGTGAGACATGAGTC
ATCTGAAAGGCCTTTTGAAAGCTATATAAATAATGTGTTTGCCACAGTGCCCCACAGCACACCT
TCAGCCACCTTGCTCCAGGGCACAAATGAACTTGTATGCCTGGTCTTTGGCTTCAGCCCTGCAT
CCATTAATATCACTTGGTATGATGGACCAAAGGAATTGTTGGACTACAACACTAGTGAGCCCCA
CAGAGGCCAGAACGGAAAGTTCAGTATCCAAAGCCACCTTCGTCTGTCCCAGGGCAACTGGTTA
CAAGGGAAAGTAATTACCTGCAGGGTGATGCATGTAAACACCACCCTATCCCTGAATATATCTA
AACCAGGCACATTGGAGCACTGTGACTTCTTTGATGACATCATGGATGCAGAT

Figure 14: Area within the transcriptome where the confirmed IgD sequence is found. The confirmed sequence was aligned to the transcriptome sequence using ClustalX and added together, removing the transcriptomic sequence where it overlapped the confirmed sequence. The highlighted region is the remaining unconfirmed transcriptomic sequence and the non-highlighted sequence is the confirmed region.

3.2.5 RACE PCR

Using the confirmed sequence data, primers were designed in an attempt to amplify the 3' end of the IgD gene. RNA from spleen, liver and kidney tissue was prepared for 3' RACE PCR. The first round of RACE PCR used the primer slIgD-3'F1 and produced a number of bands in each lane at varying sizes (Figure 15). This was followed by the second round of PCR which used the primer slIgD-3'F2 and produced a consistent sized band from each tissue sample which was approximately 600 bp. A second, smaller band in the spleen and kidney sample was also produced which was approximately 500 bp (Figure 16). The single band that was produced from the liver was gel purified and ligated into a vector.

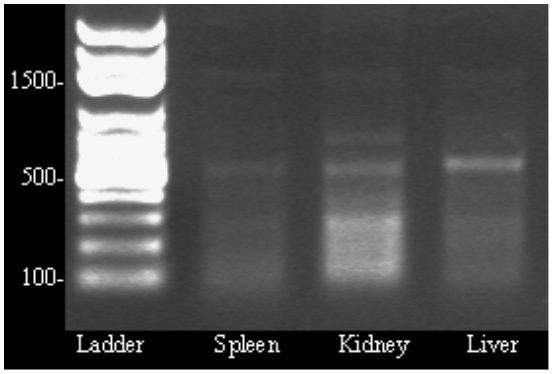

Figure 15: First round 3' RACE PCR for IgD. A number of bands of different sizes were seen in each lane after the first round of RACE PCR. Each PCR product was used for a second round of nested PCR to isolate a single band.

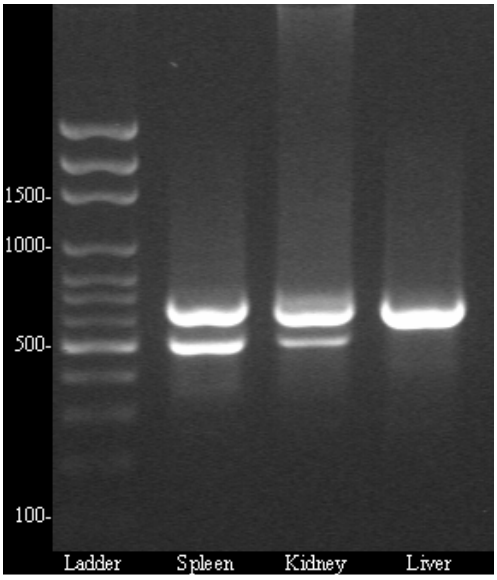

Figure 16: Second round of 3' RACE PCR for IgD. A consistent band was seen in each lane at approximately 600 bp. Two smaller bands at approximately 500 bp were also found in the spleen and kidney samples.

3.2.6 Colony PCR for IgD 3' RACE

Upon successful growth of potentially transformed colonies, a colony PCR was used to detect the insertion of the 3' RACE product into the vector. The colony PCR (Figure 17) showed a band with an approximate size of 800 bp in the first five colonies and a smaller band in colony six of approximately 400 bp. Each colony was used for further analysis, despite the smaller band seen in colony six.

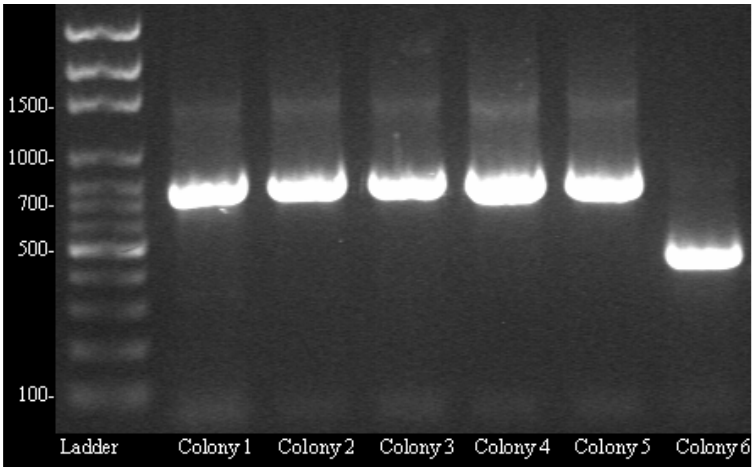

Figure 17: Colony PCR of 3' RACE IgD insert. Bands at approximately 700 bp were seen in the first five colonies and a band at approximately 500 bp was seen in colony 6. All colonies were used for further analysis.

3.2.7 Restriction Digest of Plasmids with 3' RACE IgD Insert

Plasmids were extracted from the bacterial cells containing the insert and digested. The restriction digest (Figure 18) produced two bands in colonies one, two, four and six, a large band of approximately 3000 bp and a smaller band at approximately 550 bp. However, colony three and colony five did not contain the smaller band. Despite this, all plasmids were prepared and sent for sequencing.

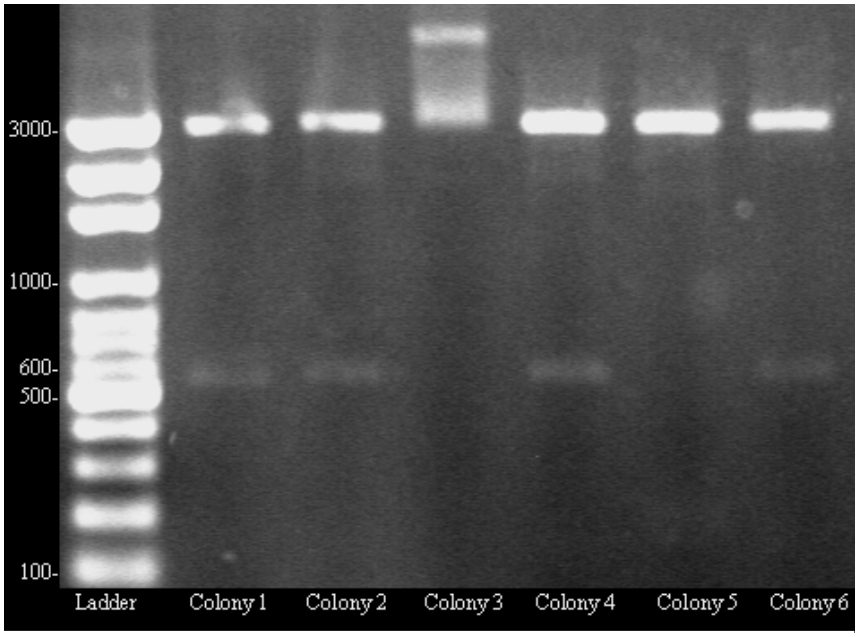

Figure 18: Restriction digests for 3' RACE IgD. A band at approximately 3,000 bp was seen in each lane, representing the digested plasmid. A band at approximately 550 bp was seen in colony one, two, four and six which represented the digested insert. No other bands were detected in the lanes for colony three and five. All plasmids were prepared and sent for sequencing.

3.2.8 Sequencing results

Plasmids were analysed using the Nanodrop 2000 to determine sample concentration and purity (Table 11).

Table 11: Nanodrop results for plasmids containing IgD insert. Concentration and purity of the plasmids were determined and used to prepare the quantities needed for sequencing.

Sample	Concentration	260/280	230/280
Colony 1	84.9 ng/µL	1.86	2.04
Colony 2	122.2 ng/µL	1.76	1.19
Colony 3	80.3 ng/µL	1.88	2.24
Colony 4	155.8 ng/µL	1.76	1.25
Colony 5	142.5 ng/µL	1.84	1.57
Colony 6	89.9 ng/µL	1.85	1.82

All plasmids were prepared and sent away for sequencing. Once sequencing data were returned, the forward primer and 3' RACE adaptor sequences were searched for and nucleotides before and after the primers that belonged to the vector were omitted from the sequence. Primer sequences could not be found within the sequencing results obtained from colony five and were therefore not used for further analysis. The remaining sequences were aligned and a consensus sequence was determined (Figure 19), which was added to the previously confirmed IgD sequence and the unconfirmed transcriptomic sequence (Figure 20). This completed the 3' end of the heavy chain IgD sequence from *S. lalandi*. The translation obtained from the IgD confirmed and transcriptomic sequence was aligned with other perciformes to confirm its identity and show conserved domains of the protein (Figure 21). For the confirmed sequence, excellent homology was seen in the areas where these domains were found. A phylogenetic tree was then constructed using a number of species to show the evolutionary relationship of *S. lalandi* IgD with other species (Figure 22). The *S. lalandi* IgD grouped very closely with other Perciformes, showing its clear relationship to other IgD sequences.

Figure 19: Alignment for 3' RACE IgD sequencing results. The sequencing results show high similarity between each clone. A consensus sequence was produced with any nucleotide differences being resolved by taking the most common nucleotide. An asterisk represents complete identity.

NNNNNNNNNNNGGCCAAGGCGACACATCACATTGTASSCYRWSKSGGAAGGTGAATTCGGGGCC
TCACCAGTCAGACTCATCTGCACCCTTAGTGGCTTCTCTCCAGAGGAATTGAATGTGGAGTGGC
AACAGGAAARCAAATCTCTACATACTGTACCAANTCAAAGAAAGCTGCNAGAGTGTGGACGGAG
ACAATAAAACCTTCAGTCTAATCAGTGAGATTCAGCCAAATATGACAGAGTGGAAAAGAGGCTT
GAGTTTTACATGCAAGGCCATTCATGACAAAAAGAATTTGANNNGGCCAAGGCGGGGAAGGTCT
TTACTTGTGAACTGTCTGACAGCTCTCTGAATGAAACTGTCAGCAAGAACATCAGCCTGTGTTC
AGTTTATTCTAGCGCTGCTCCTTCAATTCACGTGGAGATTCCCAGCTTCCAGACTGTRRYSAMR
GCGAGAATCTGATGTGATTGCAACATGTTCTGTCCTCACTGGTTTTGATGTCAAAATGACTTGG
CTGATGGATGGACTGAATCGCACAGGNGAAAGACGATGCAAACACAACTCATAAAATCAGTCAT
GTGACAGTTCCCTTAAGAGACTGGAAACAACTCAAGATGATAACATGTAAAGCTGACCATAAGT
GTTTTCTCATCCAACGGGACAGTAAATGTCGCAGGGGCTGCAGTTACGGTTCCGTCAGTGGAAA
TCAAGAGATCTTTCCCAGATTTGCTGAAGGGGGAACTTGTGCTCAGTGTCACANNNGGCCAAGG
CGCAGATTTGCTGAAGGGGGAACTTGTGCTCGAGTGTCACATCACACAACTCTCCTCAAGTGAC
CTCTACGTCACCTTTCAGGCCAACAATGTTGASRYCWMKGMSAGAGAGTATGTTGATCTTCCTG
AAGCCGTGGGCCTTCATTCAATCACTAGACGCTTCACTGTCCCTAAAAGTCACTTCAAAAAAGG
CACGAATTTCACCTGCAAAGTCAATCAAGGCTTCTCCAGCAGCTTCGAGTCAAACACAACAGGC
GATATTTTTGGGGACCCATCTATGGAACTTCTTTTGGTCCCCNNNGGCCAAGGCGTGAACCCAT
CTATGGAACTTCTTTTGGTCCCCAGTGAAGAGTCAGGACCACAGAGACTCTTGTGCTCTGGATG
GGGCTTCAATCCTCAAATTAAATGGCTTTCTGACTCTCATCAAGGACCTTATTCAAACCATGAG
TTCACCATGRGYSMAGRYGGACGTGTGGCAGTGACCAGTRAACTTMMWATCMCTCAAAAAGAGT
GGAAAACMGGGAAGGTCTTYACTTGTGAASTGTCTGACAGCTCTCTGWATGNAAACTGTCAGCA
AGAACATCAGCCTGTGTTCAGTAACTCCAGCATCATCTCAGGTAGTTGGTGTATATGTTCAGGG
ACCACCACTCCAGGAGAGTTCAGAACANNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGCCAAG
GCGGGTCGGCCCTCATCTTAATGATTTCTCCATCACCTGTAAAGTAGATGGGGACATACAGCTC
CAAAATTTCTATATTGAGCCATTAGTGAGTCACAGTAACGGCGCCATTAGACCATTAGTGAGTC
ACAGTAACGGCACAGAGACTTTGCAGAGCTTCTTCAATATGTCAGCAGAGGACTGGCACGCATA
TAAAAAAGTGTCTTGTGAAGCAAAGCACCGGTGCTCCATCCAGGGCTACAAACACCATATAAGC
AAAAGCAGAGATCAACCAACAGTGAAGATAATGCAACCAACTCCCTCTGAGCTCTCTACGTCAG
ACGTCCTCACACTAATTTGCCTAGTTTCTGGATATTCTCCATCCAACATCATAGTGTACTGGGA
AGAGAATGGCCAGAGACTGCAACCAACTCGCTACACCAACAGTGCTGCATGGAAATACCCAGGG
AGCAGCACTTATTCAATGAGCAGCAGACTAAACGTATCCAAAACTGAAGACAAGAATTCTACAT
ATTCTTGTGCTGTGAGACATGAGTCATCTGAAAGGCCTTTTGAAAGCTATATAAATAATGTGTT
TGCCACAGTGCCCCACAGCACACCTTCAGCCACCTTGCTCCAGGGCACAAATGAACTTGTATGC
CTGGTCTTTGGCTTCAGCCCTGCATCCATTAATATCACTTGGTATGATGGACCAAAGGAATTGT
TGGACTACAACACTAGTGAGCCCCACAGAGGCCAGAACGGAAAGTTCAGTATCCAAAGCCACCT
TCGTCTGTCCCAGGGCAACTGGTTACAAGGGAAAGTAATTACCTGCAGGGTGATGCATGTAAAC
ACCACCCTATCCCTGAATATATCTAAACCAGGCACATTGGAGCACTGTGACTTCTTTGATGACA
TCATGGATGCAGATGTGAACGAAGACATGGGTGTCGAAAGCTGGTATATAGCTTTCACTTTCCT
CCTCTTTTTCCTCATCTCTTTCATCTATGGTGTCTTGACTACCATAATCAAGACT**TAA**TGATGA
TGATCACAGGACAAATCCTATTTCATGATTCATATGAGGACTTTTGAAGACATATGGTCTGATT
AGTTTTAGATTCCTTTTTTTTTCAAAATTTCTTTTTCATTTTCTTTTCTTTTTTTATACTTTTG
TTATCATGGAATTTTATGGAGGTGGTTTATTCTATGTTTATTTCTTTTTATCCTTCAGTGTATT
GGCTGTGGAATGTTGTAACGCAAATGTGTGAGAGGAAAAAAGACAA**AATAAA**TGAAGCTCCAAA
AAAAAAAA

Figure 20: Confirmed 3' RACE sequence for IgD added to the sequence obtained from the initial PCR and the unconfirmed transcriptomic sequence. The 3' RACE sequence was added to the original confirmed sequence and the unconfirmed transcriptomic sequence (highlighted). The predicted stop codon (TAA) is underlined and as is the polyadenylation site (AATAAA). This completed the 3' end of the IgD heavy chain sequence of *S. lalandi*.

```
S.chuatsi          MFSVALLLLLAAG-CVKCEQLTQPASVTVQPGQRLTITCQVS-YSVSSYRTAWIRQPAGK
L.sanguineus       MFSVVLLLLLAAG-CVKCEQLTQPASVTVQPGQRLTITCQVS-YSVSSYYTAWIRQPAGK
E.coioides         MFSVALLLLLAAG-CVKCEQLTQPASVTVQPGQRLTITCQVS-YSVSGYYTAWIRQPAGK
O.niloticus        MFPVALLLLAAGS-CVKCEQLTQPASVIVQPGQRLTITCQVS-YSLG-WYTAWIRQPAGK
P.olivaceus        MFPVAVLLLLAAGSYVKCQTLTQPASATVQPGQPLTISCQVS-YSMSSYSTAWIRQPAGK
S.lalandi          ----------------------------XXXXPRRHITLXXX----------------------
I.punctatus        MMLGHCILFLLIS-YSYGQSLTSSASVVKRPGESVTLSCTVSGFSMGSNYMHWIRQKPGK
                                                  * . :*:
S.chuatsi          GLEWIGIRRVGYTTYYKDSLKNKFSIDLDSSSNTVTLNGQNVQPEDTAVYYCAR-----E
L.sanguineus       RLEWIGMR-VGSTSYYKDSLKNKFSIDLDSSSNTVTLNGQNVQPEDTAVYYCAR-----E
E.coioides         GLEWIGMKSTGYS-YYKDSLKNKFSISLDSSSNTATLNGQNVQPEDTAVYYCARGAAGTV
O.niloticus        GLEWIGMKHTSTS-YYKDSLKNKFSIDLDTSSNTVTLNGQNVQPEDTAVYYCAR----RA
P.olivaceus        GLEWIGAKYTGAS-YYKESLKNKFSIDLDTSSNTATLKGQNMQPGDTAVYYCAR---DRG
S.lalandi          ------------------------------------------------------------
I.punctatus        GLEWIGRIDRGTGTTFAQSLQGQFSITKDTNKNMLYLEVKSLKAQETAVYYCAG-ENIVM
```


S.chuatsi ILY--WAFDYWGKGTTVTVTSATSTGPT-VFPLMQCGSGTGDTVTLGCLATGFTPSS-LT
L.sanguineus IRSGHYAFDYWGKGTMVTVTSATSQGPT-VWPLTQCGSGAGETVTFGCFATGFSPSS-VT
E.coioides YYSGHGAFDYWGKGTTVTVTSATPNAPT-VFPLMQCGSGTGNTVTLGCLATSFTPSS-LT
O.niloticus SSSWGHSFDYWGKGTMVTVSSATSTAPT-VFPLVPCGTESGEMVTLGCLATGFNPPA-VT
P.olivaceus LAGTGYYFDYWGQGTKVTVTSATPAIPT-LFPVMPCGSGTGQTVTLGCLATGFTPSS-LT
S.lalandi ----------XEGEFGASPVRLICTLSGFSPEE-LN
I.punctatus TGGGDWAFDYWGKGTAVTVTSAVQSAPKSLFPVWQCGSASDGLVTLGCVTRDLASADGVS
S.chuatsi FTWSK-NGAALTDFIQYPPVQKGDVYTGISQIHVRRQDWDARESFQCAVTHPAGNVQTNF
L.sanguineus YSWTK-NGAAQTDFIQYPPVQKGDTYTGVTQIKVPRQEWG-KATFKCLATHTAGNGQATI
E.coioides YSWNIVNGAALTDFIQYPPVLKDNLYTGISQVKVSKQGWDALKSFRCDVRHTAGDQHVII
O.niloticus FSWTK-GGAALTDFIQYPAVQKGNVYTGVSQVRVRRQDWNAQQNLQCAVTHAAGNAQTIV
P.olivaceus FAWDK-NGAALTDAIQYLSVLKGDFYTGVSQIRVPRQEWDNSRPFKCTVTHEAGSPQITL
S.lalandi VEWQQ----------EXKSLHTVPXQRKLX----------ECGRRQ-NL
I.punctatus FIWKDASGSALTDVVQYPAVQATGGYTSVSHVRVKASDWNGNKKFTCEVKNGLGSKDASL
S.chuatsi IKPS----QRVDSPNITLHPVWEG-EFGASPVRLICTLSGFFPDKLNVTWQRDNRALD--
L.sanguineus PGPPP--VPRVVSPNITIHPVWNG-EVGASQVKLICTLSGYFPDKLSVKWKRDNQPINY-
E.coioides TKPK----ERVVPPNITLHPVWDNVPVGASSVRLVCTLSGYFPDELSVEWKRDNQRLQP-
O.niloticus TPPPPPPLPRIVSPTITLYPVWDG-EFGISAVRLICTLSGFFPDNLILKWVQDEQPLNQN
P.olivaceus QKPK----SRVVSPNITLYPVWEG-EFGVSPVRLICTLSGFFPDQLQVEWHKDNHLLHI-
S.lalandi QSNQ----------DSAKYDRVEKRLEFYMQGHS----------
I.punctatus QKPA----QRVTEPNITMST-----NTMDNNVNLLCWLDGFSPKKISVEWYKGNTLHTK-
S.chuatsi -GQIQRKLQSVEGVEKTFSLSSEIEPNMTEWADGSSFTCMSIHN--NSEFRKTISICQIH
L.sanguineus -APIERKLQSLDKTEKTFSLSSEITPNMKQWAAGSKFTCKATHN--DAEFVKTISICQVH
E.coioides -AKSPTKLQSADGTGKTFSLTSEIEPKTTEWERGSNFTCKSTHK--DQEYKKTISICQIH
O.niloticus NEANTRKLQSPEQGMKTFSLSSELWPNMTLWAEGSTFTCMADHN--GVKYIKTINICHTL
P.olivaceus -TPTQTKLQSMQGEEKTFSLISDIEA--VNWKSNSTFTCKAQHNN-DKKFTKTVNFVQIC
S.lalandi ----------QKEFXX----------AKAGKVFTCELSDSSLNETVSKNISLCSVY
I.punctatus -KTTMKIFESLNNGEKTFGALSQLSINAEQWNEGTEFTCKATHIS--KIFSQTWSKCKAE
S.chuatsi SRAAPSIHVEIPSFKTVMMSA--SEVKATCSLRTVFDAKVTWLMDGKLPSRDQVNKVTNT
L.sanguineus ARTLPSIHLEIPRFKTVMMAN--SQVKATCSVHTLFDAKVTWQMNGTAPHSTNVNQIRNT
E.coioides ESSPPSIHVEIPSFKTVMMAGG-STVKATCLVRSDFDAKLTWQINGEDAPRDQVTQDKNS
O.niloticus PSSPPFIQVEISSFKTAITSN--TDVQATCLVHTYFDAMITWLIDG-TAKSERVTQEKNA
P.olivaceus SSAAPSIDVEIPSFRTVMTES---AVTAKCSVNNRFGAKVTWLMDGKAESKYIVTSNANQ
S.lalandi SSAAPSIHVEIPSFQTXXXARI-CDCNMFCPHWF-HHCQNDLADGWTESHRXKTMQTQLI
I.punctatus PTSKPLIRLEKPGLMSVLTDS---EVTASCVVETVHNTKVSWFVDG-KEKTDRVTLKTLD
S.chuatsi THLISTLTVSLNQWKQLKLLNCKAEHRCFSSAEETT---NVSRPAVTAPSVEIRRSLPDL
L.sanguineus THLISTLTVDLSRWKRLKLVKCKAEHPCLKSIEETEAEEKVSGPAVTAPSVEIRRSLPDL
E.coioides TYIFSTVTVSSTEWKKIKLITCKAEHQCFSATEETV---NVAGPAVTTPVVTIRRSLNEL
O.niloticus THIISNLTVPSTQWEKVKNVTCKAEHKCFTSAEKTI---NIAGCPVNTPLVEIRRSLTDL
P.olivaceus THTISEVAVPPSQWKQLKSITCKADHQCFSTQRTVN----VAGPAVTNASVEIKRSFPDL
S.lalandi KSVM-QFP---ETGNNSR--HVKLTISVFSSNGTVN----VAGAAVTVPSVEIKRSFPDL
I.punctatus GRTVSNLTISTNDWKNWQTIKCTAAHLCFGTVEKTIN---ILEPVQKTPTVVIRRNLADI
S.chuatsi LKGN----------SAVLECDITQLSSSDLYVTFQANSVDISDKQYVDLPE-DP
L.sanguineus LKGN----------SSVLECSITELSSSDLYVTFQANSVDISDKVYVDLPE-TP
E.coioides LNGQ----------SAVLECDVTGLSSRDLYITFQAGTSDISEKLYVDLPE-AP
O.niloticus LKRD----------RAVFECDIQKLSSCDLFIALEVNHGQMLEKKHYNFAEGSV
P.olivaceus LKGQ----------SAVLECDITKLSSSDLYVTFQANDKDISEKQYVVLPE-AP
S.lalandi LKGELVLSVTXGQGADLLKGELVLECHITQLSSSDLYVTFQANNVXXXXREYVDLPE-AV
I.punctatus LKGD----------SAVLECAARDLPSGELSVILQANGIRVFEPQYVDLPK---
S.chuatsi GLHSVSRRFTVPPSHWKKDTSFTCKVNQGFSSNFESNSTGKIFVDPSVELLLVP------
L.sanguineus ERHSISRRFTVPREYWTKDKRFTCKVNQGFTGSFKSQPTGNIFVDPSVELLLDP------
E.coioides GLHSISRSFPVPPEHLKKDKSFTCKTIS-LIDCFLPN-----LADPSMKLLLAP------
O.niloticus VNLSISSQFIVPQKFWMKDQTFTCKVNQGFSQTFHSNSISNIFVDPSMELLLVP------
P.olivaceus DPQSITRRFSIPSSYWKKDTTFSCKVHQGFSPSVKSKSTGNIFVDPSVELLLFP------
S.lalandi GLHSITRRFTVPKSHFKKGTNFTCKVNQGFSSSFESNTTGDIFGDPSMELLLVPXGQGVN
I.punctatus GVDTLTARFTVSTTQRNKNQRFTCQIQQSRSKQWTSNSIENLFGDPSVELLVISS-----
S.chuatsi ---------SKESGPQRLVCSGWGFDPQIKWFSESQQRSPSTN-DISMGADGRVAVTSQL
L.sanguineus ---------SEESDTQRLLCSGWGFNPQIKWFSESKQRSSSTV-DISMGADGRVAVTSQL
E.coioides ---------SEESGSQTLVCSGRGFNPQIQWSSESQQRAPSTN-DISMGADGRVTVTSQF
O.niloticus ---------SKGSESKTLSCSGWGFNPNITWLSESQERSPNST-NISMSVDGHIRVTSQL
P.olivaceus ---------NELSGPQRLLCSGRGFNPKIQWLSVSN-KLPSNN-DVTMGADGRVVVTSQL
S.lalandi PSMELLLVPSEESGPQRLLCSGWGFNPQIKWLSDSHQGPYSNH-EFTMXXXGRVAVTSXL
I.punctatus -------VDKSASATQKLICAATGLNPNIKWLPESVVNALNGLSKVTVDSDGRVKVSSEI
S.chuatsi HIPQTEWKTGKGFTCKVSDKSLN----------KIVEKEISLCSVTPASSQIVGVY
L.sanguineus HIPREEWKTGKVFTCEASDKSLN----------KTDRKNISFCSVTPASSQIVGVY
E.coioides QIPHKEWSTGKVFTCQVSDKSLG----------KNIRKDISLCSVTPASSQVVGVY
O.niloticus VIDHSEWKNGNTFTCEVSDRSLN----------TRITKTISLCSETPASSQIVGVY
P.olivaceus QIPQTEWETGKVYSCEVSDTSLS----------KTVSKNISLCSVTPASSQIVGVV
S.lalandi XIXQKEWKXGKVXTCEXSDSSLXXNCQQEHQPVFSNSSIISGSWCICSGTTTPGEFRTXX

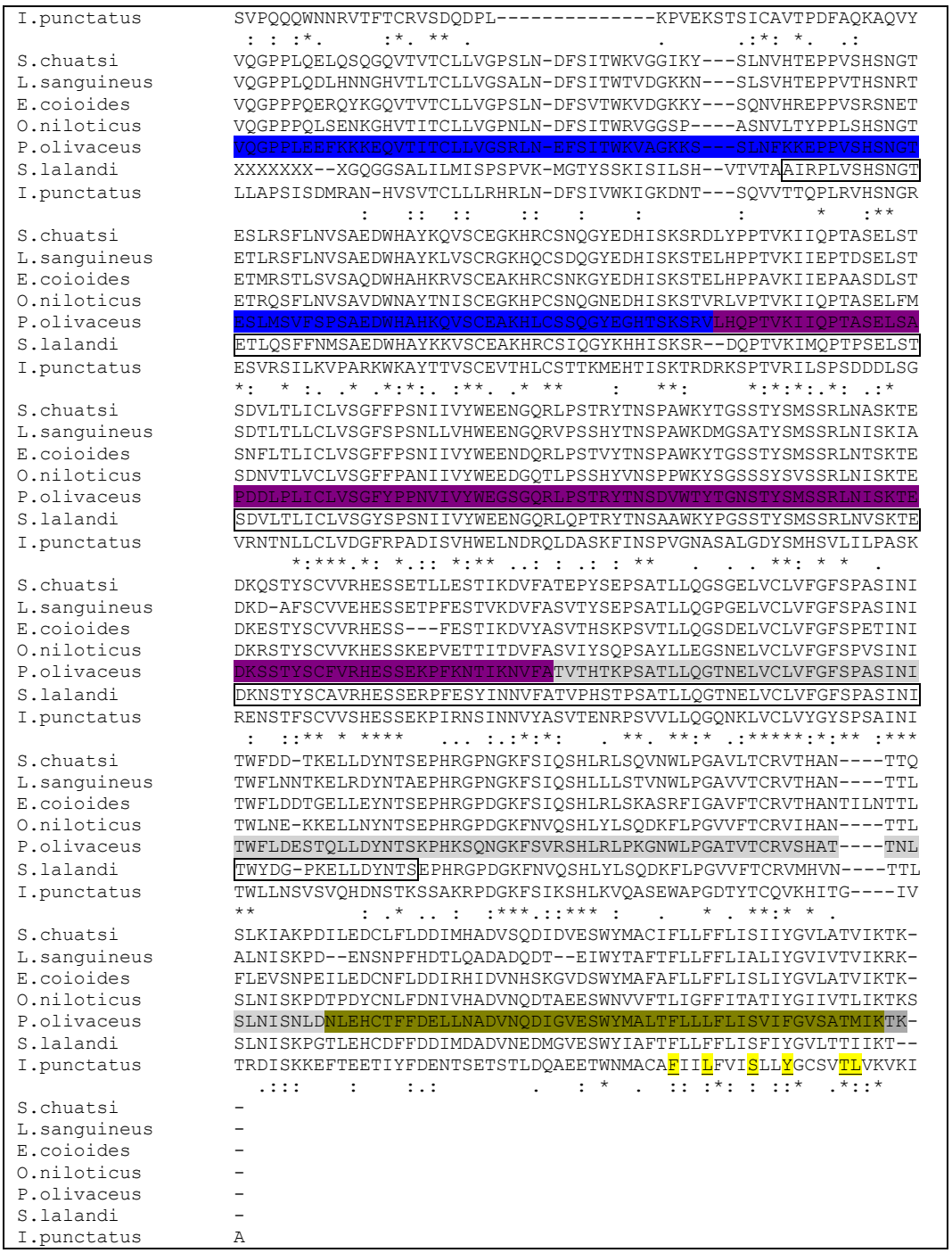

Figure 21: Alignment of S. lalandi IgD sequence with IgD from other known teleost species. Sequences are divided into regions which are distinguished by colour. Red text indicates the variable region of IgD, followed by the Cμ domain in teal. Yellow, bright green, turquoise, red, dark blue, violet, grey areas represents the Cδ regions 1-7, respectively. The dark yellow and short dark grey regions are the C-terminal transmembrane domains of IgD, δTM1 and δTM2. The amino acids highlighted in yellow and underlined are part of the conserved antigen receptor transmembrane (CART) motif. The boxed region of the S. lalandi amino acid sequence has been confirmed with the remaining amino acids being derived from the transcriptome. Sequences used in this alignment were *Epinephelus coioides* (AFI33218.1), Siniperca chuatsi (ACO88906.1), *Lutjanus sanguineus* (AIC33830.1), *Oreochromis niloticus* (AHY86392.1), *Paralichthys olivaceus* (BAB41204.1), and *Ictalurus punctatus* (T18537). An asterisk represents complete identity, a colon represents conservative substitutions and a dot represents a semiconservative substitution.

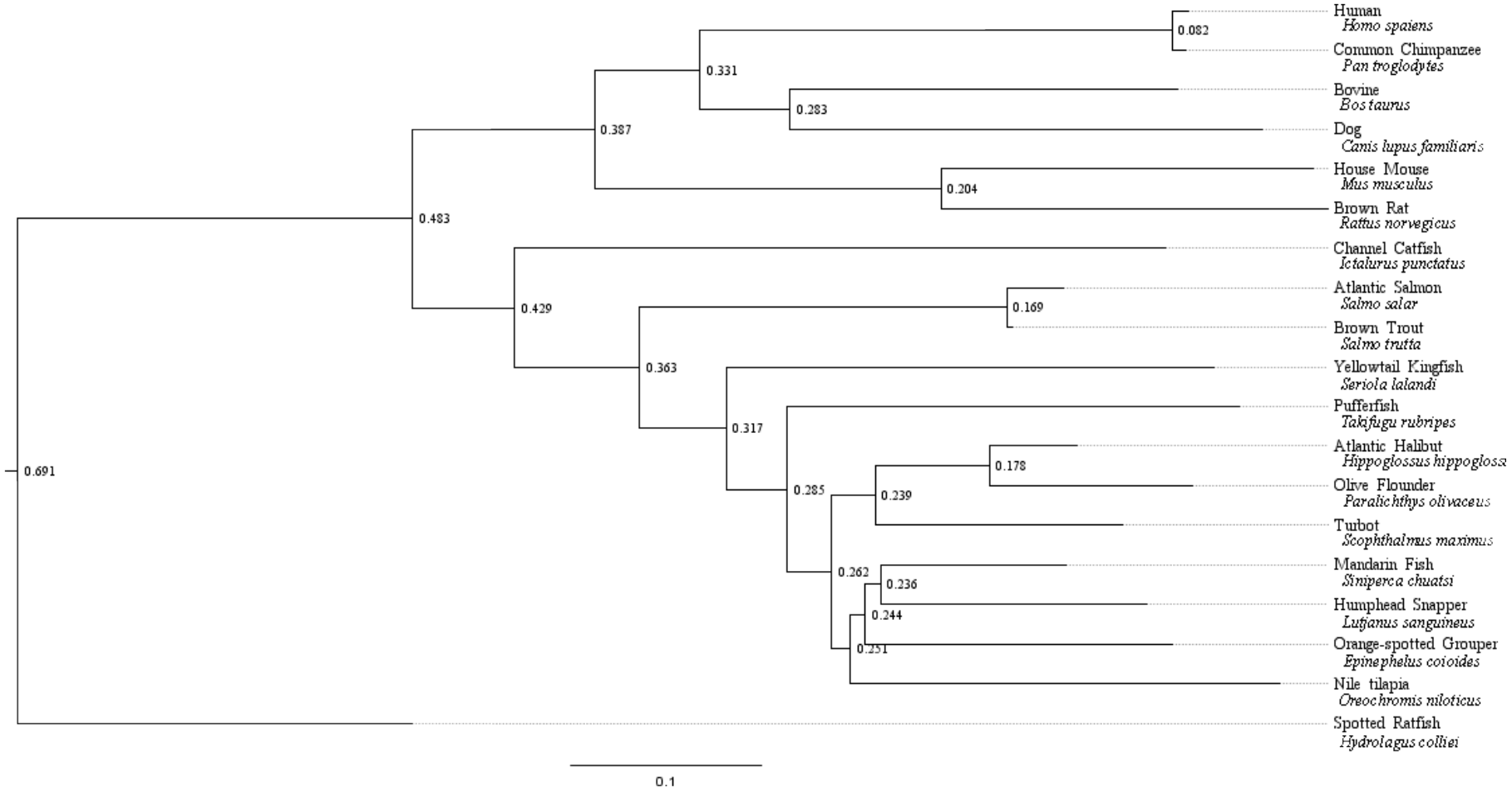

Figure 22: Phylogenetic tree of IgD sequence data from a range of species. An alignment of the sequences was first performed using ClustalX and the tree was constructed from this alignment using the Figtree software. The species were used for the alignment can be found in appendix IV.

3.3 Recombination Activating Gene 1

Initial transcriptome searches using RAG1 genes from related species obtained from the NCBI database produced no reads within Geneious. However, sequence data was found in the NCBI database for a RAG1 gene in a very closely related species, *Seriola dumerili*. Both amino acid (Accession No. AGD96067.1) and nucleotide (Accession No. JQ938283.1) sequences were identified. A multiple alignment (Figure 23) was performed that included the RAG1 amino acid sequences of the following species: *Seriola dumerili, Niphon spinosus*, *Trachinus draco*, *Serranus baldwini* and *Paralabrax clathratus*. The alignment was then used to find areas of high conservation to design primers. A nucleotide alignment for these species was also performed and can be found in Appendix I.

```
Serranus_baldwini          SGFSVMIKESCDGMGDVSEKHGVGPAVPEKAVRFSFTVMSISVLADEEEK
Paralabrax_clathratus      SGFSVMIKESCDGMGDVSEKHGGGPAVPEKAVRFSITVMSISVLADEEEK
Niphon_spinosus            SGFSVMIKESCDGMGDVSEKHGGGPAVPEKAVRFSITVMSVSILADEEEE
Trachinus_draco            SGFSVMIKESCDGMGDVSEKHGGGPAVPEKAVRFSFTVMSVSVLADDEEE
Seriola_dumerili           SGFTVMIKECCDGMGDVSEKHGGGPAIPEKAVRFSFTVMSVSVRAEEEEE
                           ***:*****.************ ***:*********:****:*: *::**:
Serranus_baldwini          EVTIFTEPKPNSELSCKPLCLMFVDESDHETLTSVLGPIVAERGAMKESR
Paralabrax_clathratus      EVTIFTEPKPNSELSCKPLCLMFVDESDHETLTAVLGPIVAERDAMKESR
Niphon_spinosus            EVTIFTEPKPNSELSCKPLCLMFVDESDRETLTAVLGPIVAERNAMKESR
Trachinus_draco            EVTIFTEQKPNSELSCKPLCLMLVDESDHETLTALLAPVVAERNAMKESR
Seriola_dumerili           DVTIFTEPRPNSELSCKPLCLMFVDESDHETLTALLGPVVAERHAMKESR
                           :****** :*************:*****:****::*.*:**** ******
Serranus_baldwini          LILSVGGLPRSFRFHFRGTGYDEKMVREMEGLEASGSTYVCTLCDSTRAE
Paralabrax_clathratus      LILSVGGLPRSFRFHFRGTGYDEKMVREXEGLEASGSTYVCTLCDSTRAQ
Niphon_spinosus            LILSLGGLPRSFRFHFRGTGYDEKMVREMEGLEASGSTYVCTLCDSTRAE
Trachinus_draco            LILSVGGLPRSFRFHFRGTGYDEKMVREMEGLEASGSTYVCTLCDSTRAE
Seriola_dumerili           LILSIGGLPRSFRFHFRGTGYDEKMVREMEGLESSGSTYVCTLCDSSRAN
                           ****:********************** ****:***********:**:
Serranus_baldwini          ASQNMVLHSVTRSHEENLDRYEIWRTNPFSESVDELRDRVKGISAKPFLE
Paralabrax_clathratus      ASQNMVLHSVTRNHDENLDRYEIWRTNPFSESVDELRDRVKGISAKPFLE
Niphon_spinosus            ASQNMVLHSVTRSHEENLDRYEIWRTNPFSESVDELRDRVKGVSAKPFME
Trachinus_draco            ASQNMVLHSITRSHEENLDRYEIWRTNPFSESVDELRDRVKGVSAKPFME
Seriola_dumerili           ASQNMVLHSITRGHEENLERYEIWRTNPFSESVDELRDRVKGVSAKPFME
                           *********:**.*:***:**********************:*****:*
Serranus_baldwini          THPTLDALHCDIGNAIEFYKIFQDEIGEMYKKVNPRREERRSWRAALDKQ
Paralabrax_clathratus      THPTLDALHCDIGNAIEFYKIFQDEIGEVYTKVNPSREERRSWRAALDKQ
Niphon_spinosus            THPTLDALHCDIGNATEFYKIFQDEIGEVYQKVNPSREERRSWRAALDKQ
Trachinus_draco            TQPTMDALHCDIGNATEFYKIFQDEIGEVYKKVNPSREERRSWRAALDKQ
Seriola_dumerili           THPTLDALHCDIGNATEFYKIFQDEIGEVYQKVNPSREERRSWRAALDKQ
                           *:**:********** ************:* **** **************
Serranus_baldwini          LRKKMKLKPVMRMNGNYARRLMTQETVEVVCELVPSEDRREALRELMRIY
Paralabrax_clathratus      LRKKMKLKPVMRMNGNYARRLMTQETVEVVCELVPTEDRREALRELMRIY
Niphon_spinosus            LRKKLKLRPVMRMNGNYARRLMTQEAVEVVCELVPSEERREALRELIRIY
Trachinus_draco            LRKKMKLKPVMRMNGNYARRLMTQEAVEVVCELVPSEERREALRELIQIY
Seriola_dumerili           LRKKMKLKPVMRMNGNYARRLMTLETVEVVCELVPSEERREALRELMRLY
                           ****:**:*************** *:*********:*:********:::*
Serranus_baldwini          LQMKPVWRATCPAKECPDQLCRYSFNSQRFADLLASTFKYRYNGKITNYL
Paralabrax_clathratus      LQMKPVWRATCPAKECPDQLCRYSFNSQRFADLLASTFKYRYNGKITNYL
Niphon_spinosus            LQMKPVWRATCPAKECPDQLCRYSFNSQRFADLLSSTFKYRYDGKITNYL
Trachinus_draco            IQMKPVWRATCPAKECPDQLCRYSFNSQRFADLLSSTFKYRYNGKITNYL
Seriola_dumerili           LQMRPVWRATCPAKECPDQLCRYSFNSQHFADLLSSTFKYRYNGKITNYL
                           :**:************************:*****:*******:*******
Serranus_baldwini          HKTLAHVPEIIEKDGSIGAWASEGNESANKLFRRFRKMNARQSKFFEL--
Paralabrax_clathratus      HKTLAHVPEIIEKDGSIGAWASEGNESANKLFRRFRKMNARQSKIFELED
Niphon_spinosus            HKTLAHVPEIIERDGSIGAWASEGNESANKLFRRFRKMNARQSKAF----
Trachinus_draco            HKTLAHVPEIIERDGSIGAWASEGNESANKLFRRFRKMNARQSK------
Seriola_dumerili           HKTLAHVPEIIERDGSIGAWASEGNESANKLFRRFR--------------
                           ************:***********************
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Serranus_baldwini          ----
Paralabrax_clathratus      VLKH
Niphon_spinosus            ----
Trachinus_draco            ----
Seriola_dumerili           ----
```

Figure 23: Amino acid alignment for RAG1. Alignment shows areas of extremely high conservation between the sequences of the RAG1 protein which is useful for the design of primers without the need for the transcriptomic data. An asterisk represents complete identity, a colon represents conservative substitutions and a dot represents a semiconservative substitution. Accession numbers of sequences used in this alignment were: *Seriola dumerili* (AGD96067.1), *Niphon spinosus* (AEK98033.1), *Trachinus draco* (AHA61994.1), *Serranus baldwini* (AEK98035.1), *Paralabrax clathratus* (AHA61997.1).

3.3.1 Amplification and Cloning of RAG1

A combination of primers was designed and used to amplify parts of the CDS regions of RAG1. The first round PCR was performed for RAG1 using different primer combinations all in spleen tissue (Figure 24) Expected product size was dependant on the combination of primers used, however the average band size was about 1,000bp. The second round of nested PCR was performed using the F1R1, F1R2 and F2R1 PCR products. Three combinations of primers were used with the original F1R1 product as a template, which were F1R2, F2R1 and F2R2 (Figure 25). Only one combination was used with the original F1R2 and the original F2R2 products which was F2R2. Non-specific bands were greatly reduced in the second round and the F2R1 product from the second round was purified and ligated into a cloning vector.

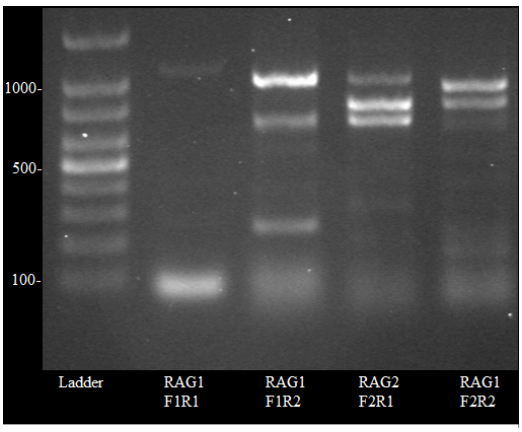


Figure 24: First round of nested PCR for RAG1. Four combinations of primers were used to amplify RAG1 in spleen which produced different band sizes that ranged from approximately 100 bp to 1200 bp. Multiple bands indicate non-specific amplification.

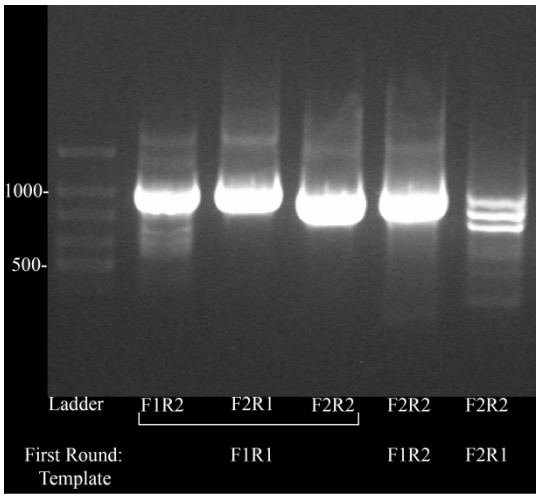


Figure 25: Second round nested PCR for RAG1 amplification. Strong bands were seen in the first four lanes at around 800-900 bp and three bands were seen in the final lane. Non-specific amplification was reduced compared to the first round. The F2R1 product gel purified and ligated for cloning.

3.3.2 Colony PCR for RAG1

Upon successful ligation and transformation of bacterial cells, a colony PCR was performed. Five colonies were screened using colony PCR and run on a gel (Figure 26). One band at approximately 1,194 bp was expected, based on the size of the insert plus the 172 bp extra added from the vector, when using the M13 primers. Non-specific bands greater than 1,500 bp were also present likely due to non-specific amplification of the vector. Colonies one, two, three and five were selected as good candidates and were grown up overnight in LB media. The faint band produced from colony four excluded it from further preparations.

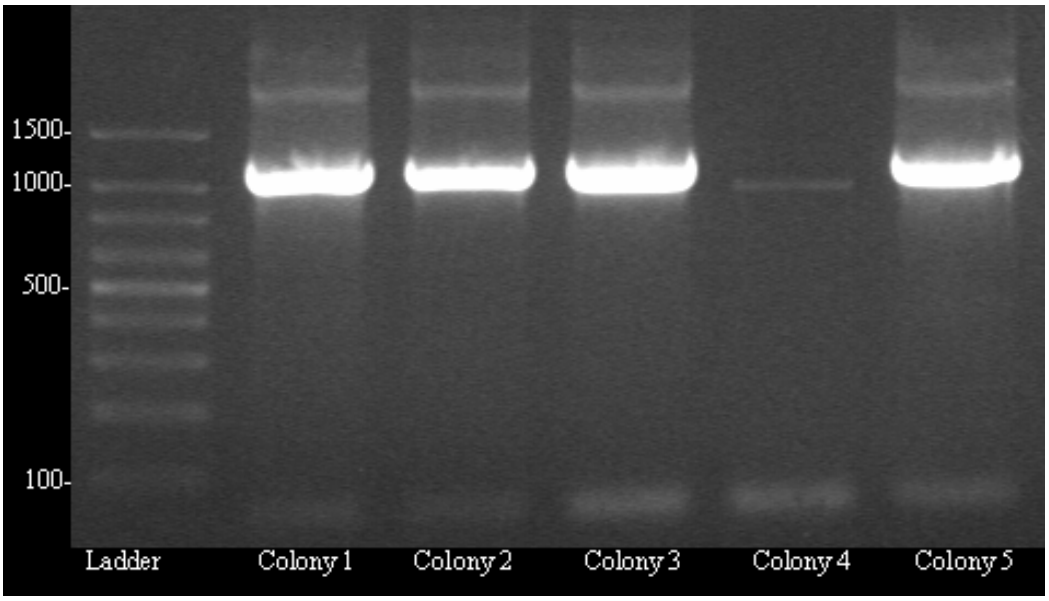


Figure 26: Gel image of colony PCR that shows the RAG1 insert. Successful insertion of RAG1 was indicated by a 1,194 bp band which was seen in each colony. Colony four was not used for further research due to the band being significantly fainter than the other bands.

3.3.3 Restriction Digest of Plasmid with RAG1 Insert

Once the plasmids were extracted, a restriction digest was used to ensure the insert was present. The restriction digest produced two bands of interest, the insert at approximately 750 bp and the remaining plasmid, which was greater than 1,500 bp (Figure 27). The presence of bands at 750 bp demonstrated the successful insertion of RAG1.

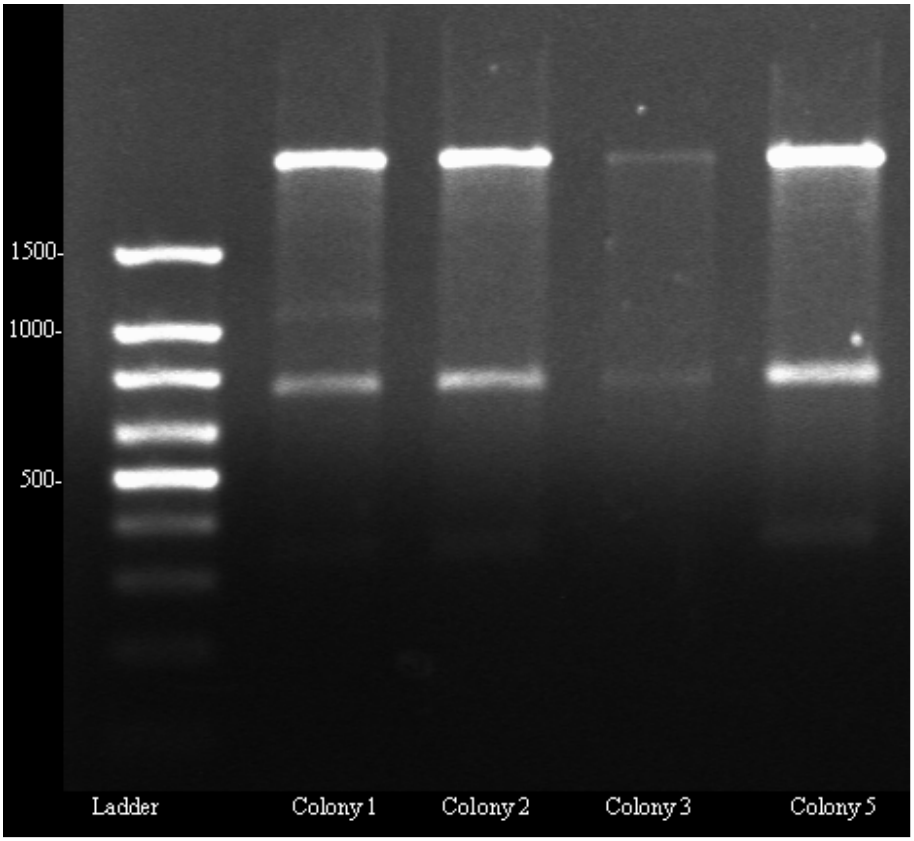


Figure 27: Gel image that shows the restriction digest of vector containing RAG1. The digest was shown by the presence of the 750 bp band. A fainter band was seen in the plasmid digest of colony three but was still prepared for sequencing as it was still indicative of a successful ligation

3.3.4 Sequencing results

Plasmids were analysed using the Nanodrop 2000 to determine sample concentration and purity (Table 12).

Table 12: Nanodrop results for plasmids containing RAG1 insert. Concentration and purity of the plasmids were determined and used to prepare the quantities needed for sequencing.

Sample	Concentration	260/280	230/280
Colony 1	221.9 ng/µL	1.84	2.13
Colony 2	294.4 ng/µL	1.87	2.20
Colony 3	124.6 ng/µL	1.82	1.81
Colony 5	318.2 ng/µL	1.87	2.22

All plasmids were prepared and sent away for sequencing. Once sequencing data were returned, the primer sequences used to amplify the product were searched for and nucleotides before the forward primer and after the reverse primer omitted from the sequence, as they were part of the vector sequence. The sequences were then aligned and a consensus sequence was determined (Figure 28). The confirmed RAG1 sequence was compared to the nucleotide sequence of *S. dumerili* used to design primers for *S. lalandi* RAG1 (Figure 29). The consensus sequence was then aligned to the *S. dumerili* nucleotide sequence to show the similarity between the two species (Figure 30).

An alignment was also performed between a number of species to identify any conserved domains or amino acids, important for its function (Figure 31). The confirmed amino acid sequence showed good homology with other sequences and amino acids important to RAG1 function were conserved. A phylogenetic tree was also constructed to show the evolutionary relationship between species using RAG1 (Figure 32). This showed a close relationship of the *S. lalandi* IL-1β sequence with other fish species, as it clearly grouped with them away from other vertebrate groups.

```
RAG1_Colony3      -CCCTTCTGGGTGATCAGCTTATGGGCGATGTCAGCGAGAAGCACGGCGGAGG--ACCAG
RAG1_Colony5      --TCGTATGGTCGATCAGCTTATGGGCGATGTCAGCGAGAAGCACGGCGGAGG--ACCAG
RAG1_Colony1      -CGTACTGGGTCGATCAGCTTATGGGCGATGTCAGCGAGAAGCACGGCGGCGCGCACCAG
RAG1_Colony2      TGGTACTGGGTCGATCAGCTTATGGGCGATGTCAGCGAGAAGCACGGCGGAGG--ACCAG
                         **  ************************************ *   *****
RAG1_Colony3      CCATTCCCGAGAAGGCTGTACGTTTCTCTTTCACTGCTATGTCTCTCTCTGTCCGGGCAG
RAG1_Colony5      CCATTCCCGAGAAGGCTGTACGTTTCTCTTTCACTGTTATGTCTCTCTCTGTCCGGGCAG
RAG1_Colony1      CCATTCCCGAGAAGGCTGTA---GTTTCTTTCACTGTTATGTCTCTCTCTGTCCGGGCAG
RAG1_Colony2      CCATTCCCGAGAAGGCTGTACGTTTCTCTTTCACTGTTATGTCTCTCTCTGTCCGGGCAG
                  ********************    * ********** ************************
RAG1_Colony3      AGGAGGAGGAGGAGGACGTTACCATCTTCACCGAGCCAAGGCCCAACTCGGAGCTATCCT
RAG1_Colony5      AGGAGGAGGAGGAGGACGTTACCATCTTCACCGAGCCAAGGCCCAACTCGGAGCTATCCT
RAG1_Colony1      AGGAGGAGGGGGGGGACGTTACCATCTTCACCGAGCCAAGGCCCAACTCGGAGCTATCCT
RAG1_Colony2      AGGAGGAGGAGGAGGACGTTACCATCTTCACCGAGCCAAGGCCCAACTCGGAGCTATCCT
                  ********* ** ********************************************
RAG1_Colony3      GTAAGCCCCTTTGCTTGATGTTTGTGGATGAGTCAGACCACGAGACACTCACAGCTCTCC
RAG1_Colony5      GTAAGCCCCTTTGCTTGATGTTTGTGGATGAGTCAGACCACGAGACACTCACAGCTCTCC
RAG1_Colony1      GTAAGCCCCTTTGCTTGATGTTTGTGGATGAGTCAGACCACGAGACACTCACAGCTCTCC
RAG1_Colony2      GTAAGCCCCTTTGCTTGATGTTTGTGGATGAGTCAGACCACGAGACACTCACAGCTCTCC
                  ************************************************************
RAG1_Colony3      TGGGGCCTGTAGTTGCAGAGCGTAATGCAATGAAAGAGAGCAGGCTCATCCTCCCCATCG
RAG1_Colony5      TGGGGCCTGTAGTTGCAGAGCGTAATGCAATGAAAGAGAGCAGGCTCATCCTCTCCATCG
RAG1_Colony1      TGGGGCCTGTAGTTGCAGAGCGTAATGCAATGAAAGAGAGCAGGCTCATCCTCTCCATCG
RAG1_Colony2      TGGGGCCTGTAGTTGCAGAGCGTAATGCAATGAAAGAGAGCAGGCTCATCCTCTCCATCG
                  ************************************************** ******
RAG1_Colony3      GCGGCCTTCCTCGCTCCTTCCGCTTCCACTTCAGGGGCACGGGATACGATGAGAAGATGG
RAG1_Colony5      GCGGCCTTCCTCGCTCCTTCCGCTTCCACTTCAGGGGCACGGGATACGATGAGAAGATGG
RAG1_Colony1      GCGGCCTTCCTCGCTCCTTCCGCTTCCACTTCAGGGGCACGGGATACGATGAGAAGATGG
RAG1_Colony2      GCGGCCTTCCTCGCTCCTTCCGCTTCCACTTCAGGGGCACGGGATACGATGAGAAGATGG
                  ************************************************************
RAG1_Colony3      TGCGAGAGATGGAAGGCCTGGAGTCCTCGGGGTCCACGTATGTCTGCACTCTGTGTGACT
RAG1_Colony5      TGCGAGAGATGGAAGGCCTGGAGTCCTCGGGGTCCACGTATGTCTGCACTCTGTGTGACT
RAG1_Colony1      TGCGAGAGATGGAAGGCCTGGAGTCCTCGGGGTCCACGTATGTCTGCACTCTGTGTGACT
RAG1_Colony2      TGCGAGAGATGGAAGGCCTGGAGTCCTCGGGGTCCACGTATGTCTGCACTCTGTGTGACT
                  ************************************************************
RAG1_Colony3      CCAGCCGGGCGAACGCCTCTCAAAACATGGTGCTACACTCCGTCACCCGCGGCCATGAAG
RAG1_Colony5      CCAGCTGGGCGAACGCCTCTCAAAACATGGTGCTACACTCCGTCACCCGCGGCCATGAAG
RAG1_Colony1      CCAGCCGGGCGAACGCCTCTCAAAACATGGTGCTACACTCCGTCACCCGCGGCCATGAAG
RAG1_Colony2      CCAGCCGGGCGAACGCCTCTCAAAACATGGTGCTACACTCCGTCACCCGCGGCCATGAAG
                  ***** ********************************************************
RAG1_Colony3      AGAACCTAGAACGTTATGAAATATGGAGAACCAACCCCTTCTCTGAGTCTGTGGACGAAC
RAG1_Colony5      AGAACCTAGAACGTTATGAAATATGGAGAACCAACCCCTTCTCTGAGTCTGTGGACGAAC
RAG1_Colony1      AGAACCTAGAACGTTATGAAATATGGAGAACCAACCCCTTCTCTGAGTCTGTGGACGAAC
RAG1_Colony2      AGAACCTAGAACGTTATGAAATATGGAGAACCAACCCCTTCTCTGAGTCTGTGGACGAAC
                  ************************************************************
RAG1_Colony3      TGCGAGACAGAGTCAAAGGTGTCTCTGCAAAGCCCTTCATGGAGACCCACCCCACGCTAG
RAG1_Colony5      TGCGAGACAGAGTCAAAGGTGTCCCTGCAAAGCCCTTCATGGAGACCCACCCCACGCTAG
RAG1_Colony1      TGCGAGACAGAGTCAAAGGTGTCTCTGCAAAGCCCTTCATGGAGACCCACCCCACGCTAG
RAG1_Colony2      TGCGAGACAGAGTCAAAGGTGTCTCTGCAAAGCCCTTCATGGAGACCCACCCCACGCTAG
                  ********************** ************************************
RAG1_Colony3      ATGCGTTGCACTGCGACATTGGCAATGCCACTGAGTTCTACAAAA--TCTTCCAGGACGA
RAG1_Colony5      ATGCGTTGCACTGCGACATTGGCAATGCCACTGAGTTCTACAAAA--TCTTCCAGGACGA
RAG1_Colony1      ATGCGTTGCACTGCGACATTGGCAATGCCACTGAGTTCTACAAAAATCTTTCCAGGACGA
RAG1_Colony2      ATGCGTTGCACTGCGACATTGGCAATGCCACTGAGTTCTACAAAA--TCTTCCAGGACGA
                  ******************************************    ************
RAG1_Colony3      -GATTGGGGAGGCGTATCAAAAGGTCAACCCCAGCCGGGAGGAAAGGCGCAGCTGGAGGG
RAG1_Colony5      -GATTGGGGAGGTGTATCAAAAGGTCAACCCCAGCCGGGAGGGAAGGCGCAGCTGGAGGG
RAG1_Colony1      AGATTGGGGAGGTGCA----------------------------------------------
RAG1_Colony2      -GATTGGGGAGGTGTATCAAAAGGTCAACCCCAGCCGGGAGGAAAGGCGCAGCTGGAGGG
                   *********** * *
RAG1_Colony3      CCGCCCTAGATAAACAGCTGAGGAAGAAGATGAAGCTTAAA-CCGGTAATGAGGATGAAT
RAG1_Colony5      CCGCCCTAGATAAACAGCTGAGGAAGAAGATGAAGCTTAAA-CCGGTAATGAGGATGAAT
RAG1_Colony1      ------------------------------------------------------------
RAG1_Colony2      CCGCCCTAGATAAACAGCTGAGGAAGAAGATGAAGCTTAAAACCGGTAATGAGGATGAAT

RAG1_Colony3      GGGAACTACGCCCGCAGGCT-AATGACCCTGGAGACTGTGGAGGTGGTGTGTGAACTGGT
RAG1_Colony5      GGGAACTACGCCCGCAGGCT-AATGACCCTGGAGACTGTGGAGGTGGTGTGTGAACTGGT
RAG1_Colony1      ------------------------------------------------------------
RAG1_Colony2      GGGAACTACGCCCGCAGGCTTAATGACCCTGGAGACTGTGGAGGTGGTGTGTGAACTGGC

RAG1_Colony3      G--CCCTCAGAGGAAAGGGAGAGAGGCCCTGAGGGAGCT-CATGAGGCTCTACCTCCAGG
RAG1_Colony5      G--CCCTCAGAGGAAAGG-AGAGAGGCCCTGAGGGAGCT-CATGAGGCTCTACCTCCAG-
RAG1_Colony1      ------------------------------------------------------------
RAG1_Colony2      TGCCCCTCAGAGGAAAGGAGAGAGGCCCCTGAGGGAGCTTCATGAGGCTCTACCTTCCAG

RAG1_Colony3      ATGAGGCCTGTGTGGCGCGCCACCT-GCCCAGCC-AAGGAAATGCCCCAGACCAGCTGTG
RAG1_Colony5      ATGAGGCCTGTGTGGCGCGCTACCT-GCCCAGCCCAAGGGAATGCCC-AGACCAGCTGTG
RAG1_Colony1      ------------------------------------------------------------
RAG1_Colony2      AATGAGCCTGTGTGGCGCGCCACCTTGCCCAGCC-AAGGGAATGCCC-AGACCAGCTGGT
```

```
RAG1_Colony3      CC-GCTACAGCTTTTAACTCGCAG---CACTTTGAC-GACCTTCTCTC-CTTCTACCTTC
RAG1_Colony5      CCCGCTACAGCTTT-AACTCGCAG---CACTTTGGCCGACCTTCCTTCTCCTCTACCTTC
RAG1_Colony1      ------------------------------------------------------------
RAG1_Colony2      GCCGCTACAGCTTTTAACTCGCAGGCACGTTTTGACCGACCCTCTCCTCCCTCTACTTCT

RAG1_Colony3      AAAATACAGG--TACGA-CGGGAAA-GATCACCCAGTTACT-TGCAC---AGACTCT---
RAG1_Colony5      AAA-TACAGG--TACGA-CGGGAAAAGATCACCAACTTACCCTGCAC---AGACTTCT-A
RAG1_Colony1      ------------------------------------------------------------
RAG1_Colony2      AGATGACCAGGTTACGAACCGGAAAGATCCACCTATTTTACTTGCAACAAGGACTCCTTG

RAG1_Colony3      AGATCTGG--AATTCCCTCTA-GAGTCGA--CTGCAGCA-TG-CAAGCCTTGGACGTAAA
RAG1_Colony5      GGATTCTG--AATCCCCTCTAAGAGTCGAACCTGCAGCAATG-CCAAGCTTGGACGTAG-
RAG1_Colony1      ------------------------------------------------------------
RAG1_Colony2      GGATTCTGGAAACTCCCTCTAGAAGTCCGAACCTTGACAAGGTCCAGGCCTCAAGGCC--

RAG1_Colony3      TCAATGGTCATAGGCCTGTCTTATGCCTGATGAG
RAG1_Colony5      TCAATGGCTCTA--TTAGCCTTTCGCT-------
RAG1_Colony1      -----------------------------------
RAG1_Colony2      TCTATGGGGAC------------------------
```

Figure 28: Alignment for RAG1 sequencing results. The sequencing results show high similarity between each colony. However colony 1 produced a short sequence. A consensus sequence was produced with any nucleotide differences being resolved by taking the most common nucleotide. An asterisk represents complete identity.

```
>S_lalandi_RAG1_confirmed
ATGGAGGGGCTGAGAGAGAGCGGGATGGAAGACAGTGCTTGCACCTCAGGCTTTACTGTCATGATCAAGGAATGCTGTGA
TGGCATGGGCGATGTCAGCGAGAAGCACGGCGGAGGACCAGCCATTCCCGAGAAGGCTGTACGTTTCTCTTTCACTGTTA
TGTCTCTCTCTGTCCGGGCAGAGGAGGAGGAGGAGGACGTTACCATCTTCACCGAGCCAAGGCCCAACTCGGAGCTATCC
TGTAAGCCCCTTTGCTTGATGTTTGTGGATGAGTCAGACCACGAGACACTCACAGCTCTCCTGGGGCCTGTAGTTGCAGA
GCGTAATGCAATGAAAGAGAGCAGGCTCATCCTCCCCATCGGCGGCCTTCCTCGCTCCTTCCGCTTCCACTTCAGGGGCA
CGGGATACGATGAGAAGATGGTGCGAGAGATGGAAGGCCTGGAGTCCTCGGGGTCCACGTATGTCTGCACTCTGTGTGAC
TCCAGCCGGGCGAACGCCTCTCAAAACATGGTGCTACACTCCGTCACCCGCGGCCATGAAGAGAACCTAGAACGTTATGA
AATATGGAGAACCAACCCCTTCTCTGAGTCTGTGGACGAACTGCGAGACAGAGTCAAAGGTGTCTCTGCAAAGCCCTTCA
TGGAGACCCACCCCACGCTAGATGCGTTGCACTGCGACATTGGCAATGCCACTGAGTTCTACAAAATCTTCCAGGACGAG
ATTGGGGAGGTGTATCAAAAGGTCAACCCCAGCCGGGAGGAAAGGCGCAGCTGGAGGGCCGCCCTAGATAAACAGCTGAG
GAAGAAGATGAAGCTTAAACCGGTAATGAGGATGAATGGGAACTACGCCCGCAGGCTAATGACCCTGGAGACTGTGGAGG
TGGTGTGTGAACTGGTGCCCTCAGAGGAAAGGAGAGAGGCCCTGAGGGAGCTCATGAGGCTCTACCTCCAGATGAGGCCT
GTGTGGCGCGCCACCTGCCCAGCCAAGGGAATGCCCAGACCAGCTGTGCCGCTACAGCTTGATCACCAATTACCTGCACA
AGACTCTGGCCCATGTGCCTGAAATCATAGAGAGAGATGGATCCATAGGAGCCTGGGCCAGTGAGGGGAACGAGTCGGCA
AACAAACTGTTCAGGCGTTTCCGG
```

Figure 29: Confirmed RAG1 sequence in S. lalandi. Sequence data was found for RAG1 in *S. lalandi*, the unconfirmed sequence shown in blue represents the *S. dumerili* RAG1 sequence.

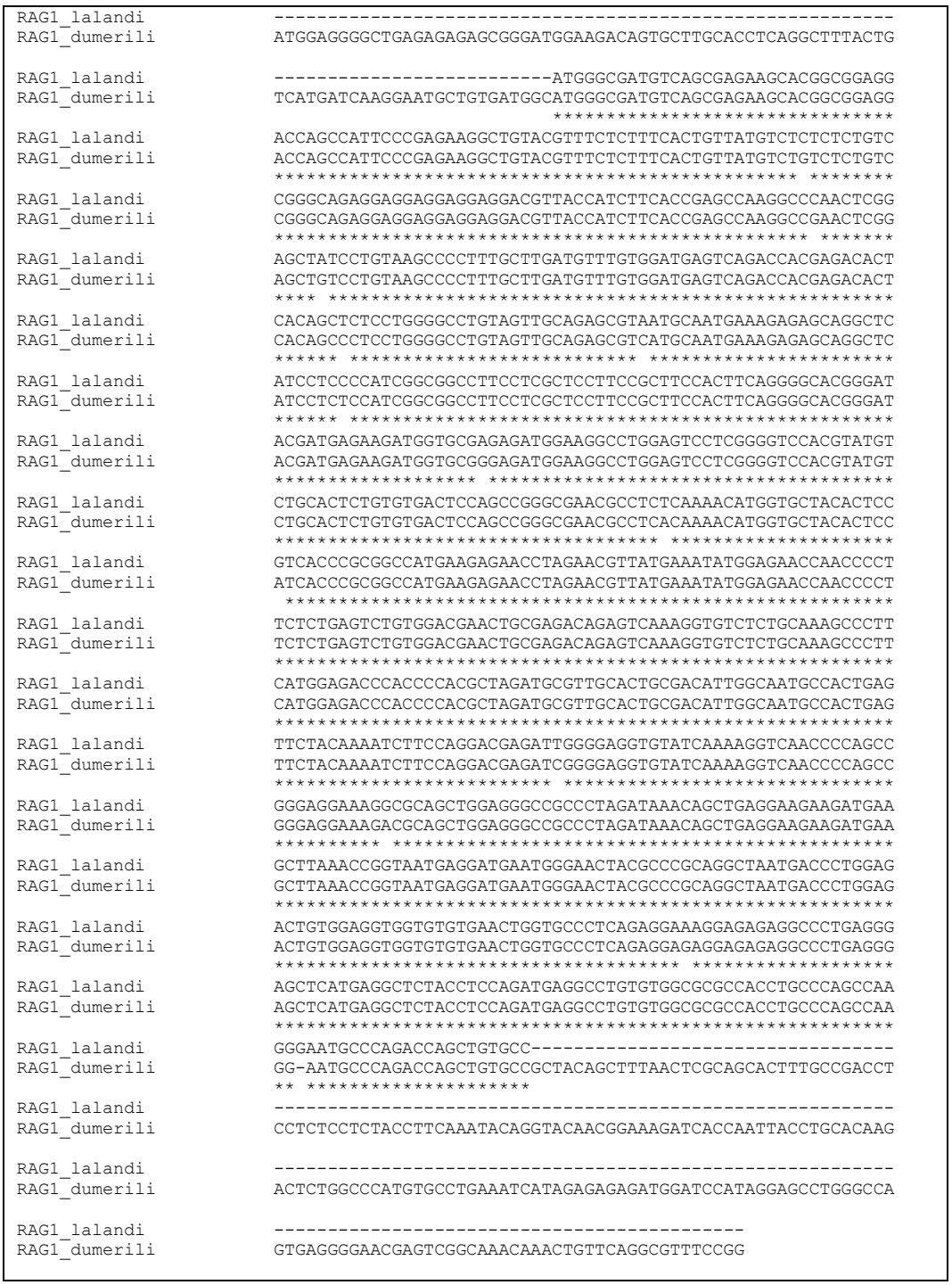
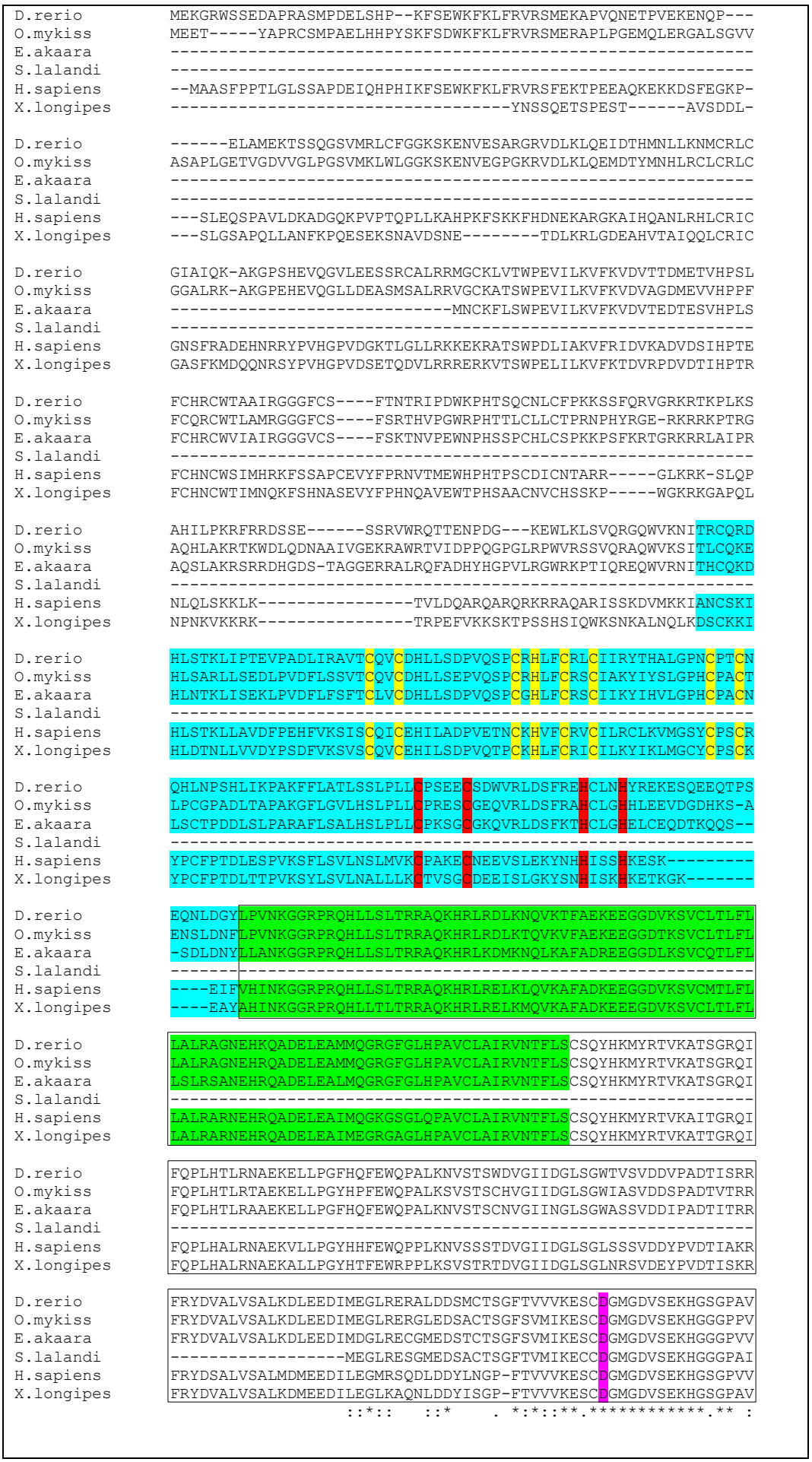


Figure 30: Nucleotide alignment of RAG1 in S. lalandi and S. dumerili. Alignment was used to compare the confirmed RAG1 sequence from *S. lalandi* to the *S. dumerili* RAG1 sequence taken from the NCBI database.


D.rerio MEKGRWSSEDAPRASMPDELSHP--KFSEWKFKLFRVRSMEKAPVQNETPVEKENQP---
O.mykiss MEET-----YAPRCSMPAELHHPYSKFSDWKFKLFRVRSMERAPLPGEMQLERGALSGVV
E.akaara ----------
S.lalandi ----------
H.sapiens --MAASFPPTLGLSSAPDEIQHPHIKFSEWKFKLFRVRSFEKTPEEAQKEKKDSFEGKP-
X.longipes ----------YNSSQETSPEST------AVSDDL-
D.rerio ------ELAMEKTSSQGSVMRLCFGGKSKENVESARGRVDLKLQEIDTHMNLLKNMCRLC
O.mykiss ASAPLGETVGDVVGLPGSVMKLWLGGKSKENVEGPGKRVDLKLQEMDTYMNHLRCLCRLC
E.akaara ----------
S.lalandi ----------
H.sapiens ---SLEQSPAVLDKADGQKPVPTQPLLKAHPKFSKKFHDNEKARGKAIHQANLRHLCRIC
X.longipes ---SLGSAPQLLANFKPQESEKSNAVDSNE--------TDLKRLGDEAHVTAIQQLCRIC
D.rerio GIAIQK-AKGPSHEVQGVLEESSRCALRRMGCKLVTWPEVILKVFKVDVTTDMETVHPSL
O.mykiss GGALRK-AKGPEHEVQGLLDEASMSALRRVGCKATSWPEVILKVFKVDVAGDMEVVHPPF
E.akaara ----------MNCKFLSWPEVILKVFKVDVTEDTESVHPLS
S.lalandi ----------
H.sapiens GNSFRADEHNRRYPVHGPVDGKTLGLLRKKEKRATSWPDLIAKVFRIDVKADVDSIHPTE
X.longipes GASFKMDQQNRSYPVHGPVDSETQDVLRRRERKVTSWPELILKVFKTDVRPDVDTIHPTR
D.rerio FCHRCWTAAIRGGGFCS----FTNTRIPDWKPHTSQCNLCFPKKSSFQRVGRKRTKPLKS
O.mykiss FCQRCWTLAMRGGGFCS----FSRTHVPGWRPHTTLCLLCTPRNPHYRGE-RKRRKPTRG
E.akaara FCHRCWVIAIRGGGVCS----FSKTNVPEWNPHSSPCHLCSPKKPSFKRTGRKRRLAIPR
S.lalandi ----------
H.sapiens FCHNCWSIMHRKFSSAPCEVYFPRNVTMEWHPHTPSCDICNTARR-----GLKRK-SLQP
X.longipes FCHNCWTIMNQKFSHNASEVYFPHNQAVEWTPHSAACNVCHSSKP-----WGKRKGAPQL
D.rerio AHILPKRFRRDSSE------SSRVWRQTTENPDG---KEWLKLSVQRGQWVKNITRCQRD
O.mykiss AQHLAKRTKWDLQDNAAIVGEKRAWRTVIDPPQGPGLRPWVRSSVQRAQWVKSITLCQKE
E.akaara AQSLAKRSRRDHGDS-TAGGERRALRQFADHYHGPVLRGWRKPTIQREQWVRNITHCQKD
S.lalandi ----------
H.sapiens NLQLSKKLK----------TVLDQARQARQRKRRAQARISSKDVMKKIANCSKI
X.longipes NPNKVKKRK----------TRPEFVKKSKTPSSHSIQWKSNKALNQLKDSCKKI
D.rerio HLSTKLIPTEVPADLIRAVTCQVCDHLLSDPVQSPCRHLFCRLCIIRYTHALGPNCPTCN
O.mykiss HLSARLLSEDLPVDFLSSVTCQVCDHLLSEPVQSPCRHLFCRSCIAKYIYSLGPHCPACT
E.akaara HLNTKLISEKLPVDFLFSFTCLVCDHLLSDPVQSPCGHLFCRSCIIKYIHVLGPHCPACN
S.lalandi ----------
H.sapiens HLSTKLLAVDFPEHFVKSISCQICEHILADPVETNCKHVFCRVCILRCLKVMGSYCPSCR
X.longipes HLDTNLLVVDYPSDFVKSVSCQVCEHILSDPVQTPCKHLFCRICILKYIKLMGCYCPSCK
D.rerio QHLNPSHLIKPAKFFLATLSSLPLLCPSEECSDWVRLDSFREHCLNHYREKESQEEQTPS
O.mykiss LPCGPADLTAPAKGFLGVLHSLPLLCPRESCGEQVRLDSFRAHCLGHHLEEVDGDHKS-A
E.akaara LSCTPDDLSLPARAFLSALHSLPLLCPKSGCGKQVRLDSFKTHCLGHELCEQDTKQQS--
S.lalandi ----------
H.sapiens YPCFPTDLESPVKSFLSVLNSLMVKCPAKECNEEVSLEKYNHHISSHKESK---------
X.longipes YPCFPTDLTTPVKSYLSVLNALLLKCTVSGCDEEISLGKYSNHISKHKETKGK-------
D.rerio EQNLDGYLPVNKGGRPRQHLLSLTRRAQKHRLRDLKNQVKTFAEKEEGGDVKSVCLTLFL
O.mykiss ENSLDNFLPVNKGGRPRQHLLSLTRRAQKHRLRDLKTQVKVFAEKEEGGDTKSVCLTLFL
E.akaara -SDLDNYLLANKGGRPRQHLLSLTRRAQKHRLKDMKNQLKAFADREEGGDLKSVCQTLFL
S.lalandi ----------
H.sapiens ----EIFVHINKGGRPRQHLLSLTRRAQKHRLRELKLQVKAFADKEEGGDVKSVCMTLFL
X.longipes ----EAYAHINKGGRPRQHLLTLTRRAQKHRLRELKMQVKAFADKEEEGDVKSVCLTLFL
D.rerio LALRAGNEHKQADELEAMMQGRGFGLHPAVCLAIRVNTFLSCSQYHKMYRTVKATSGRQI
O.mykiss LALRAGNEHRQADELEAMMQGRGFGLHPAVCLAIRVNTFLSCSQYHKMYRTVKATSGRQI
E.akaara LSLRSANEHRQADELEALMQGRGFGLHPAVCLAIRVNTFLSCSQYHKMYRTVKATSGRQI
S.lalandi ----------
H.sapiens LALRARNEHRQADELEAIMQGKGSGLQPAVCLAIRVNTFLSCSQYHKMYRTVKAITGRQI
X.longipes LALRARNEHRQADELEAIMEGRGAGLHPAVCLAIRVNTFLSCSQYHKMYRTVKATTGRQI
D.rerio FQPLHTLRNAEKELLPGFHQFEWQPALKNVSTSWDVGIIDGLSGWTVSVDDVPADTISRR
O.mykiss FQPLHTLRTAEKELLPGYHPFEWQPALKSVSTSCHVGIIDGLSGWIASVDDSPADTVTRR
E.akaara FQPLHTLRAAEKELLPGFHQFEWQPALKNVSTSCNVGIINGLSGWASSVDDIPADTITRR
S.lalandi ----------
H.sapiens FQPLHALRNAEKVLLPGYHHFEWQPPLKNVSSSTDVGIIDGLSGLSSSVDDYPVDTIAKR
X.longipes FQPLHALRNAEKALLPGYHTFEWRPPLKSVSTRTDVGIIDGLSGLNRSVDEYPVDTISKR
D.rerio FRYDVALVSALKDLEEDIMEGLRERALDDSMCTSGFTVVVKESCDGMGDVSEKHGSGPAV
O.mykiss FRYDVALVSALKDLEEDIMEGLRERGLEDSACTSGFSVMIKESCDGMGDVSEKHGGGPPV
E.akaara FRYDVALVSALKDLEEDIMDGLRECGMEDSTCTSGFSVMIKESCDGMGDVSEKHGGGPVV
S.lalandi ----------MEGLRESGMEDSACTSGFTVMIKECCDGMGDVSEKHGGGPAI
H.sapiens FRYDSALVSALMDMEEDILEGMRSQDLDDYLNGP-FTVVVKESCDGMGDVSEKHGSGPVV
X.longipes FRYDVALVSALKDMEEDILEGLKAQNLDDYISGP-FTVVVKESCDGMGDVSEKHGSGPAV
::*:: ::* . *:*::**.***********.** :

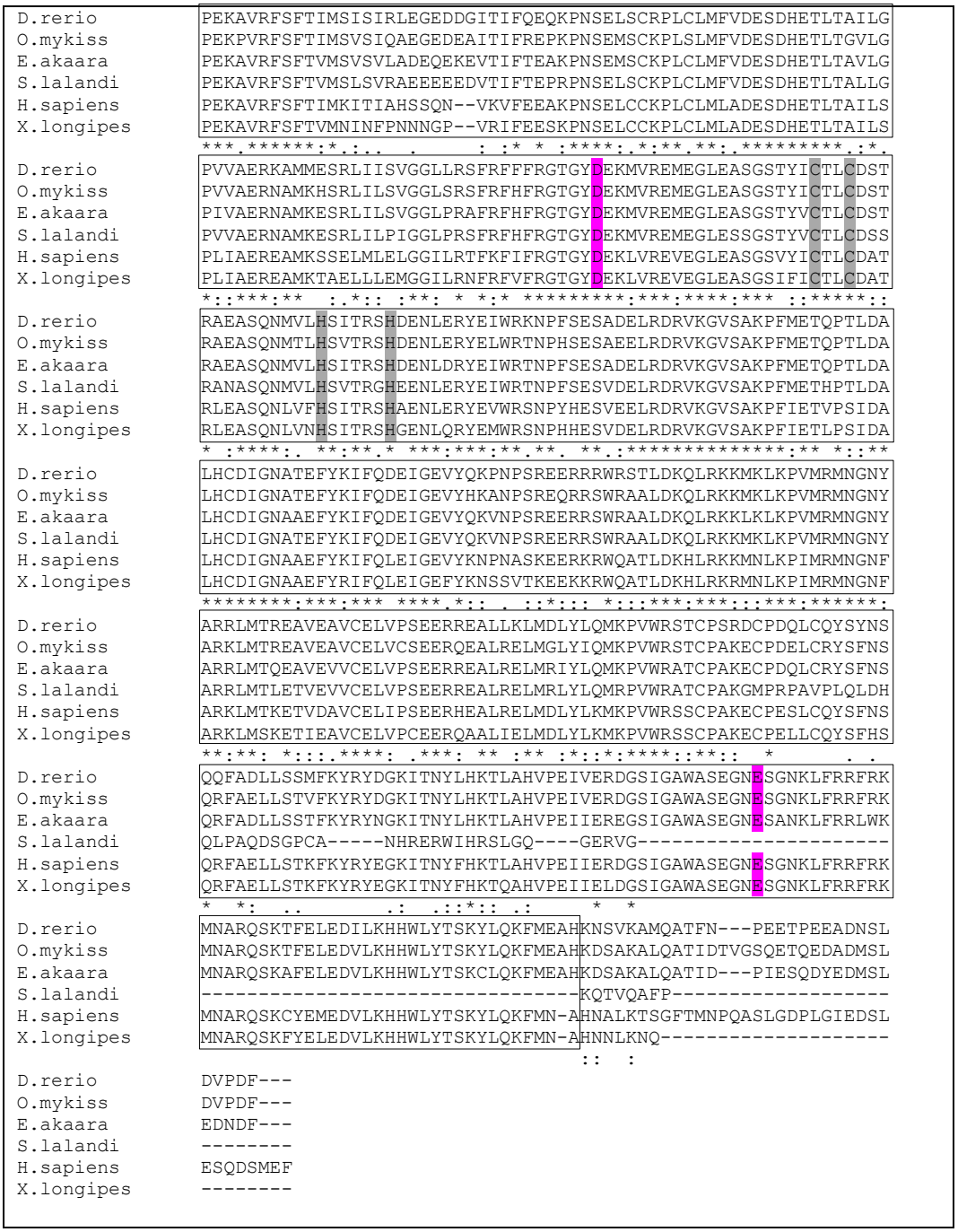


Figure 31: Alignment showing the conserved domains of IgD. Sequences are divided into regions, previously characterised in other fish species, which are distinguished by colour. The blue region is the zinc-binding dimerization domain with the yellow amino acids make up the RING finger motif and the red amino acids make up zinc-binding finger A motif. The boxed region is the core RAG1 region with the green section showing the nonamer binding region, the grey showing the amino acids that make up zinc finger B motif and the purple amino acids representing the DDE motif. Accession numbers of sequences used in this alignment were: *Homo sapiens* (AAA60248.1), *Xenopus longipes* (ABS00359.1), *Epinephelus akaara* (AEK82141.1), *Danio rerio* (NP_571464.1), *Oncorhynchus mykiss* (NP_001118209.1) and the confirmed sequence from *Seriola lalandi*. An asterisk represents complete identity, a colon represents conservative substitutions and a dot represents a semiconservative substitution.

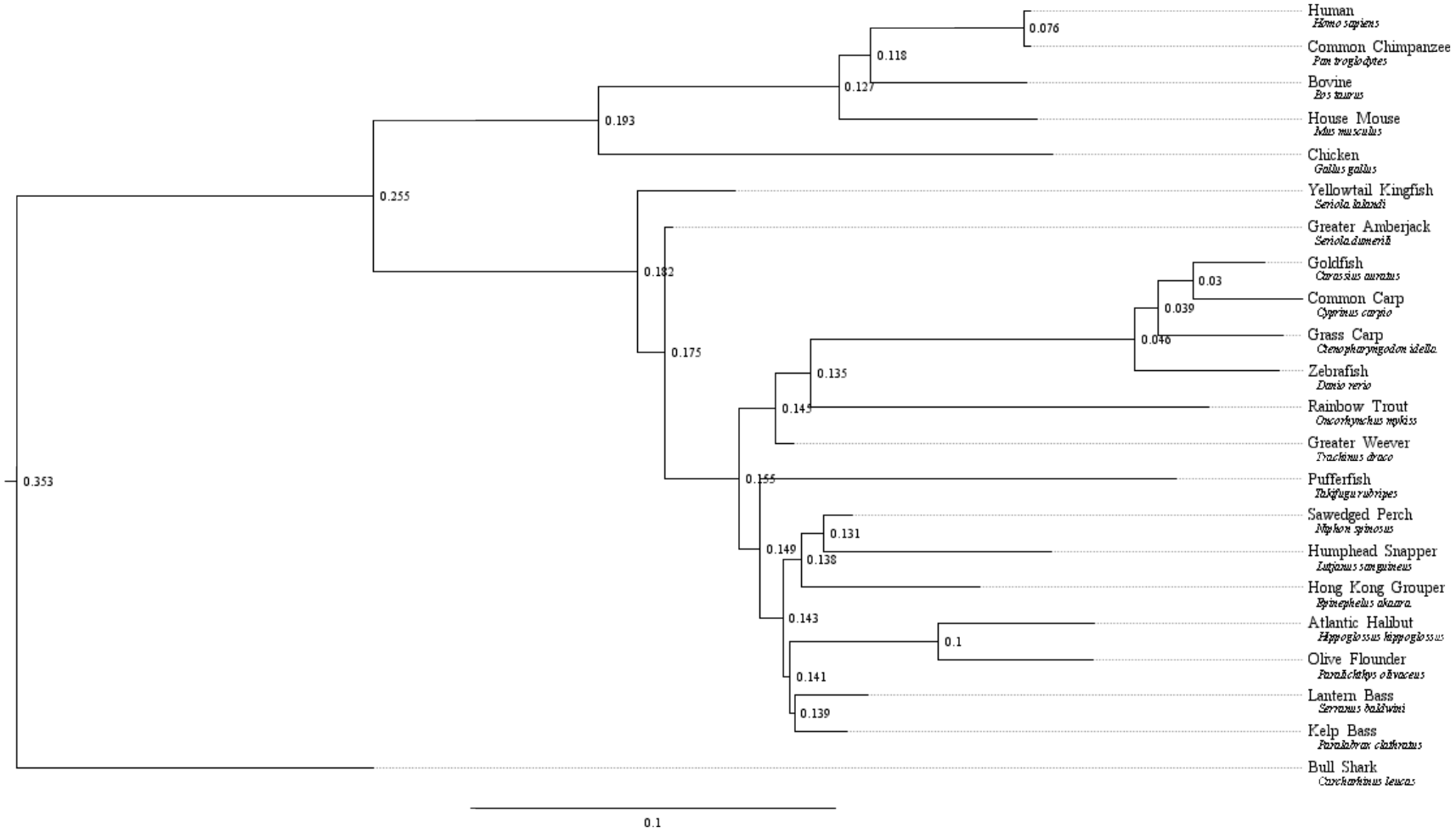


Figure 32: Phylogenetic tree of RAG1 sequence data from a range of species. An alignment of the sequences was first performed using ClustalX and the tree was constructed from this alignment using the Figtree software. The species were used for the alignment can be found in appendix IV.

3.3.5 RACE PCR for RAG1

RACE PCR to amplify the 3' end of RAG1 was attempted. Primers were designed from the confirmed RAG1 sequence and liver, kidney and Gill tissue was used to source RNA which was used for cDNA synthesis. The first round of 3' RACE PCR produced a number of bands from each sample that were approximately 500 bp or smaller (Figure 33). These products were then used as a template for the second round PCR.

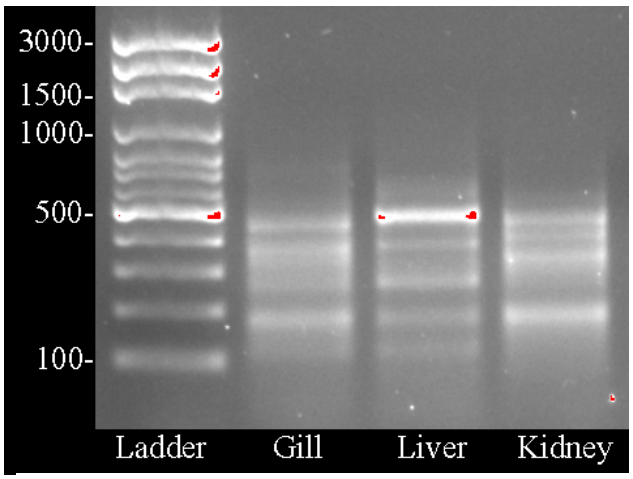


Figure 33: Gel analysis of first round 3' RACE for RAG1. Bands at approximately 500 bp and smaller were seen from each sample. Each sample was prepared for the second round of PCR.

The round PCR produced fewer bands from each tissue. Again, the band sizes were approximately 500 bp or less (Figure 34). The bands were faint and smaller than expected. No further work was performed on these products due to the faintness and small size of the bands. Due to time constraints, no further optimisation was carried out.

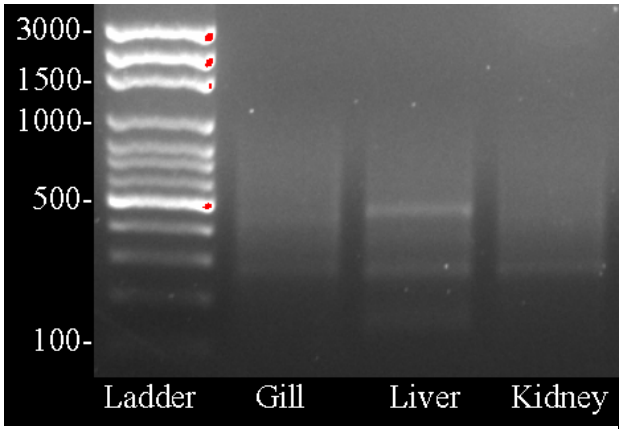


Figure 34: Gel analysis of second round 3' RACE PCR for RAG1. Faint bands were seen from each sample. The bands were very faint and smaller than expected. Further work with these products was not performed.

3.4 Interleukin-1β

The *Epinephelus coioides* (ABV02594.1) amino acid sequence was found in the NCBI database and used to search the transcriptome library for IL-1 □ using the Geneious software. The nucleotide sequences obtained from the tblastn search were assembled and a consensus sequence was determined. The consensus sequence was translated using ExPASy and aligned to the *E. coioides* IL-1β amino acid sequence which gave a 24.2% identity (Figure 35). Primers were designed to be used in amplification of the *S. lalandi* IL-1β sequence

```
S_lalandi       MESEMKCNLSEMWSSKMPKGLDLEISCNPKTMKSVATLVMTVNRMK--KSPTRSSWELRG
E_coioides      MESQMTCNVSEMWSSRMPEGLELEISHHPLTMKHVANLIIATQRFKGIISESVLGTEFRD
                ***:*.**:******:**:**:**** :* *** **.*::::.:*:*   * :  . *:*.

S_lalandi       DELCSVIMXSLVDET-VVKTVADATTGQRXIKXVRARSEECTXSDTQKKDIICTPGDLKL
E_coioides      EHLLSIMLESIVEERNVFGCEATPPTDEDMIT--RTREYDCTVEDEEKRSLVRVNNSLVL
                :.* *::: *:*:*  *.   * ..*.:  *.  *:*. :** .* :*:.:: . ..* *

S_lalandi       QAVTLKGGYADRXVNFKLVKYISCGAGQTFVLSIXNDHSHXFYLSCXKNSNNAELHLEKC
E_coioides      HAVMLQGGTDLKQVKLNMSMYLHPAPSVEGRTVALGIKGTQYYLTCRKDGTQPTLHLETI
                :** *:**   : *::::  *:  ...        . :.  :**:* *:..:. ****.

S_lalandi       SED-----DIRNDMDRFLFEXKXXXXSQXSFESVKHRGWFISTSESENQPXELCQIDSAQ
E_coioides      TKDSLASIDPNSDMVRFLFYKQISGVNVSTLMSVAHPNWYISTAEADNMPVEMCQES-TS
                ::*     * ..** ****  :    .  :: ** * .*:***:*::* * *:** . :.

S_lalandi       RVTSFNVSSKKPLIG-SSCVQLQFNVSRC-GYXFILKLFYST-PLDGVITCRMIIIQCNL
E_coioides      RYRAFTFSAIKEETPTA-------------------------------------------
                *  :*..*: *     :

S_lalandi       LKETLTLYYFTLRQNDVKFVSSLLENVTGYS-NLLPFLIIISH-LNFIIDCPLLLNMPLS
E_coioides      ------------------------------------------------------------


S_lalandi       FLSACRKLRYYLLF--TSNMCS-KKKNL--VVLIRIREAEFQHTGRRY-
E_coioides      ---------------------------------------------------
```

Figure 35: Amino acid alignment for IL-1β from the reference species and the *S. lalandi* transcriptome. A pairwise alignment was performed using ClustalX using E. coioides (ABV02594.1) IL-1β sequence and IL-1β translated from the *S. lalandi* transcriptome. An asterisk represents complete identity, a colon represents conservative substitutions and a dot represents a semiconservative substitution.

3.4.1 Amplification and Cloning of IL-1β

Primers for the amplification of the CDS region of IL-1β were designed. The tissues used for this amplification were liver and spleen. The primers combinations used were F1R1, F2R1 and F2R2 which were predicted to produce products of 1,049 bp, 1,025 bp and 906 bp, respectively. One significant band was seen in the IL-1β amplification in liver tissue after using the F1R1 primers (Figure 36). No other significant bands were seen using the F2R1 and F2R2 primers. No significant bands were seen from the spleen tissue from any primer combinations (Figure 37). There was a faint smear in the F1R1 lane which begins at approximately 450 bp, however the F1R1 band expected size was 1,049 bp. The F1R1 liver sample was ligated into a cloning vector despite the smaller size of the band, to determine what had been amplified. However attempts at growing bacteria transformed using this vector failed as colonies did not grow on the LB+ plates and due to time constraints, no further attempts were made using this approach.

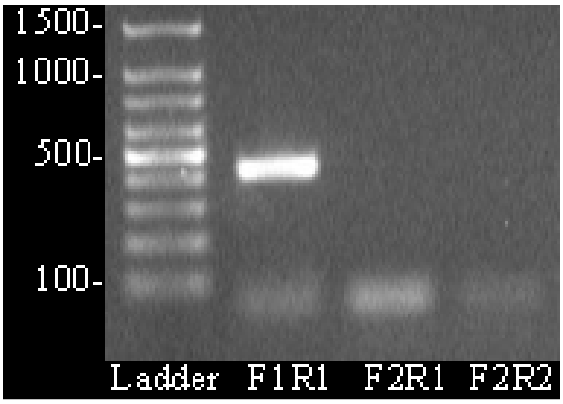


Figure 36: Amplification of IL-1β in liver tissue. One band was seen in the F1R1 lane at approximately 450 bp, however the expected size was 1,049 bp. The F1R1 product was ligated into a cloning vector despite the small size of the band.

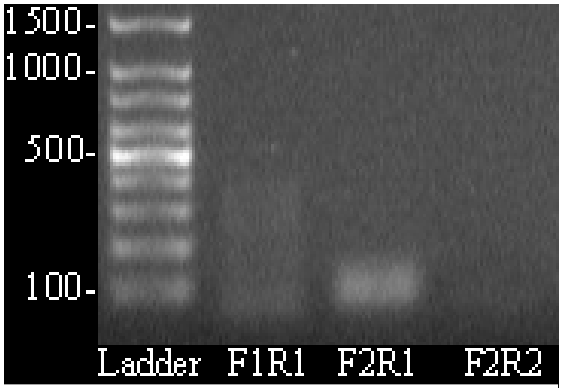


Figure 37: Amplification of IL-1β in spleen tissue. No bands of significant size or intensity were seen on this gel. A faint smear was seen in the F1R1 lane of the gel which begins at about 450 bp.

3.4.2 RACE PCR

Despite the failure to amplify anything from the CDS region of IL-1β, RACE primers were designed to target the 3' end of the mRNA. RNA from spleen, liver and kidney tissue was prepared for 3' RACE PCR. The first round of RACE PCR produced a large smear in the spleen and kidney and a number of bands were seen in liver (Figure 38). This was followed by the second round of PCR which produced fewer bands. Two bands were seen in spleen, a large smear was seen in kidney and one band was seen in liver (Figure 39). The larger band that was produced from the spleen was gel purified and ligated into a vector.

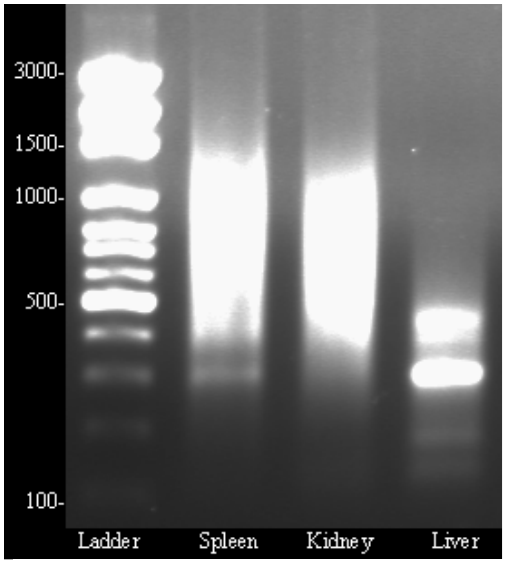


Figure 38: First round 3' RACE PCR for IL-1β. Large smears were seen in the spleen and kidney samples and a number of different sized bands were seen in liver. Each sample after the first round of RACE PCR was used in the second round. Each product was used for a second round of PCR in an attempt to isolate a single band.

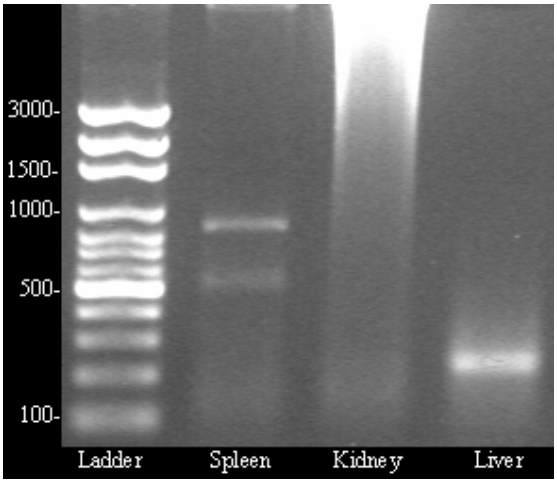


Figure 39: Second round of 3' RACE PCR for IL-1β. Two bands were seen in the spleen sample and one band was seen in liver. A large smear was also seen in the kidney. The large band in spleen at approximately 900bp was gel extracted and ligated.

3.4.3 Colony PCR for IL-1β 3' RACE

Upon the successful growth of potentially transformed colonies, a colony PCR was used to detect the insertion of the 3' RACE product into the vector. The colony PCR (Figure 40) showed a band with an approximate size of 700 bp in the first five colonies and a smaller band in colony six of approximately 400 bp. Each colony was used for further analysis with the exception of colony six, which was deemed too small.

3.4.4 Restriction Digest of Plasmids with 3' RACE IL-1β Insert

Plasmids were extracted from the bacterial cells that contained the insert. Successful restriction digest produced two bands, a large band of approximately 3000 bp and a smaller band at approximately 550 bp (Figure 41). This was true of colonies one, two, four and six. However, colony three and colony five did not have the smaller band and colony three also had a >3000 bp product. All plasmids with the smaller 550bp band were prepared and sent for sequencing.

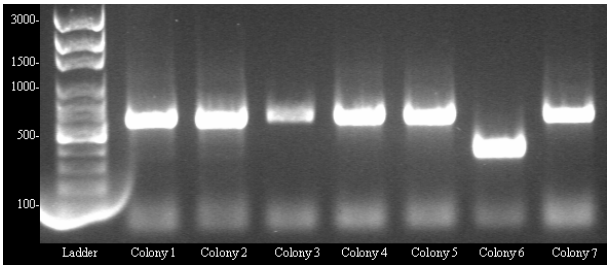


Figure 40: Colony PCR of 3' RACE IL-1β insert. Bands at approximately 700 bp were seen in the first five colonies and colony seven. A smaller band was seen in colony six. Colonies one to five and colony seven were grown up for plasmid extraction.

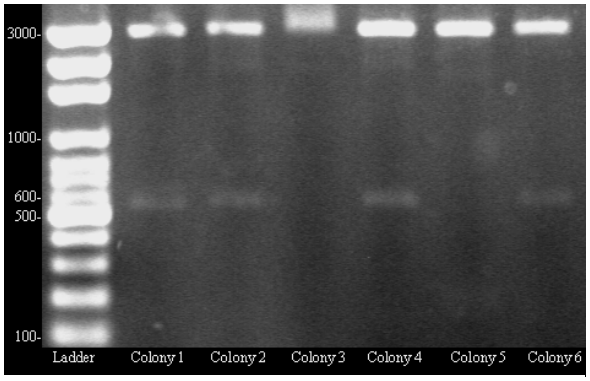


Figure 41: Restriction digests for 3' RACE IL-1β. A band at approximately 3,000 bp was seen in each lane which represented the digest plasmid. A band at approximately 600 bp was seen in colony one, two, four and six which represented the digested insert. No other bands were detected in the lanes for colony three and five. All plasmids were prepared and sent for sequencing.

3.4.5 Sequencing results

Plasmids were analysed using the Nanodrop 2000 to determine sample concentration and purity (Table 13).

Table 13: Nanodrop results for plasmids containing IL-1β insert. Concentration and purity of the plasmids were determined and used to prepare the quantities needed for sequencing.

Sample	Concentration	260/280	230/280
Colony 1	105.4 ng/µL	1.88	2.05
Colony 2	141.8 ng/µL	1.89	2.16
Colony 3	134.2 ng/µL	1.86	2.06
Colony 4	126.1 ng/µL	1.88	2.18
Colony 5	129.2 ng/µL	1.90	2.17
Colony 7	139.5 ng/µL	1.85	1.77

Plasmids from colonies one, two, three, four and seven were sent for sequencing. Once sequencing data were returned, the primer sequences were searched for nucleotides before the forward primer and after the 3' RACE primer and omitted from the sequence. The sequences were aligned (Figure 42) and a consensus sequence was determined (Figure 43). The consensus sequence from the sequencing results was found not to be related to IL-1β and was consider a failed result.

```
3_IL1b2          TTGAGCGAGATGTGGAGCTCCAAGATGTCCTAAAAAGTCATAATACAGTTTGGCTAAAAA
3_IL1b3          TTGAGCGAGATGTGGAGCTCCAAGATGTCCTAAAAAGTCATAATACAGTTTGGCTAAAAA
3_IL1b4          TTGAGCGAGATGTGGAGCTCCAAGATGTCCTAAAAAGTCATAATACAGTTTGGCTAAAAA
3_IL1b1          TTGAGCGAGATGTGGAGCTCCAAGATGTCCTAAAAAGT-AAAATACAGTTTGGCTAAAAA
                 *********************************** * ******************
3_IL1b2          AGTCATAAAAACGACATTGTATAGTACGGCAAAAAAAGTCAAAAAATTACATTGTATAAT
3_IL1b3          AGTCATAAAAACGACATTGTATAGTACGGCAAAAAAAGTCAAAAAATTACATTGTATAAT
3_IL1b4          AGTCATAAAAACGACATTGTATAGTACGGCAAAAAAAGTCAAAAAATTACATTGTATAAT
3_IL1b1          AGTCATAAAAACGACATTGCATAGTATGGCAAAAAAAGTCAAAAAATTACATTGTATAAT
                 ****************** ****** ********************************
3_IL1b2          AATGCTTAAAAAGTCATAAAAATGTCATAGCATAGTCAAGCACGAAAAATCATATTATAG
3_IL1b3          AATGCTTAAAAAGTCATAAAAATGTCATAGCATAGTCAAGCACGAAAAATCATATTATAG
3_IL1b4          AATGCTTAAAAAGTCATAAAAATGTCATAGCATAGTCAAGCACGAAAAATCATATTATAG
3_IL1b1          AATGCTTAAAAAGTCATAAAAATGTCATAGCATAGTCAAGCACGAAAAATCATATTATAG
                 ************************************************************
3_IL1b2          TAAGGCCTAAAAAGTCATAATATAGTATGGGAAGAAAAGTCATAAAGACGACATAGTATA
3_IL1b3          TAAGGCCTAAAAAGTCATAATATAGTATGGGAAGAAAAGTCATAAAGACGACATAGTATA
3_IL1b4          TAAGGCCTAAAAAGTCATAATATAGTATGGGAAGAAAAGTCATAAAGACGACATAGTATA
3_IL1b1          TAAGGCCTAAAAAGTCATAATATAGTATGGGAAGAAAAGTCATAAAGACGACATAATATA
                 ************************************************** ****
3_IL1b2          GTATGGTAAAAAAAAAAGTCAAAAAATGACGTACATAGTACAGTAATGCCCAAAAAAGTC
3_IL1b3          GTATGGTAAAAAAAAAAGTCAAAAAATGACGTACATAGTACAGTAATGCCCAAAAAAGTC
3_IL1b4          GTATGGTAAAAAAAAAAGTCAAAAAATGACGTACATAGTACAGTAATGCCCAAAAAAGTC
3_IL1b1          GTATGGTGAAAAAAAAAGTCAAAAAATGACATACATAGTACAGTAATGCCCAAAAAAGTC
                 ******* ********************** ****************************
3_IL1b2          AAAGCATTCAATTCAATTCAACTTTATTTGTATATTCCCAAATCATAACATACATTATCT
3_IL1b3          AAAGCATTCAATTCAATTCAACTTTATTTGTATATTCCCAAATCATAACATACATTATCT
3_IL1b4          AAAGCATTCAATTCAATTCAACTTTATTTGTATATTCCCAAATCATAACATACATTATCT
3_IL1b1          AAAGCATTCAATTCAATTCAACTTTATTTGTATATTCCCAAATCATAACATACATTATCT
                 ************************************************************
```

```
3_IL1b2         CACAGCACTTTACATGGTAATGTCAACACTTTACTTTATTATAGAGAAACCCAACATTTC
3_IL1b3         CACAGCACTTTACATGGTAATGTCAACACTTTACTTTATTATAGAGAAACCCAACATTTC
3_IL1b4         CACAGCACTTTACATGGTAATGTCAACACTTTACTTTATTATAGAGAAACCCAACATTTC
3_IL1b1         CACAGCACTTTACATGGTAATGTCAACACTTTACTTTATTATAGAGAAACCCAACATTTC
                ************************************************************
3_IL1b2         CCACAAAGAGCAAGCATTTGGCGATCGGGGGGGAGTAAAAAATCAATTTTAACAGGAAGG
3_IL1b3         CCACAAAGAGCAAGCATTTGGCGATCGGGGGGGAGTAAAAAATCAATTTTAACAGGAAGG
3_IL1b4         CCACAAAGAGCAAGCATTTGGCGATCGGGGGGGAGTAAAAAATCAATTTTAACAGGAAGG
3_IL1b1         CCACAAAGAGCAAGCATTTGGCGATCGGGGGGGAGTAAAAAATCAATTTTAACAGGAAGG
                ************************************************************
3_IL1b2         ACTGGGTTCAGGATGGGCGGCCGTCTGCCTCAACTGGTTGAGTTTGAGAGAGAGACCATG
3_IL1b3         ACTGGGTTCAGGATGGGCGGCCGTCTGCCTCAACTGGTTGAGTTTGAGAGAGAGACCATG
3_IL1b4         ACTGGGTTCAGGATGGGCGGCCGTCTGCCTCAACTGGTTGAGTTTGAGAGAGAGACCATG
3_IL1b1         ACTGGGTTCAGGATGGGCGGCCGTCTGCCTCAACTGGTTGAGTTTGAGAGAGAGACCATG
                ************************************************************
3_IL1b2         GAGAGCAGTACCGAGAAGCGTATACTATTGTAAGCATAAAAAGTCATAAAAACAACATTG
3_IL1b3         GAGAGCAGTACCGAGAAGCGTATACTATTGTAAGCATAAAAAGTCATAAAAACAACATTG
3_IL1b4         GAGAGCAGTACCGAGAAGCGTATACTATTGTAAGCATAAAAAGTCATAAAAACAACATTG
3_IL1b1         GAGAGCAGTACCGAGAAGCATATACTATTGTAAGCATAAAAAGTCATAAAAACAACATTG
                ******************* ********************************************
3_IL1b2         TATAGTAAGGCTTGAGAAGTCATGAAGATGACACAAAGGCATGAAAAGTCATAAAAACGT
3_IL1b3         TATAGTAAGGCTTGAGAAGTCATGAAGATGACACAAAGGCATGAAAAGTCATAAAAACGT
3_IL1b4         TATAGTAAGGCTTGAGAAGTCATGAAGATGACACAAAGGCATGAAAAGTCATAAAAACGT
3_IL1b1         TATAGTAAGGCTTGAGAAGTCATGAAGATGACACAAAGGCATGAAAAGTCATAAAAACGT
                ************************************************************
3_IL1b2         CATTATAGTAAGGAGTAAAATTAACATAGTATACTAAGGCATAAAAAATCATATTATATG
3_IL1b3         CATTATAGTAAGGAGTAAAATTAACATAGTATACTAAGGCATAAAAAATCATATTATATG
3_IL1b4         CATTATAGTAAGGAGTAAAATTAACATAGTATACTAAGGCATAAAAAATCATATTATATG
3_IL1b1         CATTATAGTAAGGAGTAAAATTAACATAGTATACTAAGGCATAAAAAATCATATTATATG
                ************************************************************
3_IL1b2         ACGTATTATGATATGGTGATGTTACAACTTTTTAACATCATATAAAACGTCATATAAATT
3_IL1b3         ACGTATTATGATATGGTGATGTTACAACTTTTTAACATCATATAAAACGTCATATAAATT
3_IL1b4         ACGTATTATGATATGGTGATGTTACAACTTTTTAACATCATATAAAACGTCATATAAATT
3_IL1b1         ACGTATTATGATATGATGATGTTACAACTTTTTAACATCATATAAAACGTCATATAAATT
                *************** ********************************************
3_IL1b2         CATAAAAACATCATAGTATATTAA-GGCATGAAAAGTAAAAAAAAAAAAAA
3_IL1b3         CATAAAAACATCATAGTATATTAAAGGCATGAAAAGTAAAAAAAAAAAAAA
3_IL1b4         CATAAAAACATCATAGTATATTAA-GGCATGAAAAGTAAAAAAAAAAAAAA
3_IL1b1         CATAAAAACATCATAGTATATTAAGGGCATGAAAAGTAATAAAAAAAAAAA
                ********************** ************** ***********
```

Figure 42: Alignment for 3' RACE IL-1β sequencing results. The sequencing results show high similarity between each colony. A consensus sequence was produced with any nucleotide differences being resolved by taking the most common nucleotide. An asterisk represents complete identity

```
TTGAGCGAGATGTGGAGCTCCAAGATGTCCTAAAAAGTCATAATACAGTTTGGCTAAAAAAGTC
ATAAAAACGACATTGTATAGTACGGCAAAAAAAGTCAAAAAATTACATTGTATAATAATGCTTA
AAAAGTCATAAAAATGTCATAGCATAGTCAAGCACGAAAAATCATATTATAGTAAGGCCTAAAA
AGTCATAATATAGTATGGGAAGAAAAGTCATAAAGACGACATAGTATAGTATGGTAAAAAAAAA
AGTCAAAAAATGACGTACATAGTACAGTAATGCCCAAAAAAGTCAAAGCATTCAATTCAATTCA
ACTTTATTTGTATATTCCCAAATCATAACATACATTATCTCACAGCACTTTACATGGTAATGTC
AACACTTTACTTTATTATAGAGAAACCCAACATTTCCCACAAAGAGCAAGCATTTGGCGATCGG
GGGGGAGTAAAAAATCAATTTTAACAGGAAGGACTGGGTTCAGGATGGGCGGCCGTCTGCCTCA
ACTGGTTGAGTTTGAGAGAGAGACCATGGAGAGCAGTACCGAGAAGCGTATACTATTGTAAGCA
TAAAAAGTCATAAAAACAACATTGTATAGTAAGGCTTGAGAAGTCATGAAGATGACACAAAGGC
ATGAAAAGTCATAAAAACGTCATTATAGTAAGGAGTAAAATTAACATAGTATACTAAGGCATAA
AAAATCATATTATATGACGTATTATGATATGGTGATGTTACAACTTTTTAACATCATATAAAAC
GTCATATAAATTCATAAAAACATCATAGTATATTAAGGCATGAAAAGTAAAAAAAAAAAAAA
```

Figure 43: Consensus sequence for IL-1β 3' RACE. This sequence was translated into its amino acid sequence and used to search ncbi for a related sequence using tblastn. The results of the database search did not produce IL-1β.

3.4.6 *S. lalandi* IL-1β Sequence Used for qPCR

Despite the inability to confirm any of the *S. lalandi* IL-1β sequence, the transcriptome sequence was fully analysed to identify conserved domains of the protein (Figure 36). The confirmed amino acid sequence showed good homology with other sequences and relevant amino acids important to IL-1β function were conserved. A phylogenetic tree was also constructed to show the evolutionary relationship between species using IL-1β (Figure 32). This showed *S. lalandi* IL-1β had a closer relationship with other fish species, as it clearly grouped with them away from other vertebrate groups.

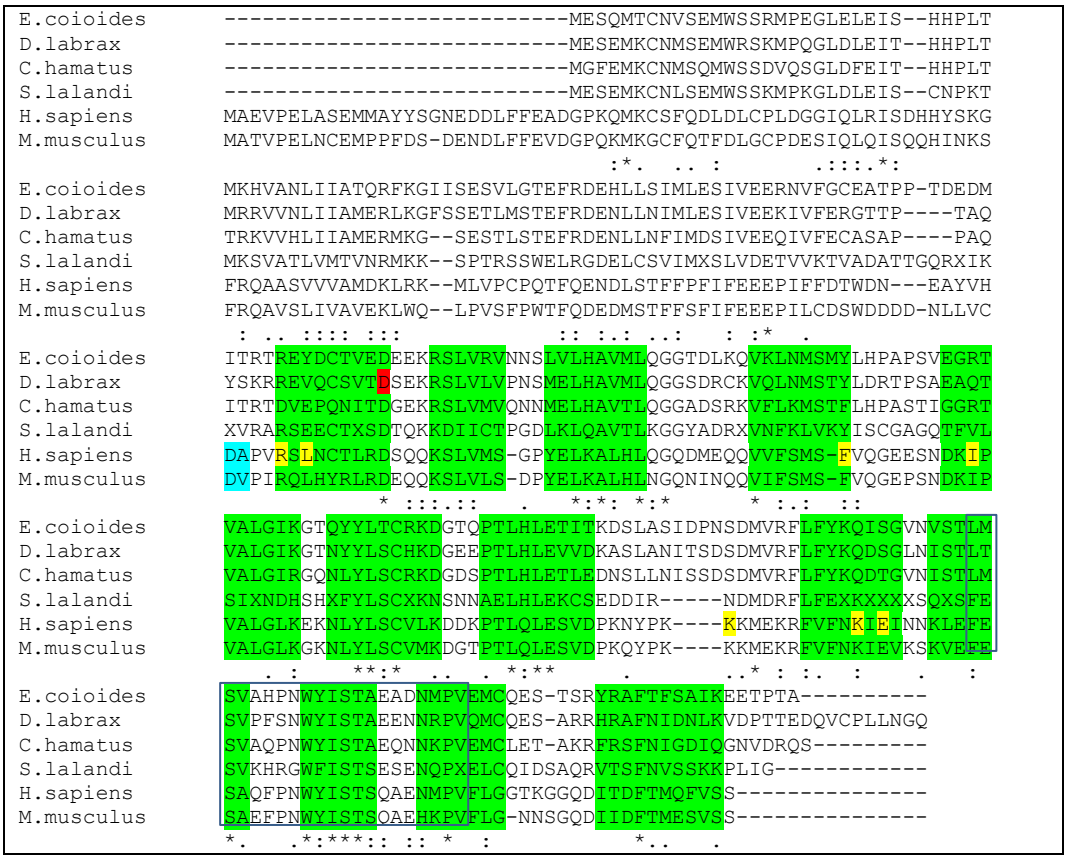


Figure 44: Conserved Domains of Interleukin-1β. Sequences are divided into regions, previously characterised in other fish species, which are distinguished by colour. The green highlighted areas represent the 12 β-sheets that make up the β-trefoil; the boxed region with bold characters that spans β-sheet 9-11 indicates the location of the family signature. The seven amino acids highlighted in yellow form the discontinuous binding site in the human IL-1β sequence. The amino acids highlighted in blue denote the ICE cut site found in humans and mice. The highlighted red amino acid represents a predicted teleost cut site. Accession numbers of sequences used in this alignment were: *Homo sapiens* (AAA74137.1), *Mus musculus* (AAH11437.1), *Epinephelus coioides* (ABV02594.1), *Chionodraco hamatus* (CAD89886.1), *Dicentrarchus labrax* (CAC41006.1), and the transcriptomic sequence of IL-1β from *S. lalandi*. An asterisk represents complete identity, a colon represents conservative substitutions and a dot represents a semiconservative substitution.

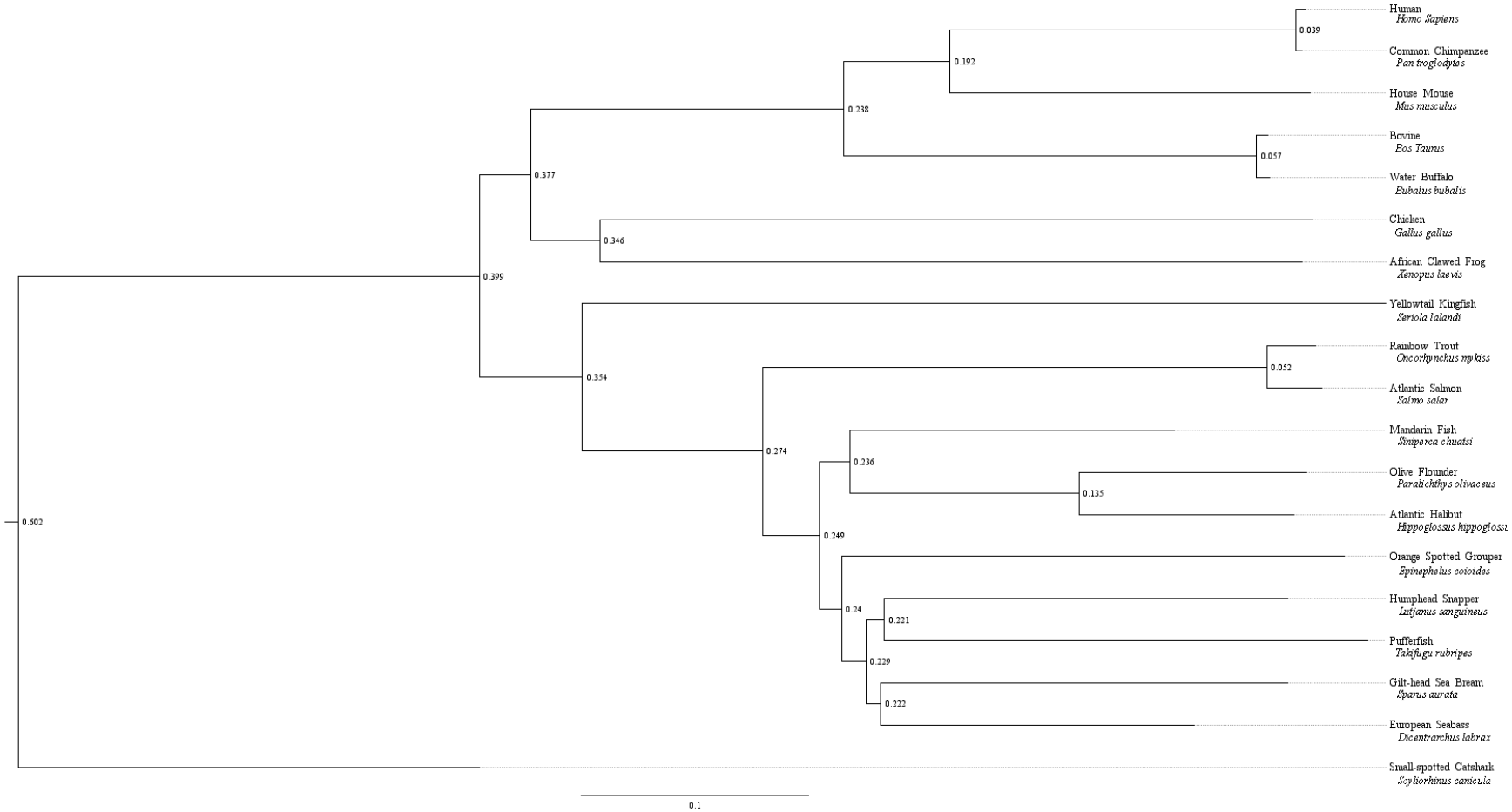


Figure 45: Phylogenetic tree of IL-1β sequence data from a range of species and the transcriptomic sequence from *S. lalandi* . An alignment of the sequences was first performed using ClustalX and the tree was constructed from this alignment using the Figtree software. The species were used for the alignment can be found in appendix IV.

3.5 Primer Efficiencies

The efficiency of each pair of primers designed for qPCR first had to be determined. At 100% efficiency the primers would be doubling the amount of template DNA; however primer pairs are often not 100% efficient. Determining the efficiency was achieved by producing a calibration curve using serially diluted temple DNA of known concentration. An example of how this was done is shown using GAPDH as an example. Using spleen cDNA at different dilutions (Figure 46) Ct values were calculated by placing a threshold line on the Y-axis of the amplification plot. The dilution series was: undiluted, 1:4, 1:16 and 1:64. The position of the line was determined by finding a region of the graph where a detectable amount of product could be seen at the early exponential phase. Following the calibration curve, a melt curve was performed (Figure 47) and the products were run on an agarose gel (Figure 48) to show the presence of only one product.

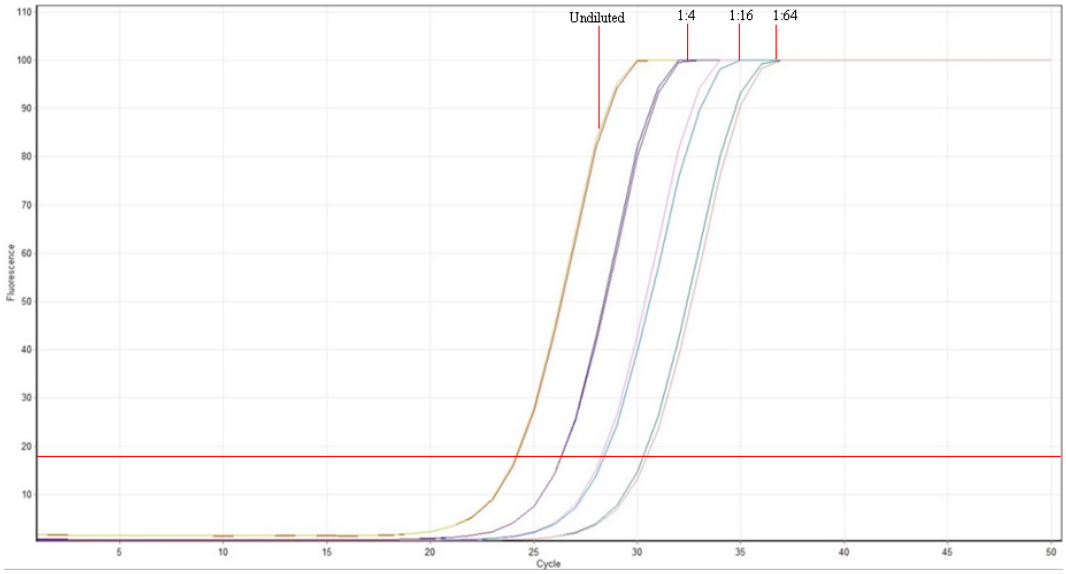


Figure 46: Calibration curve for qPCR obtained from GAPDH primers. Different dilutions of template cDNA from spleen were used and the threshold was set to obtain the corresponding Ct values. The red line indicates a region of the graph with a detectable amount of product in each sample during the early exponential phase.

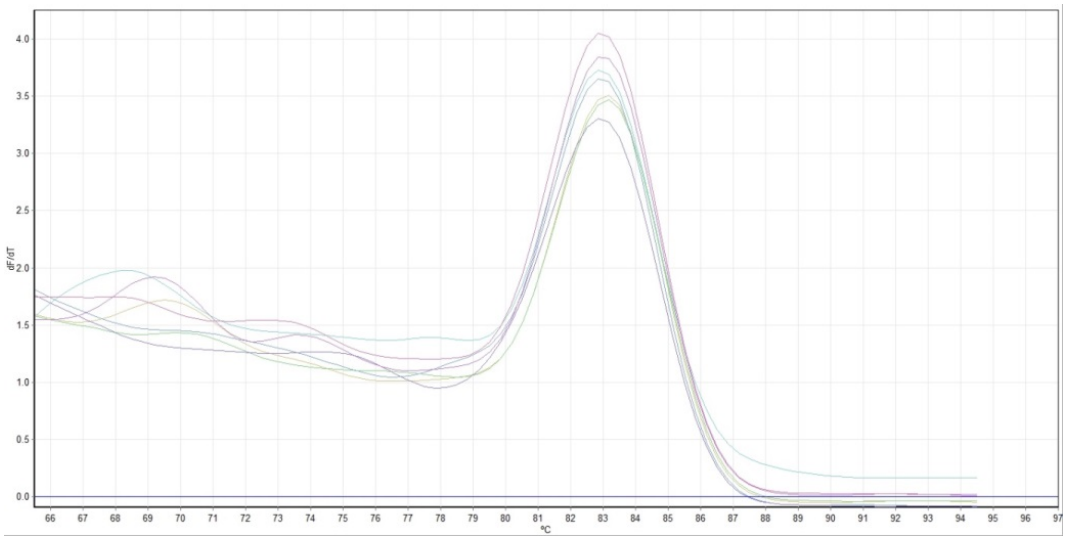


Figure 47: The melt curve obtained using GAPDH primers. The presence of one peak showed that only one product was formed during the run.

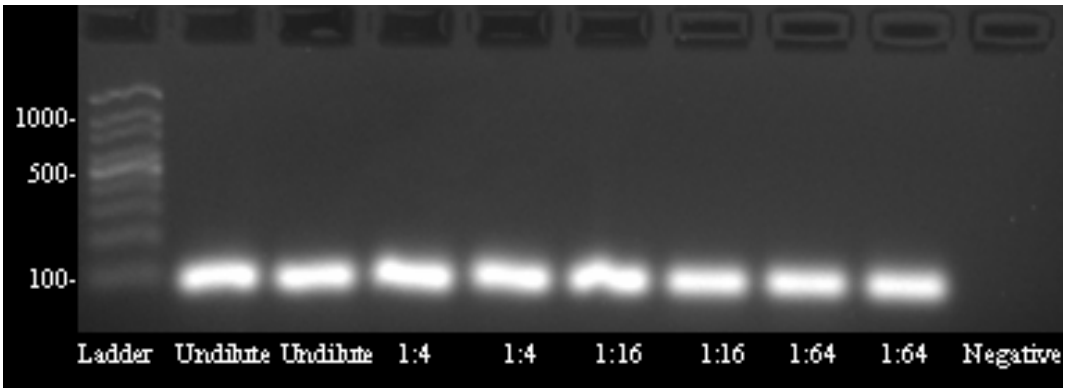


Figure 48: Gel image of the GAPDH qPCR products. Template cDNA was from kidney and each lane contained a single band and the negative control showed no band. Dilutions at undiluted, 1:4, 1:16 and 1:64 were performed in duplicate and run on the gel.

The Ct values for GAPDH were plotted on a graph against the log of the initial amount of template cDNA (Figure 49). A line of best fit was drawn through these points which produced a line equation and R^2 value. For GAPDH the line equation was **y= -3.1749x + 28.046** and the R^2 value was **0.99**. The closer the line gradient is to -3.33 shows how close the primer efficiency is to 100% and the gradient for the GAPDH primers was -3.179. Using the equation $\mathbf{E= 10^{(-1/s)}-1,}$ to determine primer efficiency, GAPDH primers were $10^{(-1/-3.1749)}$-1 = 1.06 or 106% efficient.

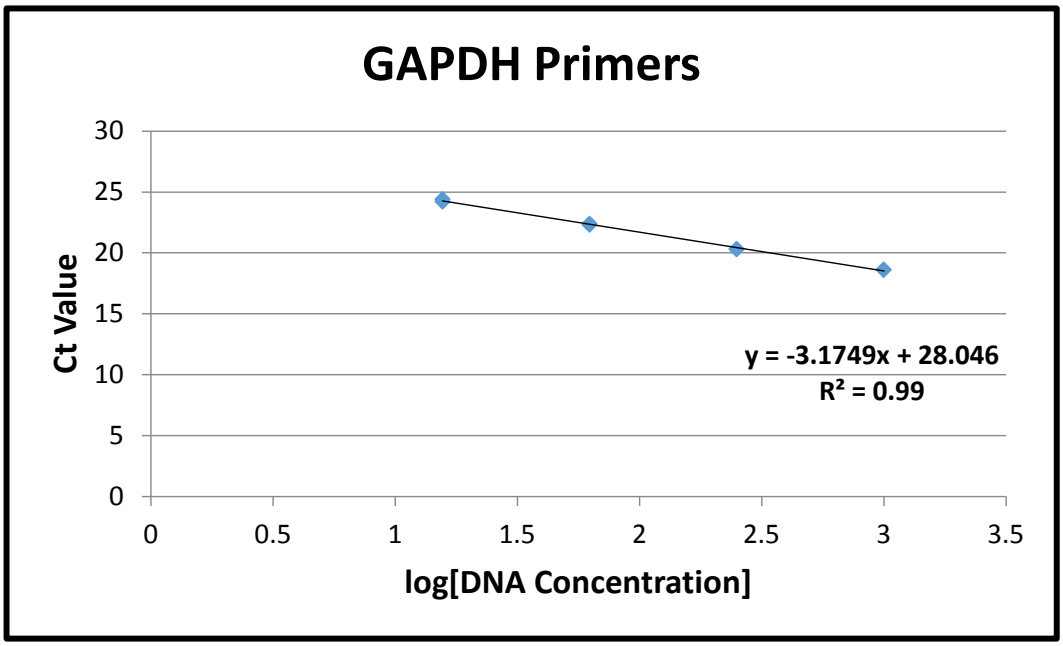


Figure 49: Standard curve for GAPDH with Ct values plotted against the log of the template concentration. The equation of the line and the R2 value are shown.

A number of primer efficiencies were then carried out on the other housekeeping gene (β-actin) and the primers for the genes of interest. The efficiency plots for β-actin (Figure 50), IgD (Figure 52), RAG1 (Figure 54) and IL-1β (Figure 56) were determined as were the melt curves for β-actin (Figure 51), IgD (Figure 53), RAG1 (Figure 55) and IL-1β (Figure 57). The efficiency plots for β-actin, IL-1β, IgD and RAG1 primers gave efficiencies of 98%, 101%, 111%, 116% respectively and each melt curve showed that only one product was being isolated.

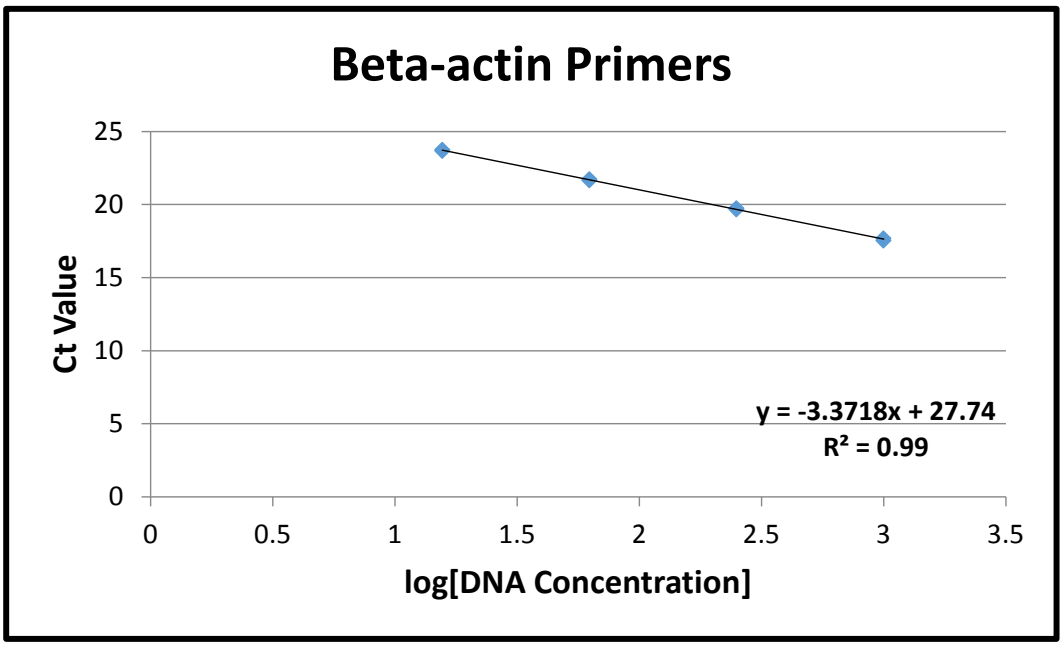


Figure 50: Standard curve for β-actin with Ct values plotted against the log of the template concentration. The equation of the line and the R^2 value are shown.

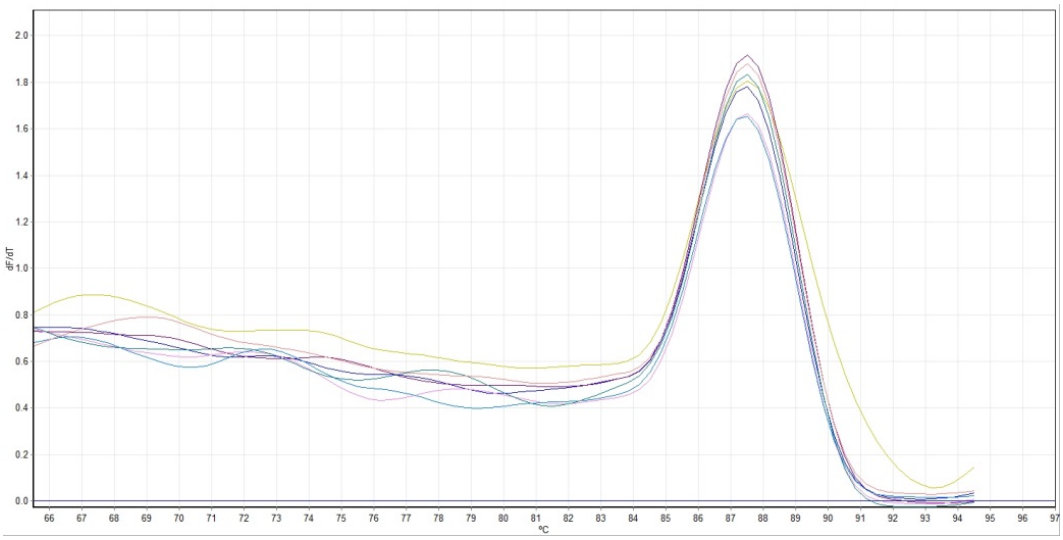


Figure 51: The melt curve obtained using β-actin primers. The presence of one peak showed that only one product was formed during the run.

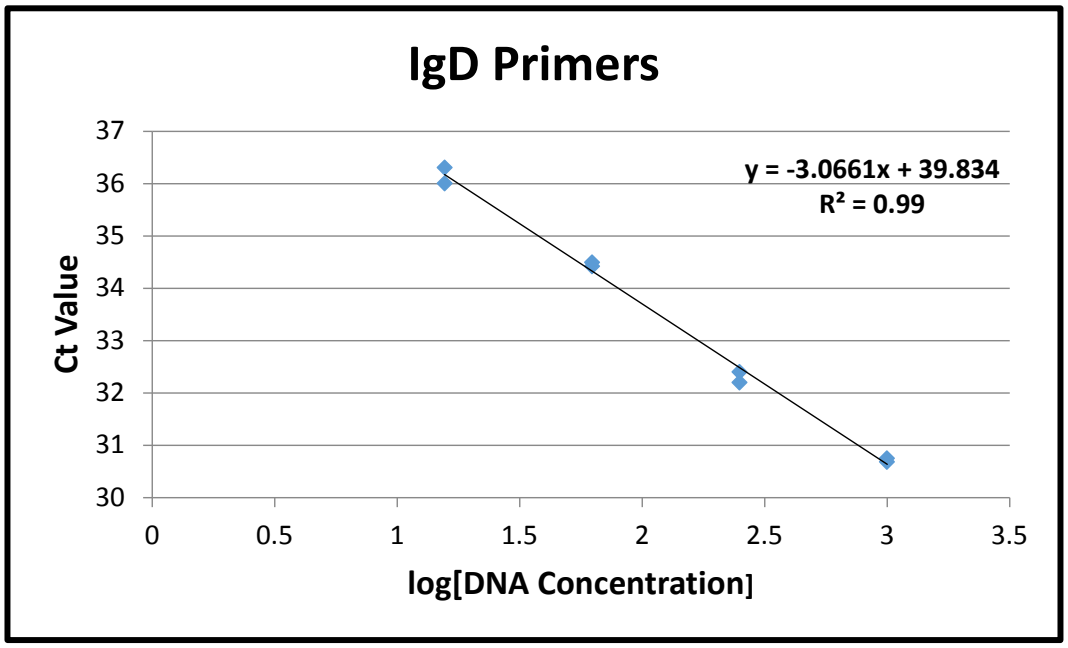


Figure 52: Standard curve for IgD with Ct values plotted against the log of the template concentration. The equation of the line and the R^2 value are shown.

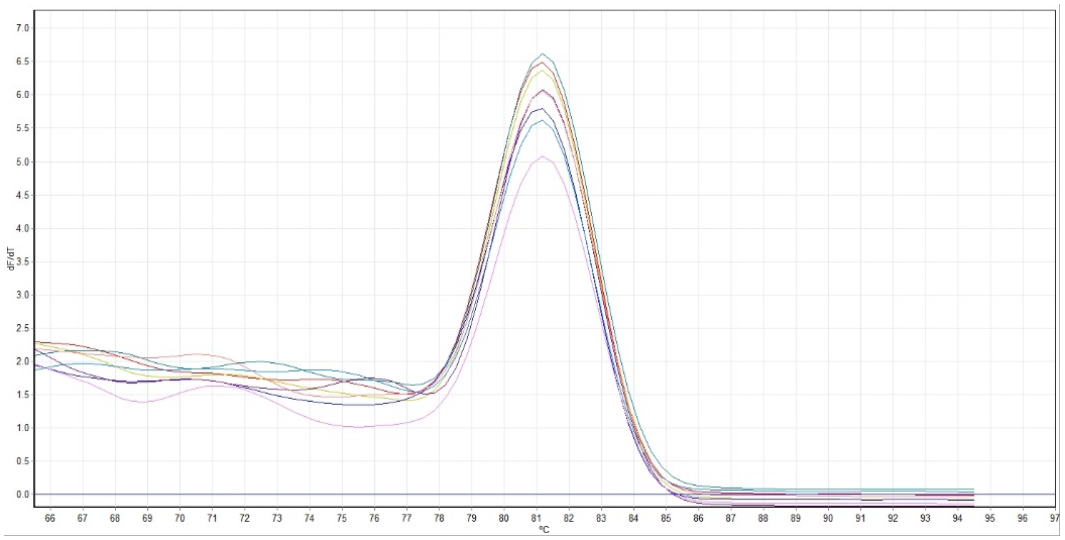


Figure 53: The melt curve obtained using IgD primers. The presence of one peak showed that only one product was formed during the run.

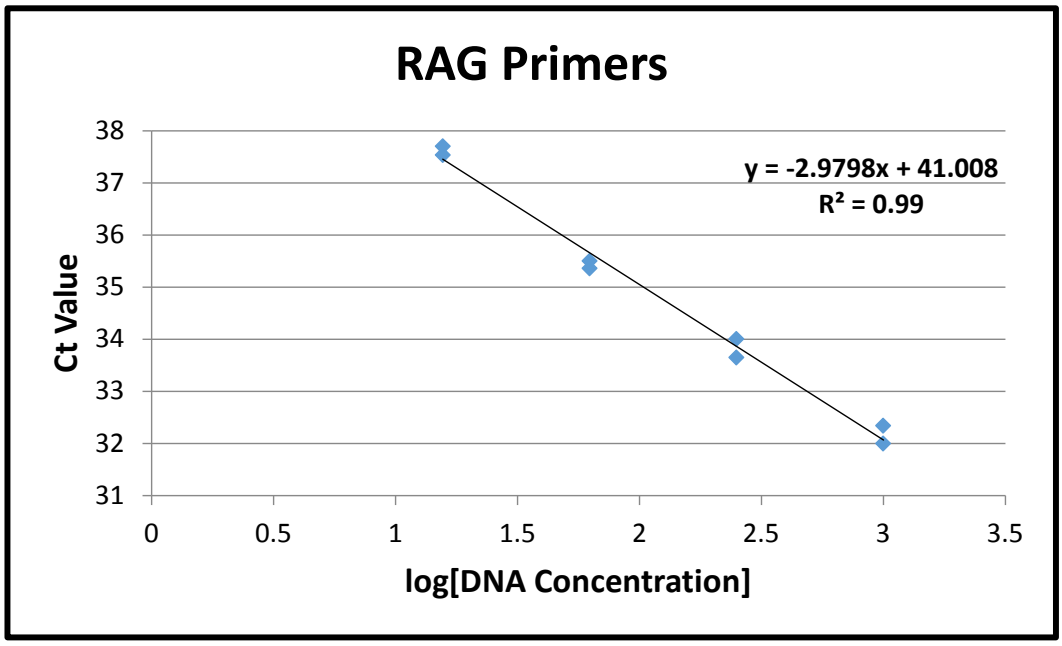


Figure 54: Standard curve for RAG1 with Ct values plotted against the log of the template concentration. The equation of the line and the R^2 value are shown.

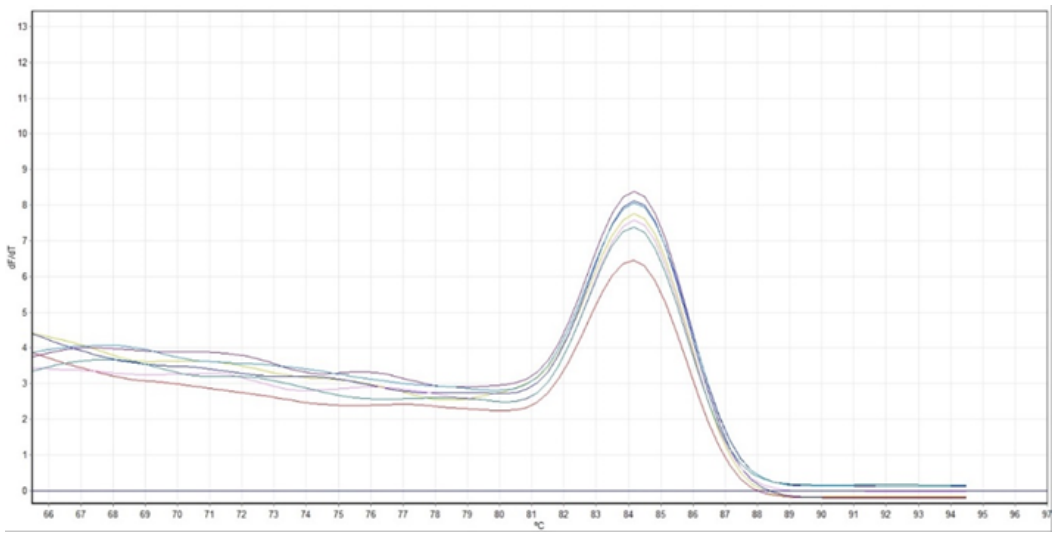


Figure 55: The melt curve obtained using RAG1 primers. The presence of one peak showed that only one product was formed during the run.

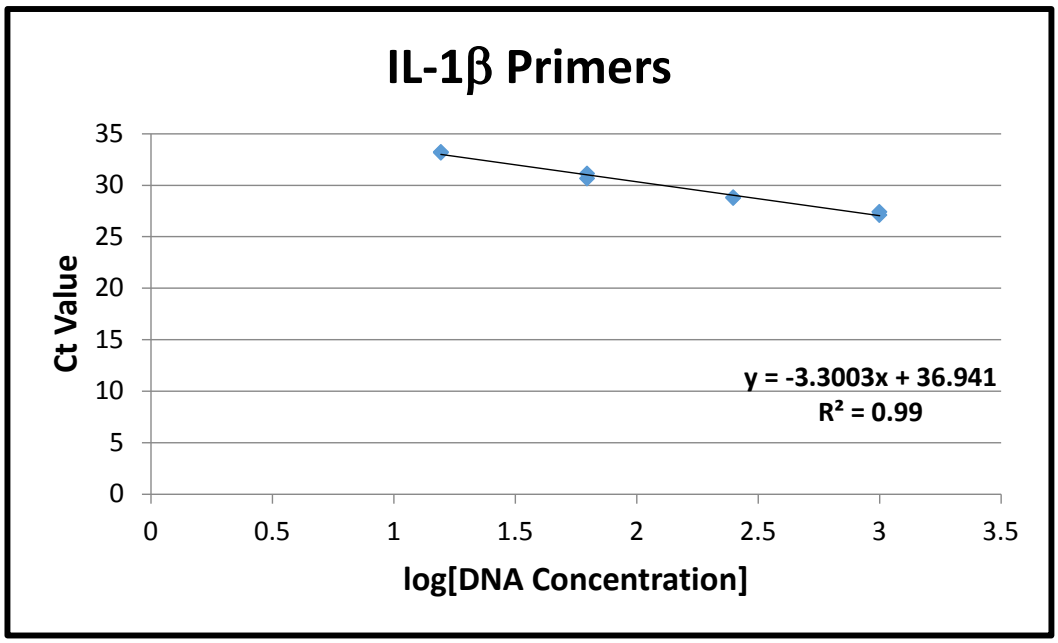


Figure 56: Standard curve for IL-1β with Ct values plotted against the log of the template concentration. The equation of the line and the R^2 value are shown.

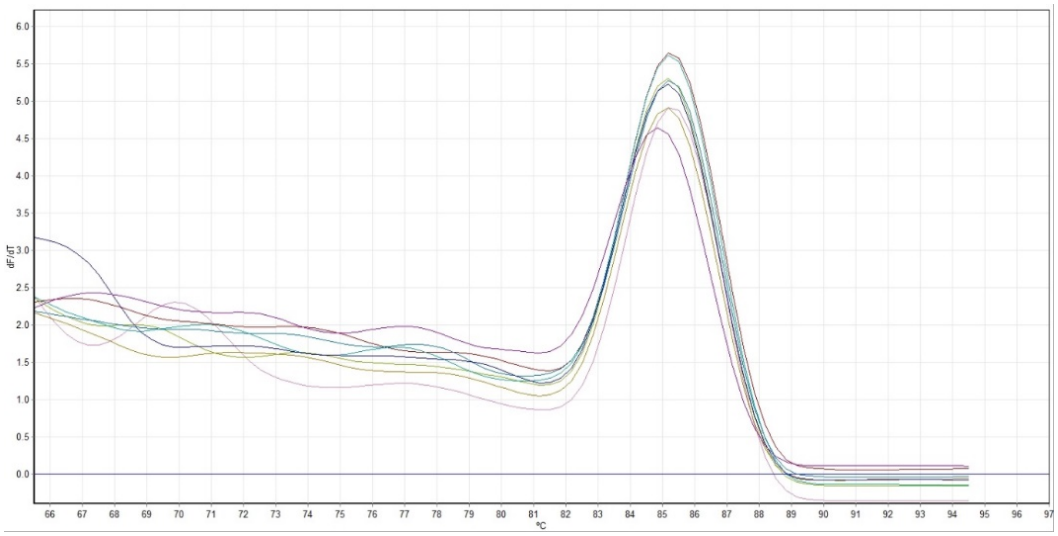


Figure 57: The melt curve obtained using IL-1β primers. The presence of one peak showed that only one product was formed during the run.

3.6 Expression of the Genes of Interest

The relative expression of the genes of interest was determined in stages of development. The data was obtained by taking the mean of four replicate qPCR runs, each containing 50 larvae. As the amplification efficiencies of each primer set was close to 2 it was found appropriate to use the $\Delta Cq = 2^{Cq(ref) - Cq(target)}$. The expression of each immune gene shows some expression at hatch, which is increased at day 12 and decreases at day 18. Analysis of the data with ANOVA shows none of these expression changes were statistically significant (Appendix VI).

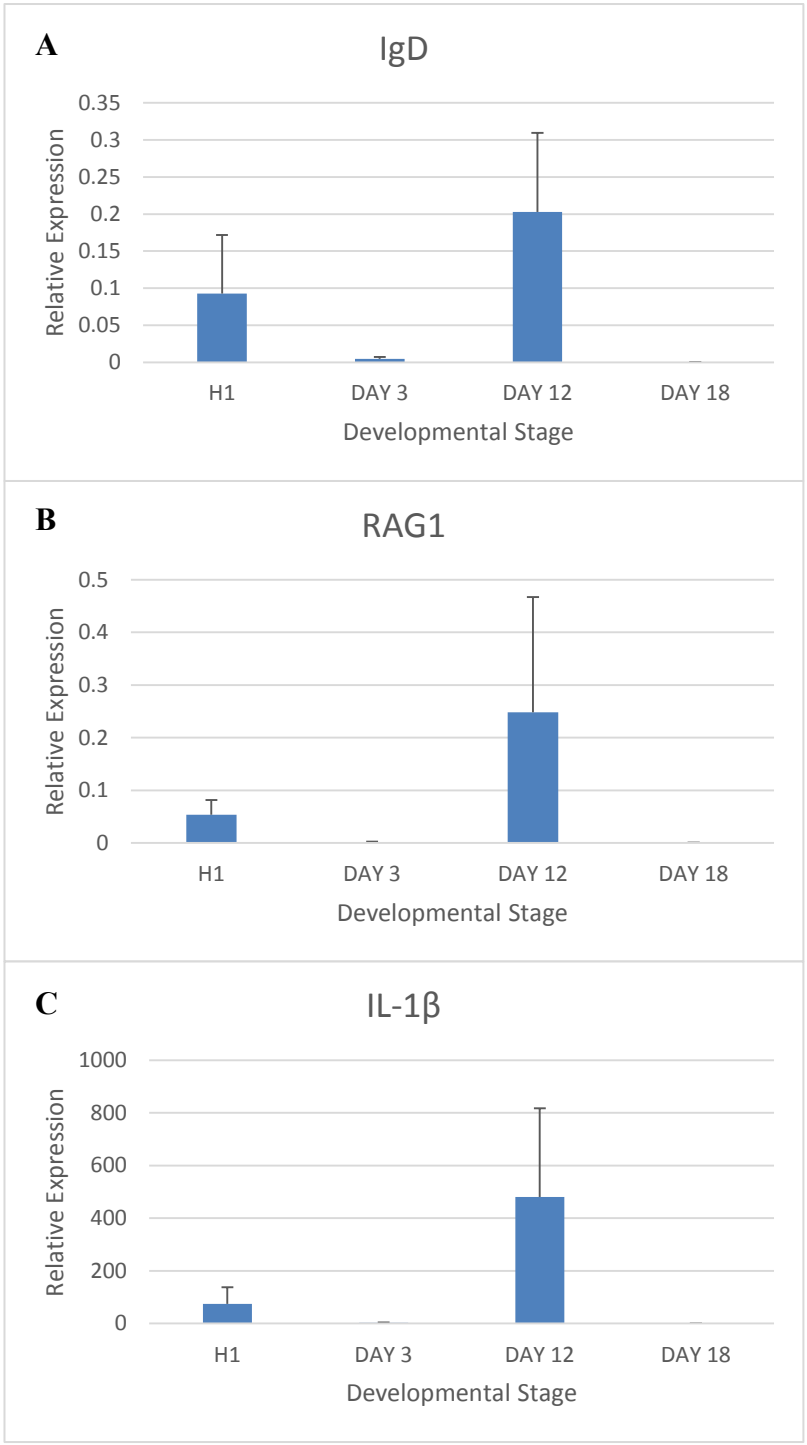


Figure 58: Relative expression of IgD, RAG1 and IL-1β (A, B, C, respectively). These data represent the expression of the genes at different stages of development. The data are the means of four replicates, each containing 50 larvae ± standard error.

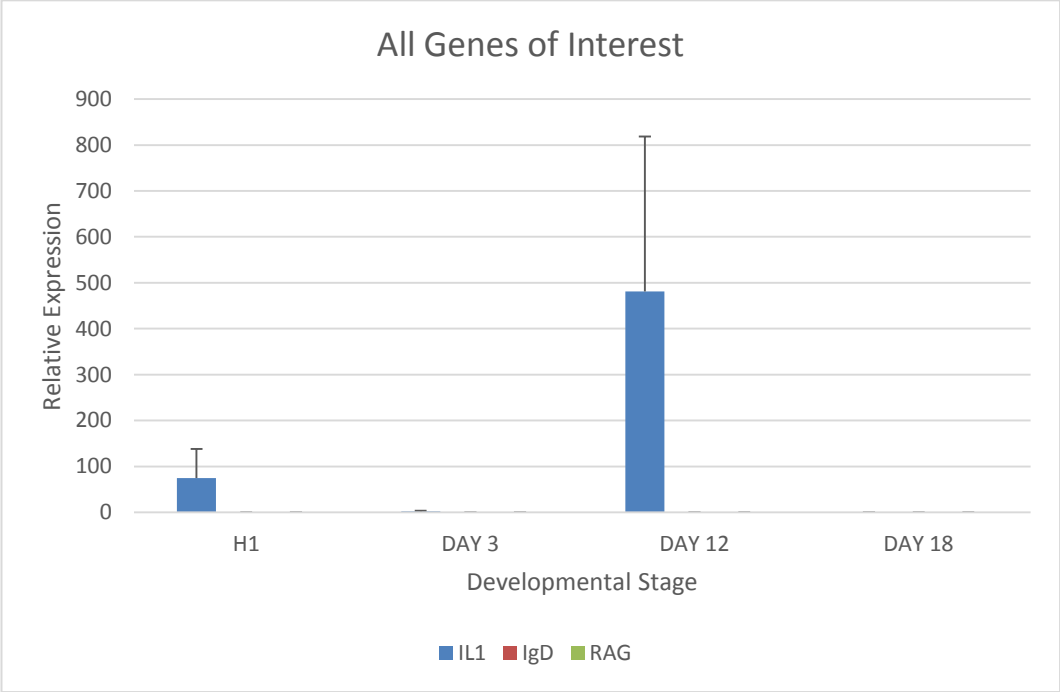


Figure 59: Relative expression of the genes of interest within larvae at different stages of development. The data are the means of four replicates, each containing 50 larvae ± standard error.

3.7 Histological Sections

Larval fish were sectioned to allow histological study and for use in *in situ* hybridization. However, due to problems with obtaining good quality sections, a lot of effort went into optimising the sectioning protocol for future work. Most sectioning took place using the wax microtome; however some sections were produced using the cryostat. Most sections were then stained using toluidine blue, with only one sample stained using haematoxylin and eosin. Following sectioning, good quality sections were imaged and internal structures were identified using a fish histology atlas (Genten et al., 2009). Most images presented were thin sections (6 µm) using the wax microtome. This is because sections below 10 µm using the cryostat were of poor quality or could not be used due to tearing or crumbling of the section (Figure 75). Similarly, poor quality sections also occurred using the wax microtome (Figure 76). However, optimization of the protocol, allowed sections to be prepared from larval fish aged 9 dph to 60 dph. In the 9 dph fish **(**Figure 60 and Figure 61) basic structures such as the oesophagus, liver and notochord could be identified and could be followed as the age of the fish increased. In 15 dph fish (Figure 62 and Figure 63), it was seen that structures within the fish had begun to develop. By 25 dph (Figure 64 and Figure 65) structures had become larger, with liver, notochord and spinal cord easily detectable. A 25 dph section produced using the cryostat (Figure 66) was included and while structures could be distinguished; the overall resolution was poorer than that of the paraffin section. Between 30 dph and 60 dph (Figure 67**,** Figure 68**,** Figure 69**,** Figure 70**,** Figure 71**,** Figure 72**,** Figure 73) the most visible change is that of size. This is especially seen at 35 dph (Figure 69) which shows a number of key structures, including liver, kidney, and gut. At 40 (Figure 70 and Figure 71) and 60 dph (Figure 72 and Figure 73) the size of the fish became too great, thus images were taken of key structures of the fish. At 40 dph (Figure 70 and Figure 71) images were taken of kidney and liver. It could be seen that kidney tissue had circular structures that could be easily identified. At 60 dph (Figure 72 and Figure 73), fish became too large for the available paraffin wax moulds and sections were taken using the cryostat. Similar structures could again be determined, including the kidney, liver, spinal cord and notochord. An image of the kidney at higher magnification did not show the previously seen circular structures. A sagittal section of a 21 dph larvae was also produced (Figure 74). This section was stained

using haematoxylin and eosin stain. The liver and kidney were clearly seen in this section as was other structures not seen in the earlier sections, such as the urinary bladder, gills and what was thought to be pancreatic tissue.

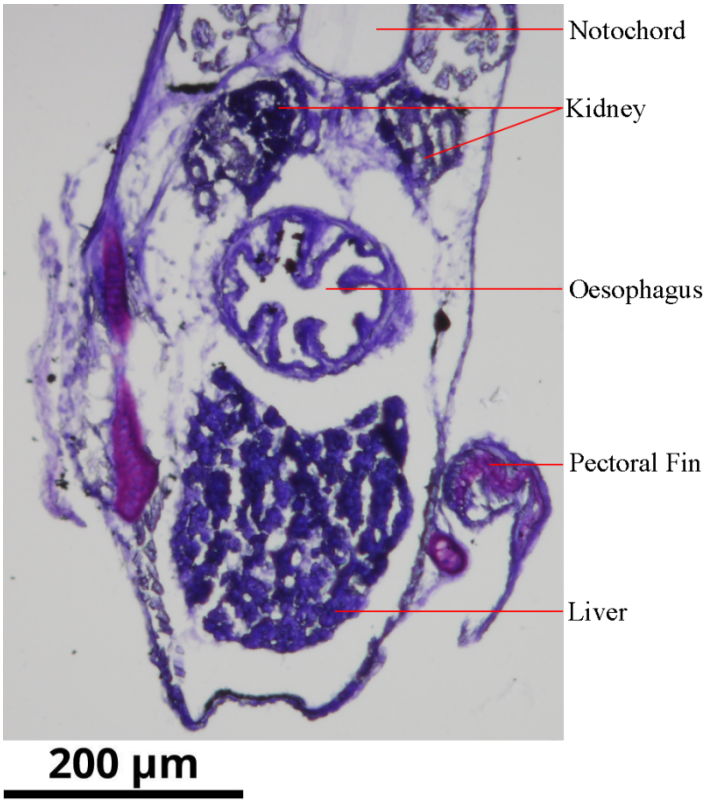


Figure 60: Photomicrograph of a larva at 9 dph. A 6 µm thick section was taken using the wax microtome mid trunk and stained with toluidine blue. Notochord, kidney, liver and oesophagus were identified. Image was taken at 10x magnification.

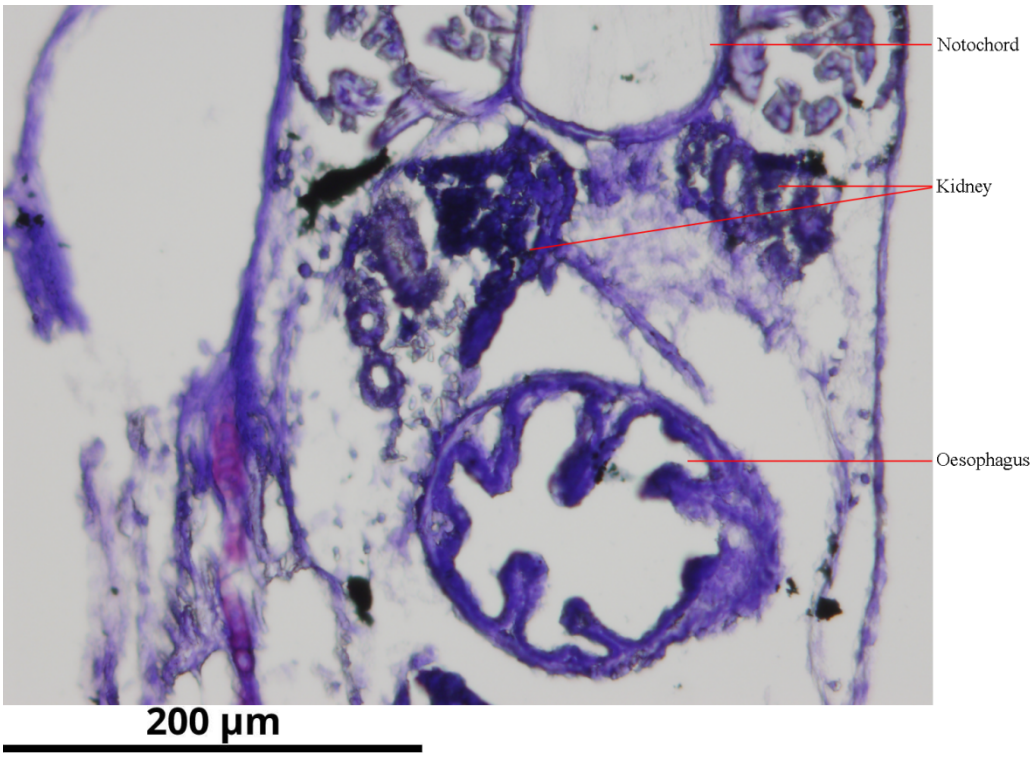


Figure 61: Photomicrograph of larvae at 9 dph. A 6 µm thick section was taken using the wax microtome mid trunk and stained with toluidine blue. Notochord, kidney, liver and oesophagus were identified. Image was taken at 20x magnification.

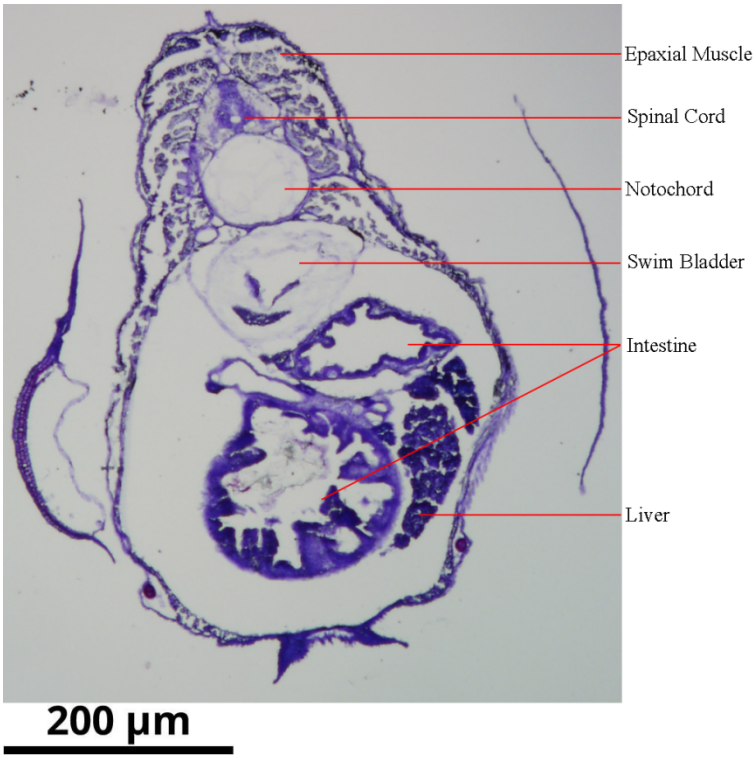


Figure 62: Photomicrograph of larvae at 15 dph. A 6 µm thick section was taken using the wax microtome mid trunk and stained with toluidine blue. Notochord, liver and intestine were identified. Image was taken at 10x magnification.

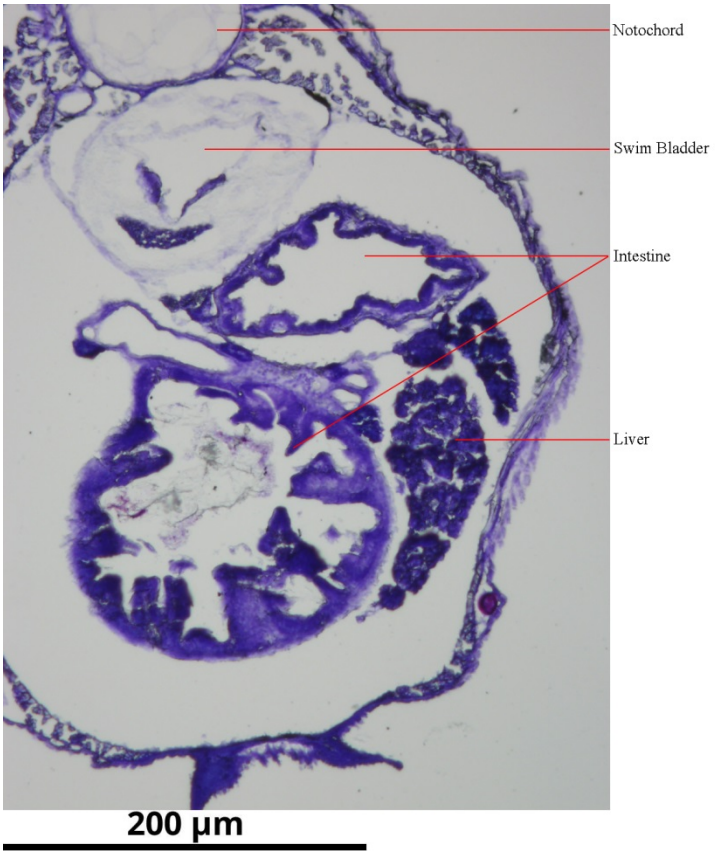


Figure 63: Photomicrograph of larvae at 15 dph. A 6 µm thick section was taken using the wax microtome mid trunk and stained with toluidine blue. Notochord, liver and intestine were identified. Image was taken at 20x magnification.

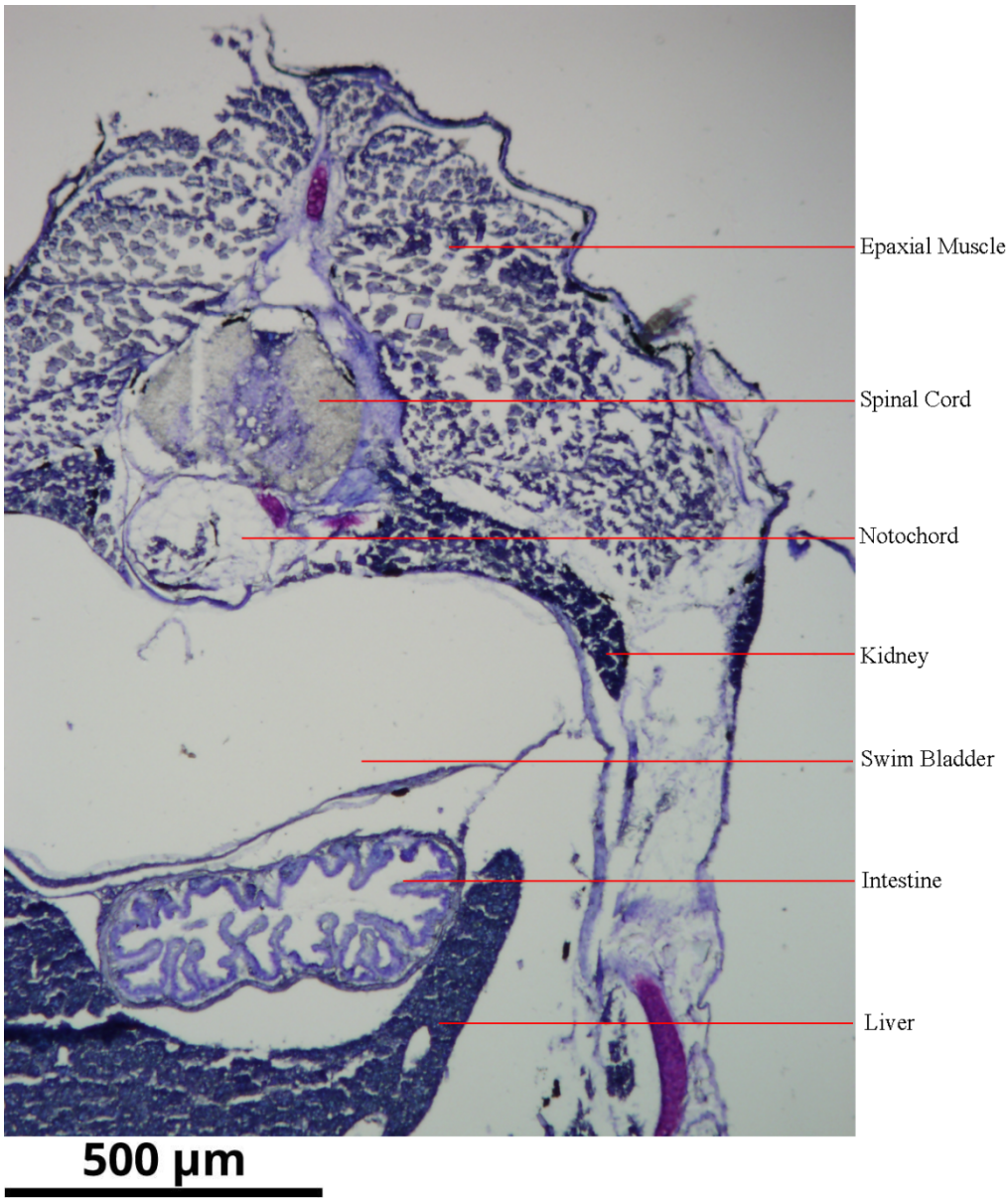


Figure 64: Photomicrograph of larvae at 25 dph. A 6 µm thick section was taken using the wax microtome mid trunk and stained with toluidine blue. Notochord, kidney, liver and intestine were identified. Image was taken at 5x magnification.

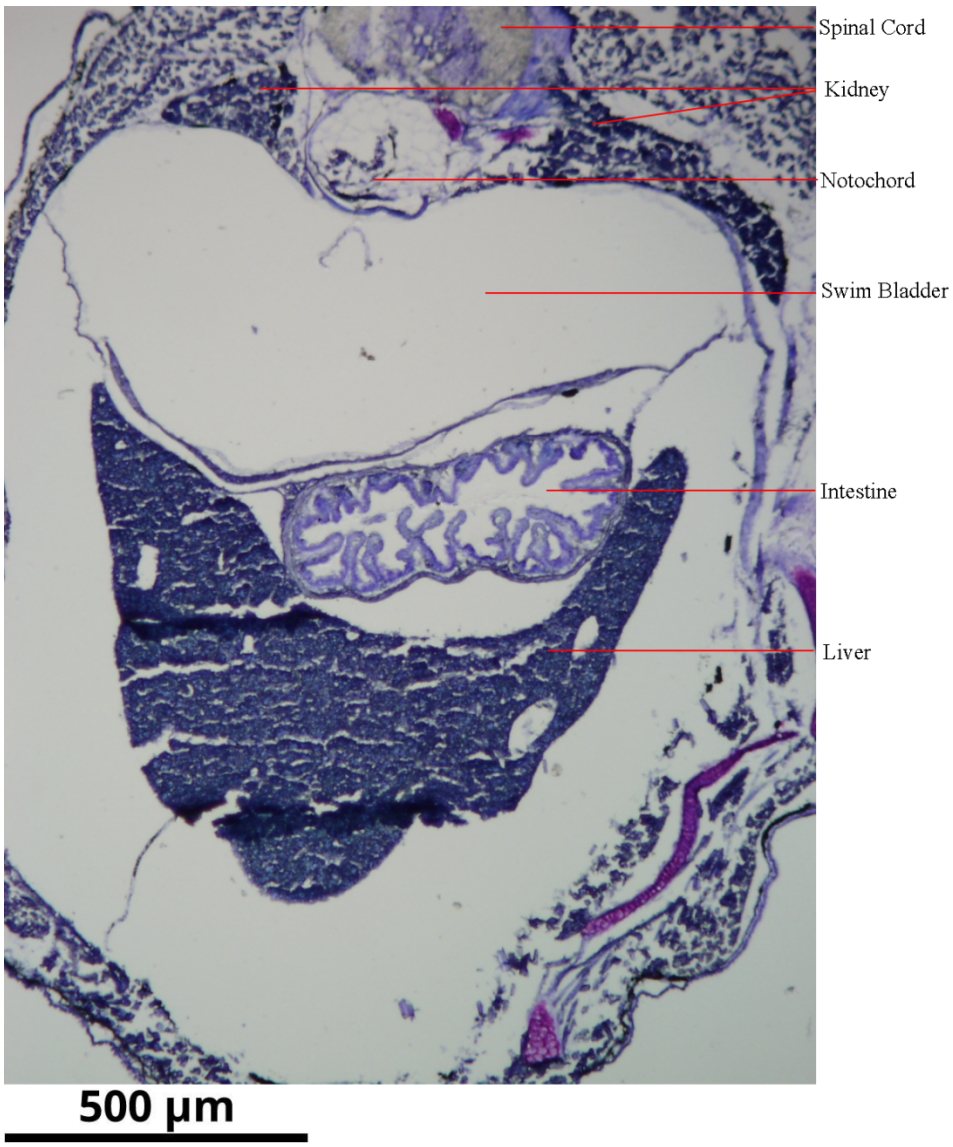


Figure 65: Photomicrograph of larvae at 25 dph. A 6 µm thick section was taken using the wax microtome mid trunk and stained with toluidine blue. Notochord, kidney, liver and intestine were identified. Image was taken at 5x magnification.

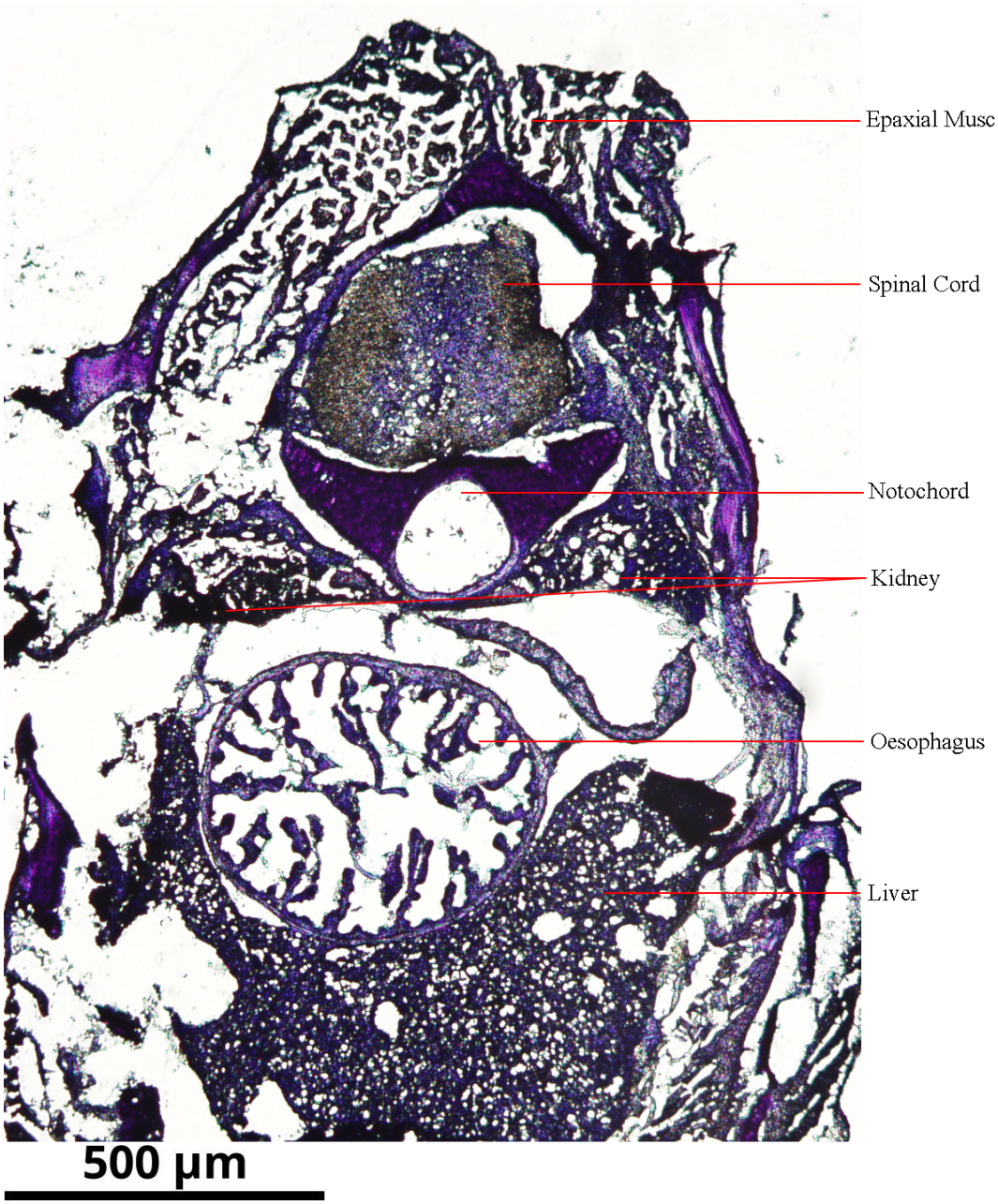


Figure 66: Photomicrograph of larvae at 25 dph. A 6 µm thick section was taken using the cryostat microtome mid trunk and stained with toluidine blue. Notochord, kidney, liver and oesophagus were identified. Image was taken at 5x magnification.

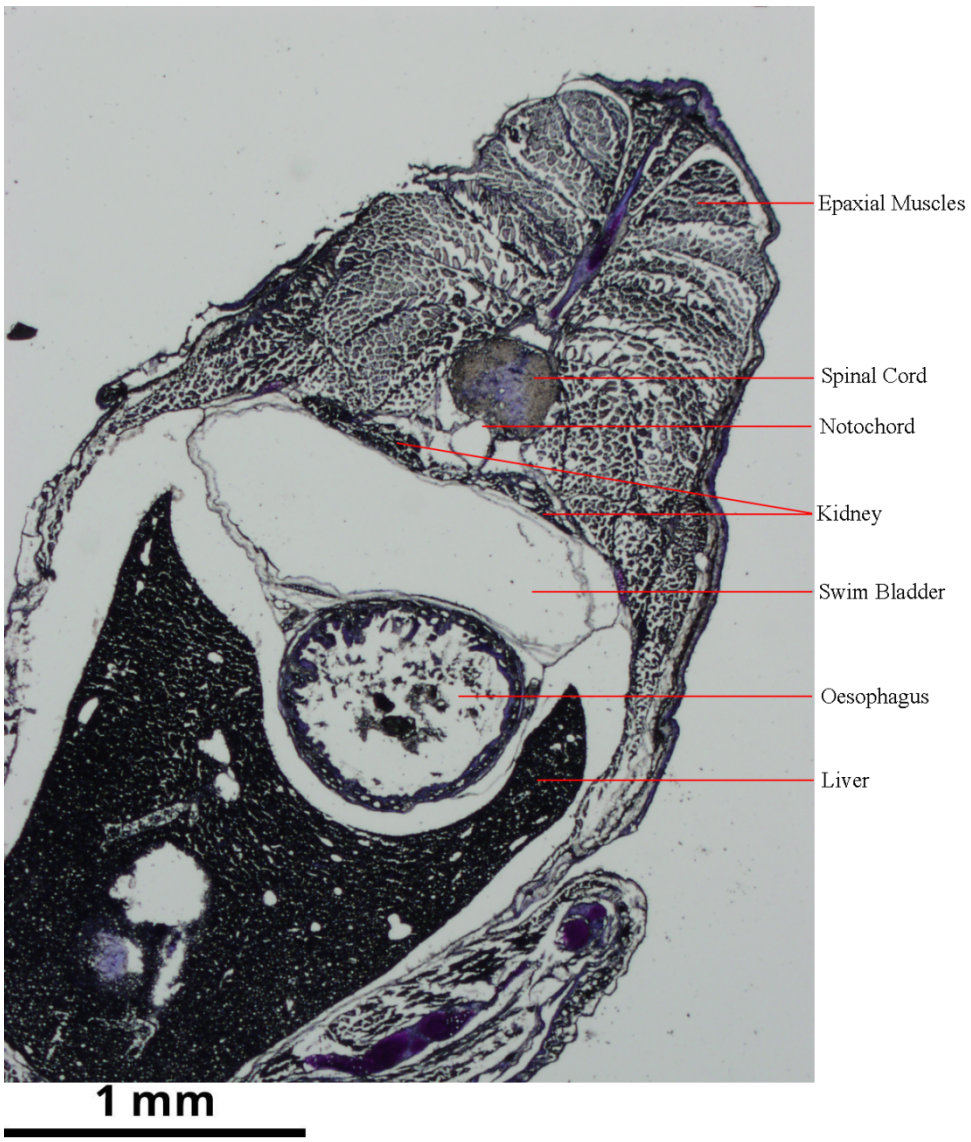


Figure 67: Photomicrograph of larvae at 30 dph. A 6 µm thick section was taken using the wax microtome mid trunk and stained with toluidine blue. Notochord, kidney, liver and oesophagus were identified. Image was taken at 2.5x magnification.

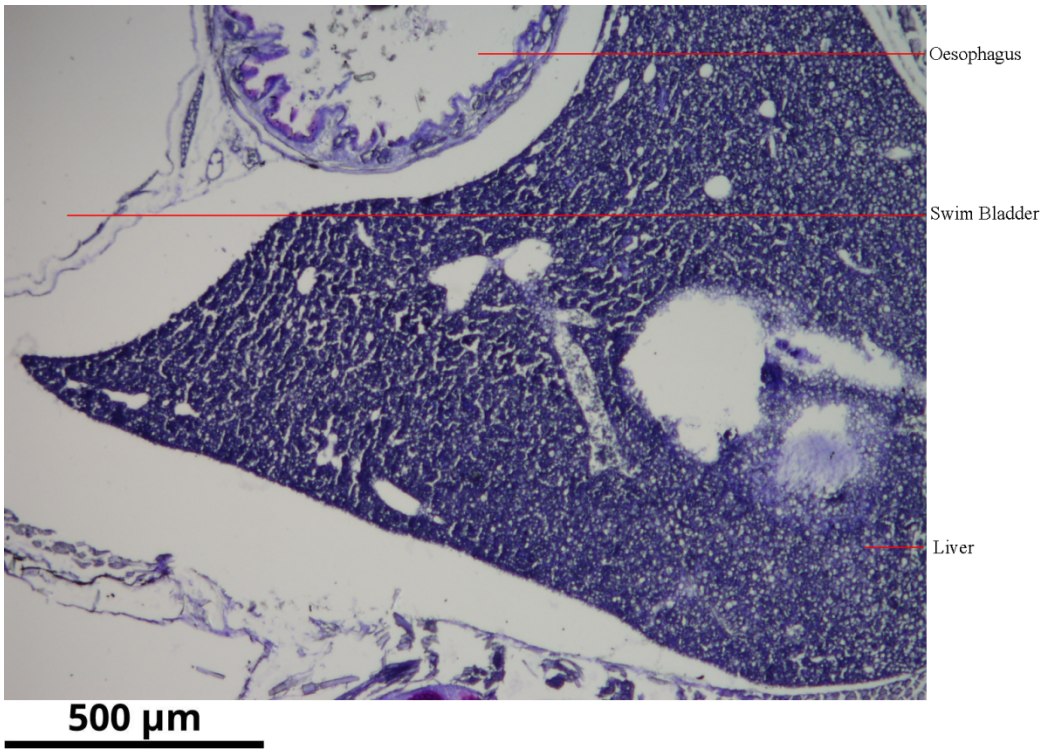


Figure 68: Photomicrograph of larvae at 30 dph. A 6 µm thick section was taken using the wax microtome mid trunk and stained with toluidine blue. Liver and oesophagus were identified. Image was taken at 5x magnification.

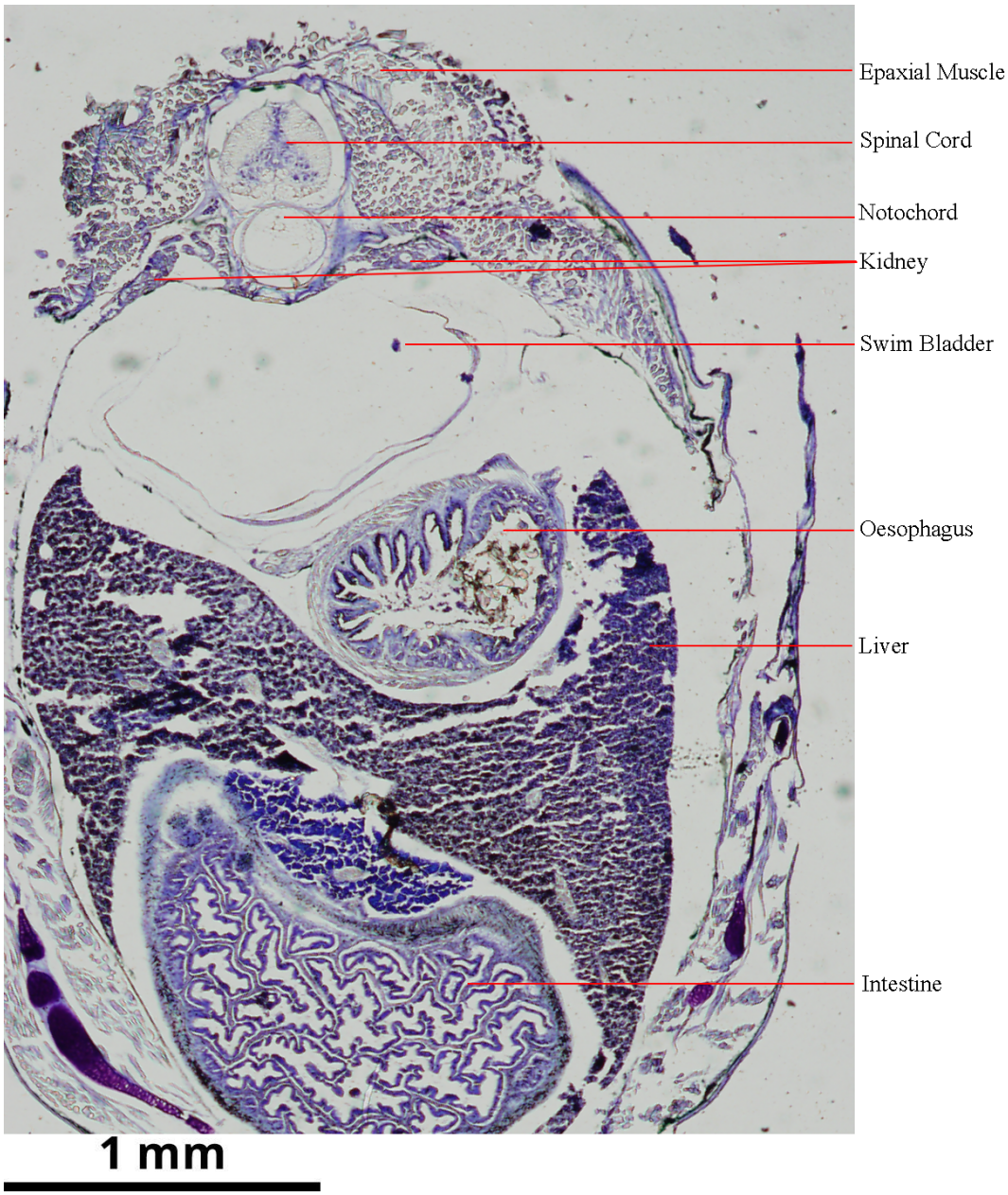


Figure 69: Photomicrograph of larvae at 35 dph. A 6 µm thick section was taken using the wax microtome mid trunk and stained with toluidine blue. Notochord, kidney, liver and oesophagus were identified. Image was taken at 2.5x magnification.

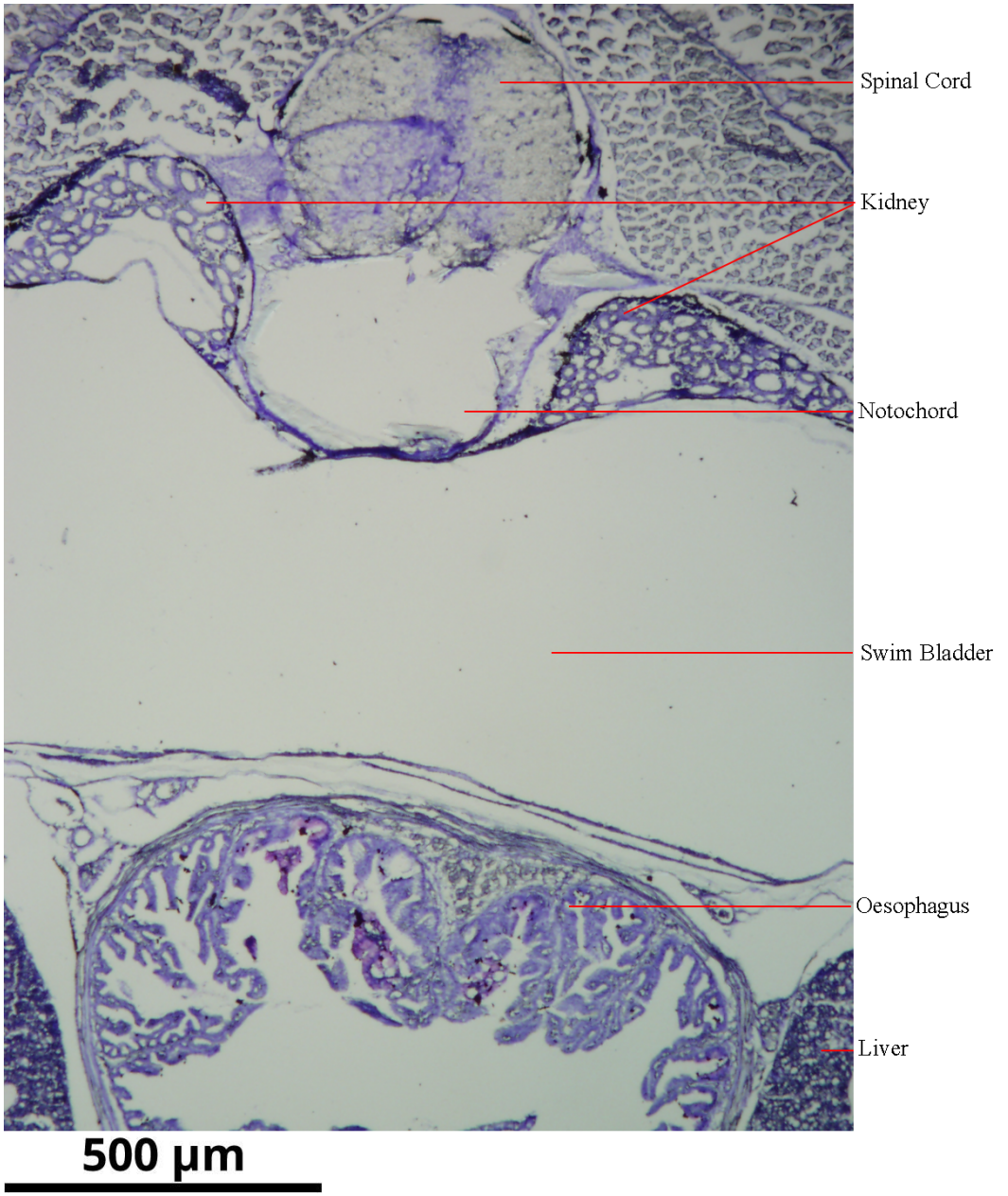


Figure 70: Photomicrograph of larvae at 40 dph. A 6 µm thick section was taken using the wax microtome mid trunk and stained with toluidine blue. Notochord, kidney, liver and oesophagus were identified. Image was taken at 5x magnification.

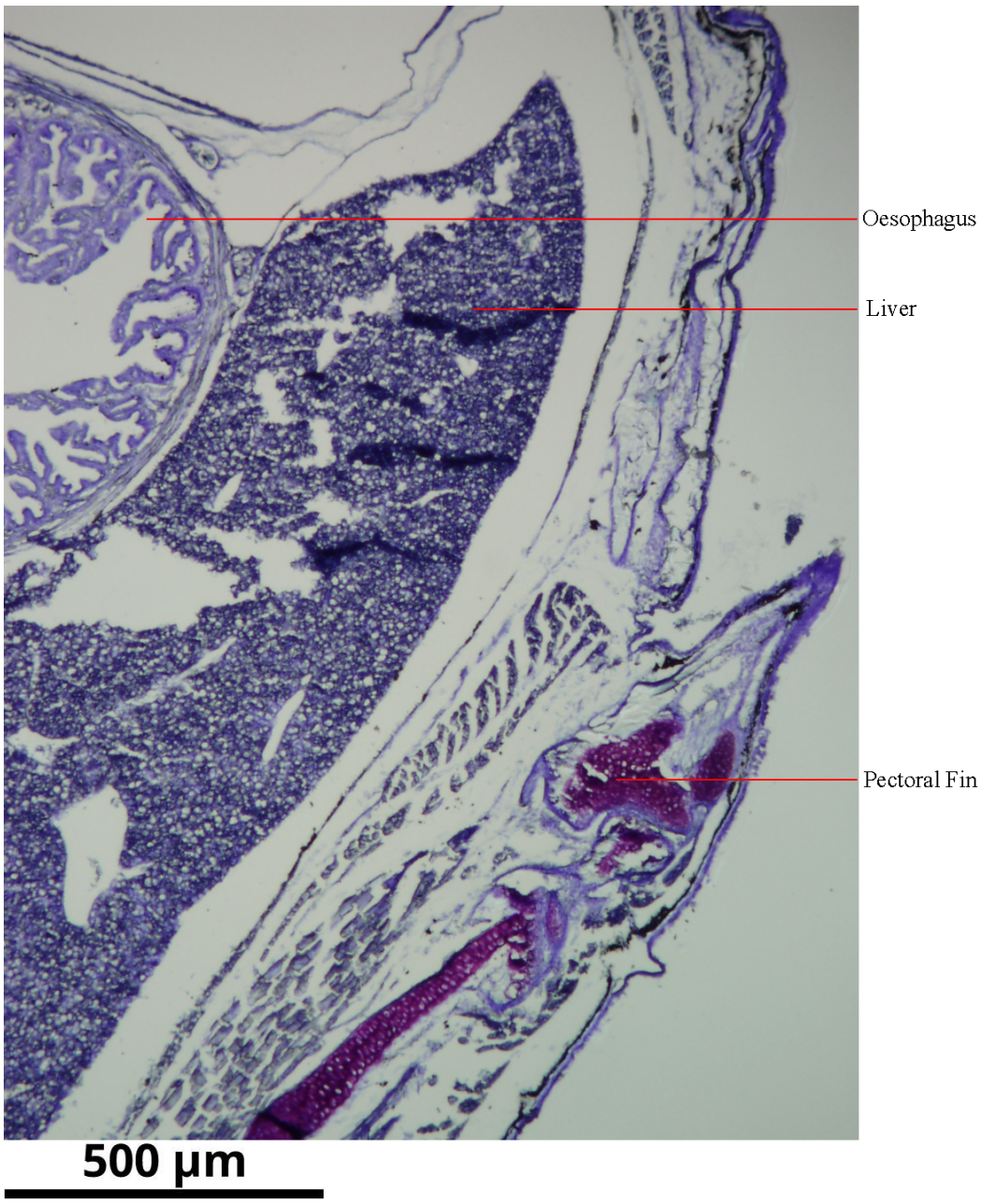


Figure 71: Photomicrograph of larvae at 40 dph. A 6 µm thick section was taken using the wax microtome mid trunk and stained with toluidine blue. Liver and oesophagus were identified. Image was taken at 5x magnification.

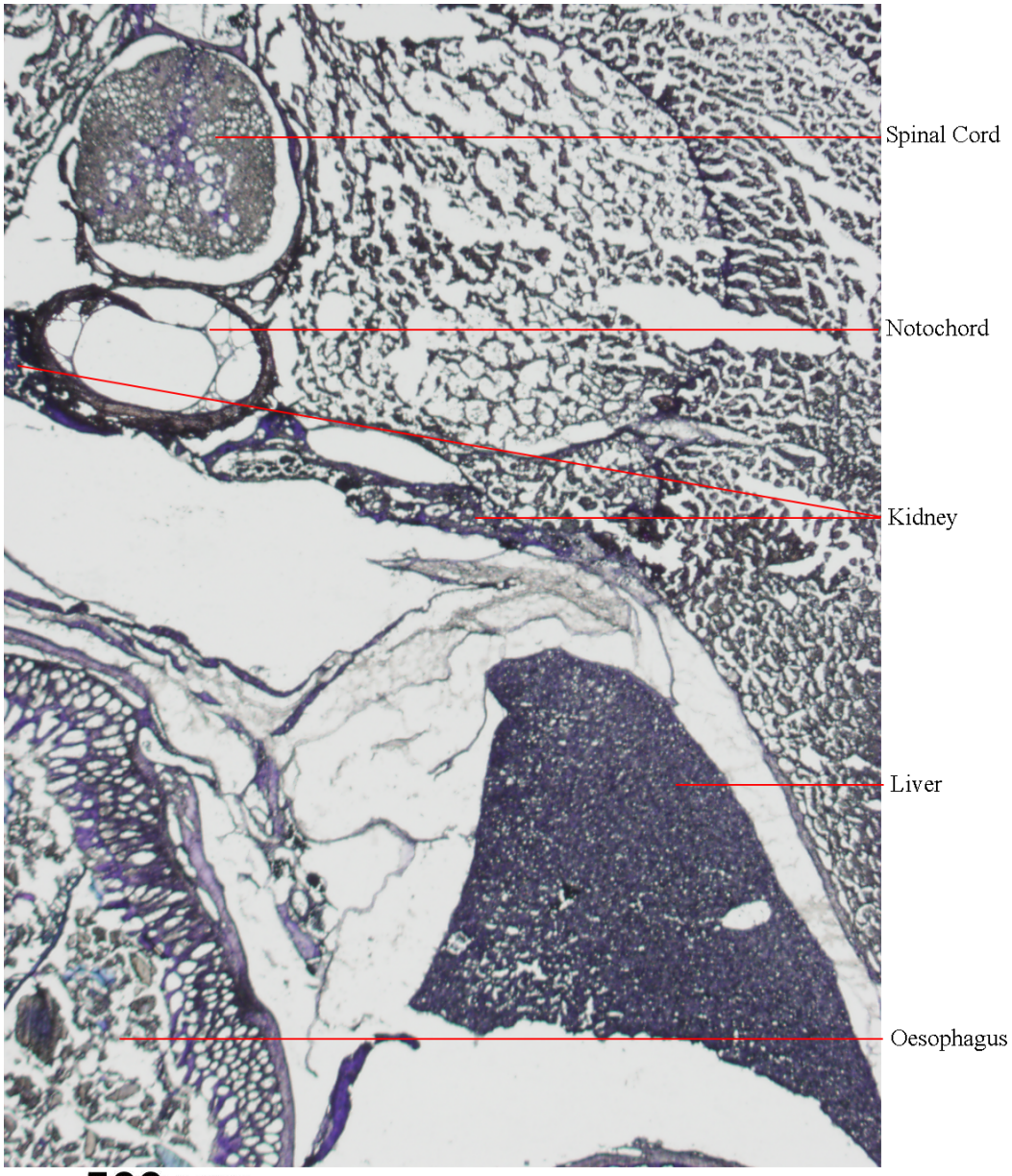


Figure 72: Photomicrograph of larvae at 60 dph. A 6 µm thick section was taken using the cryostat microtome mid trunk and stained with toluidine blue. Notochord, kidney, liver and oesophagus were identified. Image was taken at 5x magnification.

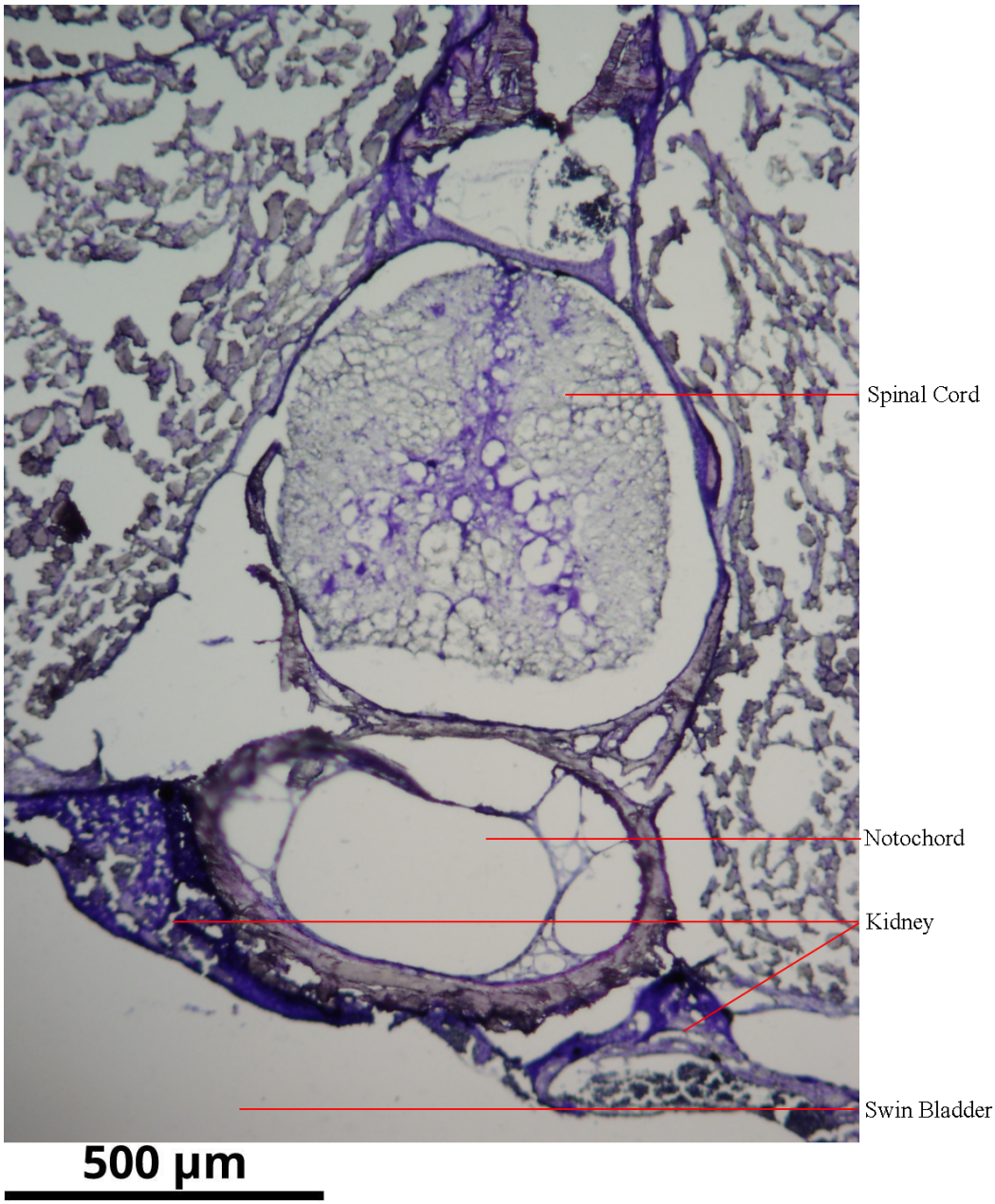


Figure 73: Photomicrograph of larvae at 60 dph. A 6 µm thick section was taken using the wax microtome mid trunk and stained with toluidine blue. Notochord and kidney were identified. Image was taken at 5x magnification.

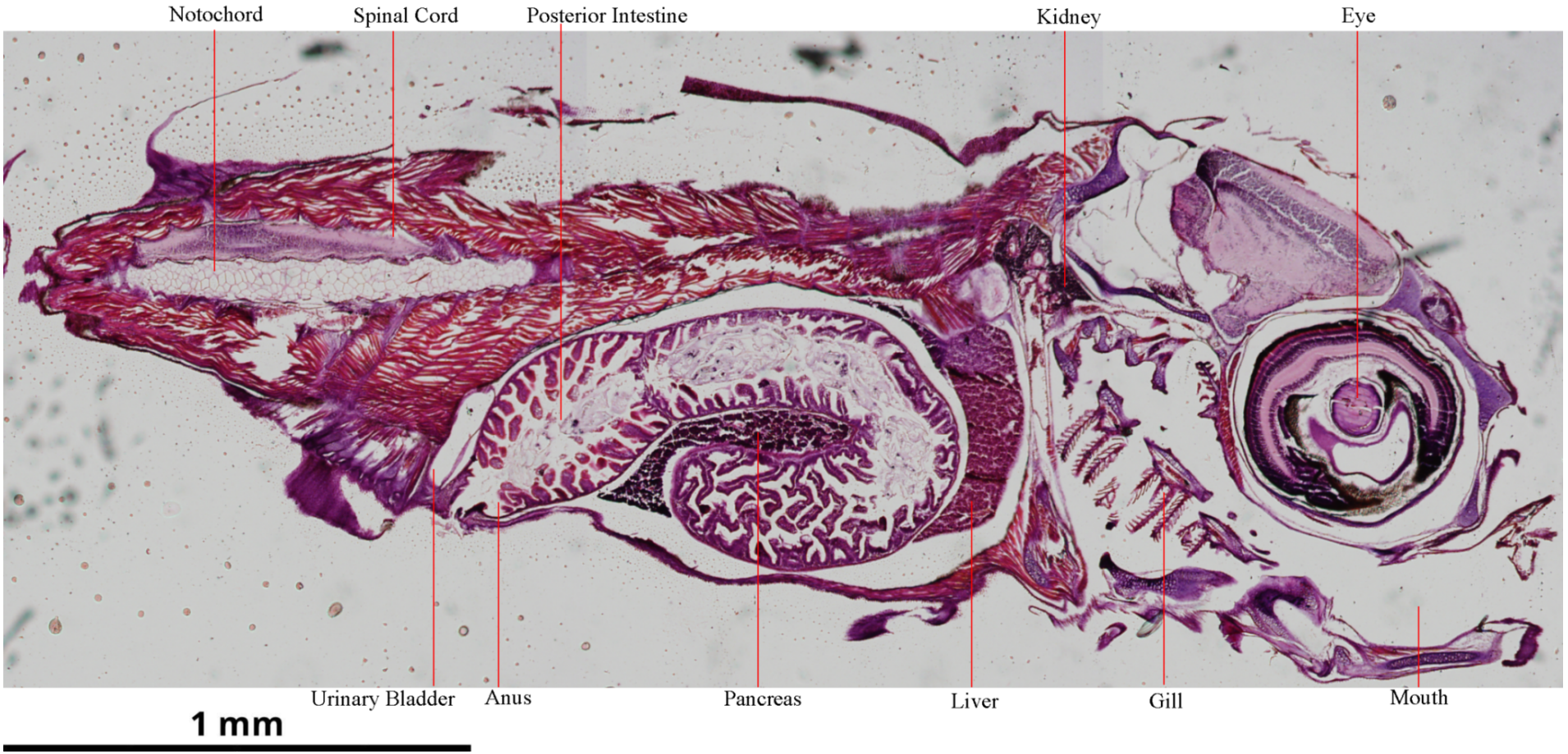


Figure 74: Composite photomicrograph of a larva at 21 dph. Three 6 µm thick sagittal sections were taken of the larvae using the wax microtome and stained with Haematoxylin and Eosin. This image is made up of three overlapping sections.

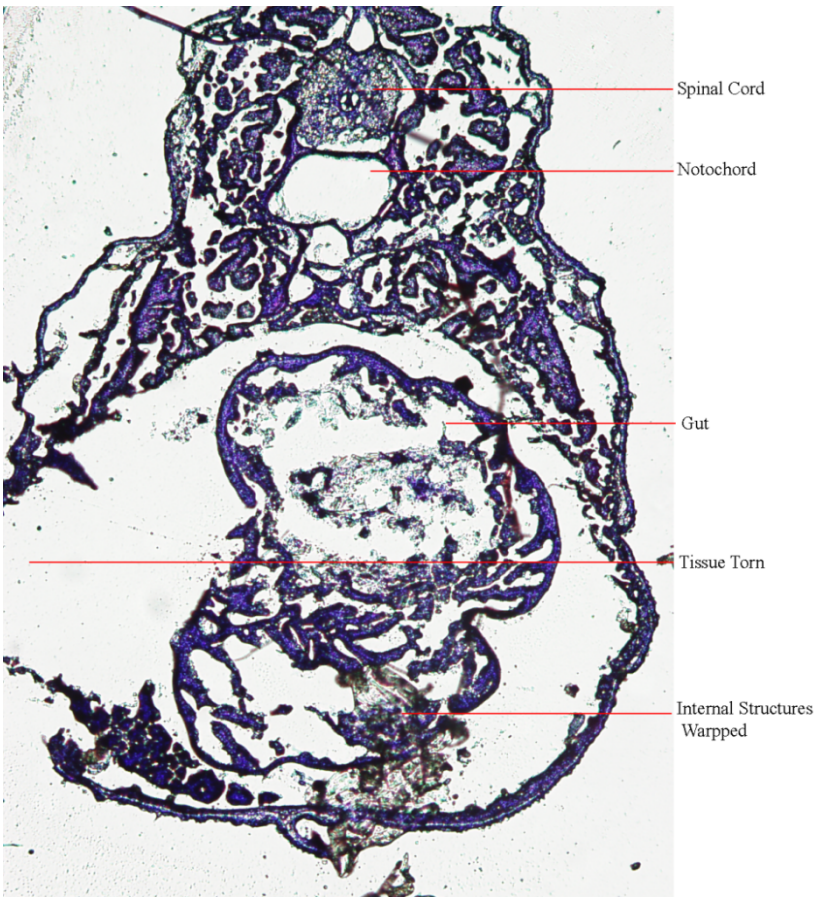


Figure 75: Poor quality section from cryostat. The internal organs and tissues have not retained their structural integrity and the outer tissue has been breached. This was common when sectioning with the cryostat.

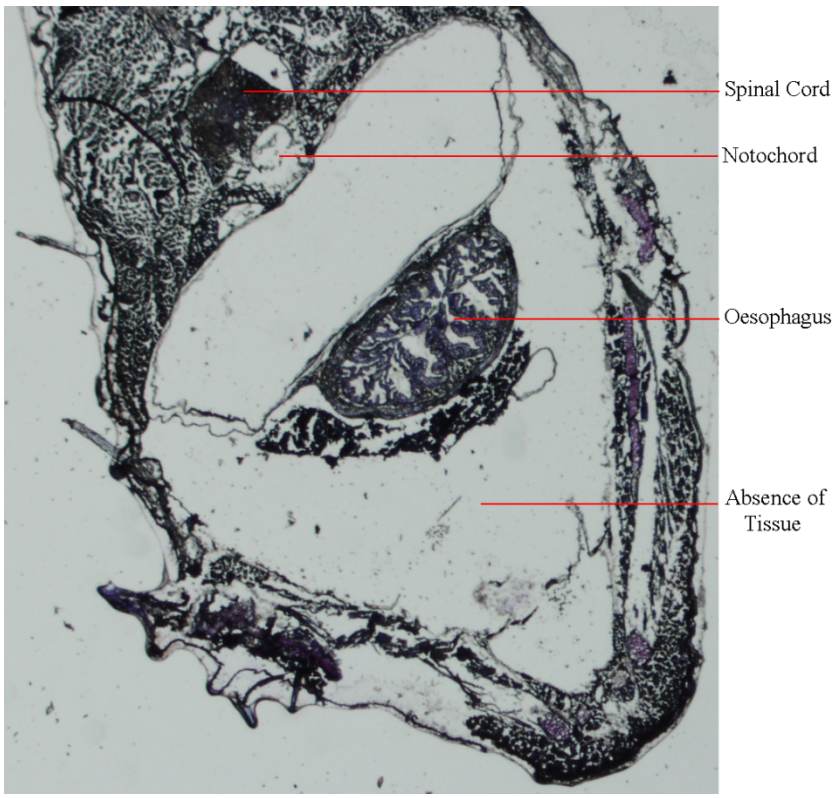


Figure 76: Poor quality section from wax microtome. Tissue was warped and most internal structures in the mid-section were missing with only the oesophagus remaining.

Chapter Four: Discussion

4. Discussion

4.1 Purpose and rational of the experiment and methods

Currently, very little is known about the immune system of the yellowtail kingfish (*Seriola lalandi*) and there has been a limited amount of work to investigate its genetic characteristics. The aim of this investigation was to identify and sequence important immune genes in *S. lalandi* and characterise their expression profiles during the development of juvenile fish. Monitoring fish health is important in an aquacultural setting and understanding the expression profile of selected immune genes provides a method for this. It also allows us to predict when components of innate and adaptive immunity become present and if therapeutic intervention is required. Initially, using bioinformatics approaches, a transcriptomic library that had been developed from spleen tissue of a seemingly healthy wild adult *S. lalandi* greater than 75 cm was searched to identify a range of immune genes. Various molecular techniques were used to confirm the predicted sequences, which were then used in quantitative PCR to examine their expression. In an attempt to use *in situ* hybridization, histological work was undertaken, in order to look closely at where gene expression was occurring in the tissues of the developing juvenile fish. Developing this extra capability would provide us with a better understanding of the role of these genes during development. However, due to technical problems with sectioning of larvae, and the time taken to optimise the embedding in paraffin, there was only time to obtain sections for histological staining. This was still beneficial as it provided a strong protocol for future preparation of larvae for *in situ* work. The work carried out in this thesis started to build a better understanding of the immune system of *S. lalandi* and has laid a foundation for further work with this fish.

4.2 Gene Selection and Justification

Initially, six genes, that included markers for innate immunity, were selected as candidates; Interleukin-1 beta (IL-1β) and Toll-like receptor 22 (TLR22) and adaptive immunity; Recombination-activating gene (RAG)-1, RAG-2, Immunoglobulin D (IgD) and IgT/Z. The IL-1β gene was selected because of its centrality in the inflammatory response of the innate immune system (Weber,

Wasiliew, & Kracht, 2010). TLR22 was selected because it is found in teleost species but not birds or mammals, and its role in pattern recognition remains unknown (Matsuo et al., 2008). RAG1 and RAG2 were selected as indicators of adaptive immune system capability. This is due to their integral role in producing the diversity of antigen recognition possessed by immunoglobulins and T cell receptors (Market & Papavasiliou, 2003). IgD is of interest as it is present in species from cartilaginous fish to mammals, suggesting that IgD has important immunological functions. However, little about its function is known, even in humans (Geisberger, Lamers, & Achatz, 2006). Lastly, IgT/Z was chosen as, compared to IgM and IgD, it is a newly discovered antibody and unique to bony fish (Y. A. Zhang, Salinas, & Oriol Sunyer, 2011). Each of these candidates was looked for within the kingfish transcriptome and three were selected for sequence confirmation.

4.3 Transcriptomic Database Mining and Bioinformatics

While the *S. lalandi* genome has not yet been sequenced, a transcriptomic library has been produced which was used to search for immune genes. To date, a number of teleosts have had their genome sequenced. This includes farmed species, such as Atlantic cod (Star et al., 2011), Atlantic salmon (Davidson et al., 2010) and European sea bass (Tine et al., 2014) and model species, such as zebrafish (*Danio rerio*) (Howe et al., 2013) and medaka (*Oryzias latipes*) (Kasahara et al., 2007). The availability of these sequences has significantly helped in gene identification, compared to the methods that had previously been available, such as Expressed Sequence Tags (ESTs) (Nagaraj, Gasser, & Ranganathan, 2007). While a complete genome database is useful, producing a transcriptome library provides an alternative method that is currently faster and cheaper than sequencing a genome. A transcriptome library is also able to be used as an extensive database of expressed genes. Transcriptomic libraries can be produced at specific points in an organism's development and from selected tissues, depending on the focus of investigation or genes of interest. Previous work has looked at brain, muscle, liver and kidney tissue in the hypoxia-tolerant crucian carp (*Carassius auratus*) (Liao et al., 2013), and understanding the effect of functional feeds in the white muscle and liver of Atlantic salmon (Tacchi, Bickerdike, Douglas, Secombes, & Martin, 2011). Transcriptomics is also beginning to play a role in the study of immune genes in fish. For example, an

early transcriptomic approach was used in an attempt to understand the expression of key immune genes in the Japanese amberjack, including IL-1β and IgM (*Seriola quinqueradiata*) (Darawiroj et al., 2008). However, with new technologies available, such as RNA-Seq, a lot more can now be achieved in a relatively short time frame (Z. Wang et al., 2009).

The transcriptomic library used in this research was derived from the spleen tissue of a wild *S. lalandi*. This tissue was selected because of the immunological properties the spleen possesses, particularly the number of immune related cells it contains and the expression of immune gene transcripts (Gradil, Wright, Wadowska, & Fast, 2014). Amino acid sequences of immune genes already characterised in other teleost species were first used to perform a tblastn search within the transcriptomic library. This approach used a protein sequence to search the transcriptome library, which is translated into all six reading frames (Gertz, Yu, Agarwala, Schaffer, & Altschul, 2006). Where available, the searches were further restricted to genes from fish in the order Perciformes, to which *S. lalandi* belongs. Using sequences from closely related fish allowed the search to be more targeted, increasing the likelihood that hits from the transcriptome were the gene of interest. Fragments of gene sequence obtained from each search then underwent *de novo* assembly, to build a consensus sequence, which was a preliminary sequence of the candidate gene in *S. lalandi*.

Searches within the transcriptomic library for the candidate genes IL-1β, TLR22, IgD and IgT/Z produced preliminary sequences. Primers were designed towards IL-1β and IgD, in an attempt to confirm the preliminary sequence. This is good practice, as errors get introduced into the sequencing of transcriptomes so it is important to validate each targeted sequence individually (Bragg, Stone, Butler, Hugenholtz, & Tyson, 2013). TLR22 and IgT/Z were not worked on during this project due to time constraints which will be discussed later. Also targeted was the recombination activating gene RAG-1 and RAG-2, which did not produce any results when the spleen transcriptome was searched. Instead, while obtaining fish protein sequences to search with, a RAG-1 sequence from the Greater Amberjack (*Seriola dumerili*) was found within the NCBI database (Accession no. JQ938283.1). *S. dumerili* is found in the Mediterranean Sea, the Atlantic Ocean, the Pacific Ocean and the Indian coasts and belongs to the same genus as *S.*

lalandi. This close relationship made it the perfect target for designing primers for isolation of the RAG-1 sequence from *S. lalandi*.

The inability to find RAG-1 within the spleen transcriptome is not a complete surprise as RAG expression is uncommon in the spleen of mammals (Hillion, Saraux, Youinou, & Jamin, 2005) and fish, such the common carp (*Cyprinus carpio*) (Huttenhuis et al., 2005) and zebrafish (*Danio rerio*) (Danilova & Steiner, 2002). However, conflicting evidence does exist in an early study of rainbow trout (Hansen, 1997), where RAG-1 was found expressed in the spleen tissue. Similar to mammals, in fish the spleen is a secondary lymphoid organ and the lack of RAG expression is possibly due to the absence of lymphopoiesis. In bony fish lymphopoiesis is mostly attributed to the kidney and thymus (Austbo et al., 2014; Boehm, Hess, et al., 2012). It is also understood that the expression of RAG-1 is terminated once a lymphocyte becomes mature (Nagaoka, Yu, & Nussenzweig, 2000). However, differences between different species of fish, their health or their age could also be contributing factors, which will require further studies to confirm. The age and overall health of the fish in this study was unknown; however it was relatively mature being above the size restriction of 75 cm and had no noticeable signs of infection.

Sequences obtained from the transcriptome were used as a scaffold to design primers to amplify RAG-1, IL-1β, and IgD from *S. lalandi* tissues for sequencing. Primers were designed within the areas of the consensus sequence that had the highest quality, i.e. those that had a large number of overlapping sequences. Designing primers in these areas reduced the chance of mismatching and preventing amplification of the product for sequencing. For the RAG-1 gene, primers were designed once the *S. dumerili* DNA sequence was aligned with RAG-1 DNA sequences of other perciformes. Within fish, RAG-1 is highly conserved (X. Wang, Tan, Zhang, Zhang, & Xu, 2014) and regions that demonstrated high conservation was chosen to help design the most specific primers possible. Typical primer design recommends oligonucleotide sequences with the following specifications: nucleotide length of 18 to 30 bases, a GC content between 40% and 60%, and a Tm between 50°C and 65°C (X. Yang, Scheffler, & Weston, 2006). The design of primers in this work had similar specifications, containing a minimum of 20 bases in length, a 50-60% GC content and a Tm between 50°C and 62°C. A GC clamp with 1-2 G or C's at the 3' end of

the primer was also incorporated where possible, as this helped the binding of the primer to the template at the 3' end, greatly increasing the likelihood of amplification (Dieffenbach, Lowe, & Dveksler, 1993). However, primers could not always be designed with all of these specifications, due to low amounts of sequence available, or low sequence quality that may occur when generating the transcriptome.

4.4 Confirmation of Gene Sequences

Following bioinformatics and primer design, RNA was extracted from *S. lalandi* tissue that was known to be important to the fish's immune system. The tissue included the spleen, liver, kidney and gills. In bony fishes, the spleen is a key secondary lymphoid organ whereas the kidney takes on a lymphopoietic role (Boehm, Hess, et al., 2012). Liver and gill are thought to have a role in immunity as they have been characterised as possessing antimicrobial peptides. All candidate tissue has also been found to express IL-1 receptors (Uribe et al., 2011). Two different methods were used for both the RNA extraction and cDNA synthesis, due to their availability in the lab during this investigation. The two RNA extraction methods, the TRIzol® and R&A-BLUE protocols (see section 2.3), follow similar steps. Both performed well, with no significant differences seen between results, with regards to RNA yield and purity. The two cDNA synthesis methods, the qScript™ Flex cDNA synthesis kit and the Tetro cDNA synthesis kit (see section 2.4), did vary in their protocols, but both also performed well with no significant differences in cDNA yield and quality detected. However, the Tetro kit did have an advantage of a faster synthesis program of 35 minutes on the thermocycler, compared to 110 minutes required by the qScript kit. To test cDNA quantity and quality, a PCR was run using primers designed to a housekeeping gene, β-actin. A housekeeping gene was used as a control as these genes are expressed constitutively in all tissues (Barber, Harmer, Coleman, & Clark, 2005). The presence of a band when run on a gel can therefore show the PCR has succeeded independent of any other bands. Primers specific to *S. lalandi* β-actin were designed and used for the PCR. Amplification of a strong 476 bp band indicated successful synthesis of cDNA of sufficient quantity and quality for subsequent PCR's. This was run each time new cDNA was synthesised.

4.4.1 Immunoglobulin Delta

A number of IgD primers were designed, with the final count of six forward primers and three reverse primers. Initial attempts at amplifying IgD was carried out using IgD-F1, F2, F3, and F4, with either IgD-R1 or IgD-R2. The largest product using F1R1 was predicted to be ~2,303bp in length and the smallest product using F4R2 was predicted to be ~1,268bp in length. None of these primer combinations produced a band close to the predicted size, despite the application of changes to a variety of PCR conditions. This failure may be due to possible discrepancies found between the transcriptome-derived sequence and the *S. lalandi* IgD sequence (as discussed earlier) or the difficulty in amplifying a large PCR product. The difficulty in amplifying large sequences can be caused by the formation of secondary structures in the template or the non-specific annealing of primers (Ekman, 1999). In response to the failure of this amplification, an additional two forward (IgD-F5 and -F6) and one reverse (IgD-R3) primer were designed that produced smaller products of ~800bp (F5R3) and ~781bp. Both these primer combinations were successful in the amplification of IgD and once the DNA was sequenced and analysed it was confirmed to be *S. lalandi* IgD. A 3' RACE PCR was then performed using 3RACE IgD F1 on spleen, kidney and liver tissue. A second round RACE PCR using 3RACE IgD F2 resulted in a single band in liver tissue. This was sequenced and determined to be sequence for the 3' end of the *S. lalandi* IgD sequence.

IgD is co-expressed on B cells with IgM, and while investigations into the exact function of IgD remains inconclusive, it is expressed in all jawed vertebrates, except birds (Sun, Wei, Hammarstrom, & Zhao, 2011). Mammals have five isotypes of immunoglobulins and IgD is least understood of them all (Rogers, Richardson, Scinicariello, & Attanasio, 2006). While the exact function of IgD is unknown, it is thought to have a similar ability to IgM with its capacity to initiate an immune response as a B-cell receptor (BCR). It was found that B cells in IgM knockout mice were still able to produce an immune response and, similarly, an IgD knockout gave the same result (Edholm et al., 2011). Like all immunoglobulins, IgD has the signature secondary structure called the immunoglobulin fold, which facilitates the interaction between proteins, particularly in terms of cell to cell contact (Geisberger et al., 2006).

In teleosts, IgD is one of three immunoglobulin isotypes with IgM and IgT/Z and has been characterised in a number of teleost species which includes Japanese flounder (*Paralichthys olivaceus*) (Hirono, Nam, Enomoto, Uchino, & Aoki, 2003), zebrafish (*Danio rerio*) (Zimmerman, Moustafa, Romanowski, & Steiner, 2011), Siberian sturgeon (*Acipenser baerii*) (L. Zhu et al., 2014), Atlantic salmon (*Salmo salar*) and Atlantic halibut (*Hippoglossus hippoglossus*) (Hordvik, 2002). IgD is the most structurally variable immunoglobulin when compared between different species with longer heavy chains found in ectothermic vertebrates as a results of exon duplications or IgD gene duplication (Edholm et al., 2011; Tadiso, Lie, & Hordvik, 2011). In human (*Homo sapiens*), bovine (*Bos* Taurus), pig, (*Sus* scrofa) and sheep (*Ovis* aries) IgD is known to have three Cδ exons compared to rat (*Rattus norvegicus*) and mouse (*Mus musculus*) which have two Cδ exons (Solem & Stenvik, 2006). Teleosts have been found to have much longer IgD heavy chains with species such as Fugu (*Takifugu rubripes*), Atlantic halibut (*Hippoglossus hippoglossus*) and Japanese flounder (*Paralichthys olivaceus*) producing IgD heavy chains with seven Cδ exons (Solem & Stenvik, 2006). The Cδ exons are typically found downstream of the Cμ exons and, in mammals, the IgD heavy chain is produced by co-transcription of these exons with the Cμ exon later spliced out (Sun et al., 2011). In teleost species, IgD is known to have the Cμ fused to the N-terminal region of the constant region (Rogers et al., 2006). Using the IgD sequence from Japanese flounder (Srisapoome, Ohira, Hirono, & Aoki, 2004) and Channel Catfish (*Ictalurus punctatus*) (Wilson et al., 1997) conserved domains of the IgD molecule were identified. This included the Cμ domain, the Cδ regions, the C-terminal transmembrane domains, δTM1 and δTM2, and the conserved antigen receptor transmembrane (CART) motif. From the alignment, within the confirmed sequence of the *S. lalandi* IgD, good homology was seen in the Cδ5, Cδ6 and Cδ7 regions, as well as the C-terminal transmembrane domains, with good conservation of the CART domain. In addition, within the unconfirmed IgD sequence some conservation could also be found in the Cμ, Cδ1, Cδ2, Cδ3 and Cδ4 regions, however this region remains to be isolated using 5'RACE.

4.4.2 Recombination Activating Gene 1

A number of RAG1 primers were designed with a final count of two forward primers and two reverse primers. Initial attempts at amplifying RAG1 was carried out using RAG1-F1 and RAG1-F2, with either RAG-R1 or RAG-R2. The largest product using F1R1 was predicted to be 1,097 bp and the smallest product using F2R2 was 953 bp. In contrast to what was found from the transcriptome, the amplification of RAG1 was obtained from spleen tissue. A gel analysis of the PCR showed bands in each lane, indicating a successful amplification. A second round of PCR reduced the number of bands and a PCR product was successfully sequenced and confirmed to be *S. lalandi* RAG1. It is not clear as to why expression of splenic RAG1 could not be found in the transcriptome. There are some limitations in using a transcriptomic approach, secondary structure of the template can inhibit sequencing or a 5' end bias can be introduced by insufficient RNA fragmentation (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008), which could have led to the inability of RAG1 to be found within the spleen library. Also, in mammals it has been shown that a very small population of immature B cells can exist in the spleen that can express RAG1 (Nagaoka et al., 2000). However, the expression would be low and may have been lost using the transcriptome approach, whereas two rounds of PCR using spleen tissue and primers specific for RAG1 is a much more targeted approach, helping to amplify genes that are expressed at low levels. It is clear that further investigation is required to understand the dynamics of RAG1 expression in the tissues of *S. lalandi*.

The variability of immunoglobulins and T cell receptors depends on the recombination activating genes, RAG1 and RAG2. These genes code for proteins that bind to the recombination signal sequence (RSS) regions of the V, D, and J gene segments and introduce double strand breaks in the DNA (Ji et al., 2010). The function of these genes has been demonstrated with knockout models of various species. This includes rats (Menoret et al., 2013), zebrafish (Petrie-Hanson, Hohn, & Hanson, 2009) and mice (Mombaerts et al., 1992). These knockout experiments of RAG1 and RAG2 were mostly characterised by largely reduced numbers of B and T cells, and the inability to mount an effective immune response. The focus of this work was on the RAG1 gene, excluding RAG2 due to failure in finding a sequence in the transcriptome, as previously discussed.

The RAG1 gene has been identified in a number of teleost species, including haddock (Corripio-Miyar, Bird, Treasurer, & Secombes, 2007), flounder (X. Wang et al., 2014), common carp (Huttenhuis et al., 2005), red snapper (X. L. Zhang, Lu, Jian, & Wu, 2012) and red spotted grouper (Mao, Lei, Alex, Hong, & Wang, 2012). These studies have shown, good conservation of characteristic domains throughout vertebrates, which are important for the activity of the RAG1 protein. The two major regions of RAG1 are the core RAG1 region and the zinc-binding dimerization domain (ZDD) (De & Rodgers, 2004). Within the ZDD there are two distinct domains, the really interesting new gene (RING) finger and the zinc finger A (ZFA) region (De & Rodgers, 2004). The RING finger is defined by the following motif C-X_2-C-$X_{(9-39)}$-C-$X_{(1-3)}$-H-$X_{(2-3)}$-C-X_2-C-$X_{(4-48)}$-C-X_2-C (also represented as C_3HC_4) where C, H and X represents cysteine, histidine and any other amino acid, respectively (Borden & Freemont, 1996). The zinc-binding finger is defined by the following motifs C-$X_{(2-4)}$-C and H-$X_{(3-5)}$-H (also represented as C_2H_2) where C and H represents cysteine and histidine, respectively (Rodgers et al., 1996). At the N-terminal section of the core RAG1 region is the nonamer-binding region (NBR) which is required for the binding of the RAG1 protein to the nonamer of the RSS (Yin et al., 2009). Also in the core RAG1 region is the zinc finger B (ZFB) which has the same C_2H_2 motif as ZFA (De & Rodgers, 2004). The DDE is comprised of two aspartic acids and one glutamic acid and has been found to form the active site in RAG1 that catalyses nicking and transesterification during V(D)J recombination (P. C. Swanson, 2001). Previous work in red spotted grouper (*Epinephelus akaara*) was used as a reference to help identify the ZDD, RING, ZFA, the NBR and the core RAG1 regions (Mao et al., 2012). In addition, the domains ZFB and DDE were identified using the human RAG1 sequence. They were also found to be highly conserved throughout the species and used for the alignment (De & Rodgers, 2004). As the *S. lalandi* RAG1 sequence confirmed during this work was not a complete sequence, only the C_2H_2 motif of the ZFB and the two aspartic acids of the DDE motif could be seen to be conserved within the alignment. The glutamic acid not confirmed due to the sequence being incomplete as a result of the failed 3' RACE. It remains to be determined if there is good conservation of the ZDD, RING, ZFA and NBR domains in the *S. lalandi* RAG1, although they are predicted to be as they are clearly seen in all fish species examined to date.

4.4.3 Interleukin-1β

A number of IL-1β primers were designed with a final count of four forward primers and three reverse primers. Initial attempts at amplifying IL-1β was carried out using IL-1β-F1 and IL-1β-F2, with either IL-1β-R1 or IL-1β-R2. The largest product using F1R1 was predicted to be 1,049 bp in length and the smallest product using F2R2 was predicted to be 906 bp in length. However, the attempt to characterise IL-1β in *S. lalandi* proved difficult and initial attempts at amplification of the gene failed as no PCR products could be identified at the predicted size. The design of more primers that produced a smaller product seemed to amplify a product; however attempts to clone the product failed. In an attempt to work through this, primers were designed for RACE PCR, IL-1β-3'F1 and -3'F2. This amplified a band that was subsequently ligated into a vector, amplified and sequenced, however the sequence amplified was non-specific and did not match to any existing fish IL-1β sequence within the BLAST search.

Even though no IL-1β sequence was confirmed, it was still included for subsequent analysis in this investigation. This is due to its importance in innate immunity, and the unconfirmed transcriptome sequences' usefulness in designing primers for qPCR. In mammals, IL-1β is a member of the IL-1 superfamily that consists of 11 members, IL-1α (IL-F1), IL-1β (IL-F2), IL-1ra (IL-F3), IL-18 (IL-F4) and IL-F5-11 (Secombes et al., 2011). IL-1α and IL-1β are the best characterised family members and originate in leukocytes as large precursor proteins and are found on B-cell and monocyte surfaces, and in the cytosol, respectively (Steve Bird et al., 2002; Huising et al., 2004). IL-1β begins as an inactive pro-peptide with a size of 31kDa and is enzymatically cleaved to give an active peptide 17kDa in size (Pelegrín, Chaves-Pozo, Mulero, & Meseguer, 2004). One enzyme in particular that is involved in this process is caspase-1, which starts the process of activation of IL-1β, which is only matured once it actually leaves the cell (Steve Bird et al., 2002). Both IL-1α and IL-1β bind the receptors, IL-1RI and IL-1RII, which are activated with the aid of the IL-1 receptor accessory protein (IL-1RAP). IL-1RI is the positive receptor whereas IL-1RII is the negative receptor, i.e. antagonises the activity of IL-1 members (L. Y. Zhu et al., 2013). Activation of IL-1RI results in endothelial cells up-regulating adhesion factors which then cause the migration of various immune cells, such as lymphocytes and phagocytes (Acuner Ozbabacan, Gursoy, Nussinov, & Keskin, 2014). IL-1β is

extremely important in innate immune responses (Steve Bird et al., 2002) and in conjunction with other regulatory cytokines, begins cellular cascades in response to pathogens (recognising bacterial LPS) and/or damage-associated molecular patterns (DAMPs). The detection of these stimuli by the immune system and the subsequent effects of the cytokines lead to inflammation (Steve Bird et al., 2002; Huising et al., 2004; Ogryzko et al., 2014). Many cell types, including monocytes, macrophages, neutrophilic granulocytes, endothelial cells and T lymphocytes secrete the IL-1 family cytokine molecules in an immune response (Huising et al., 2004; L. Y. Zhu et al., 2013).

IL-1β genes have been characterised in a number of fish species such as, gilthead seabream, haddock, Atlantic salmon, carp, rainbow trout, and in striped trumpeter(Corripio-Miyar, Bird, Tsamopoulos, & Secombes, 2007; Covello et al., 2009; Fujiki, Shin, Nakao, & Yano, 2000; Ingerslev, Cunningham, & Wergeland, 2006; Pelegrín et al., 2004; Jun Zou et al., 1999). IL-1 family members belong to the β-trefoil superfamily which is distinguished by a trefoil structure formed by 12 β-sheets (Steve Bird et al., 2002). The conserved regions for the 12 β-sheets were identified in the amino acid sequence derived from the *S. lalandi* transcriptome using the previously determined crocodile icefish (*Chionodraco hamatus*) IL-1β sequence (Francesco Buonocore et al., 2006). The IL-1 family signature found in IL-1β has been described as [FS]-x_2-[FYLIV]-[LI]-[SCA]-T-x_7-[LIVM] and was also found in the *S. lalandi* IL-1β transcriptome sequence (Overgard, Nepstad, Nerland, & Patel, 2012). The human binding site in IL-1β for IL-1R is made up of seven amino acids, as follows Arginine-4, Leucine-6, Phenylalanine-46, Isoleucine-56, Lysine-93, Lysine-103, and Glutamic acid-105 (Labriola-Tompkins et al., 1991). These are not completely conserved throughout teleost species, but highlight areas that may have an effect in receptor binding. Lastly, most fish examined so far, including the unconfirmed transcriptome sequence from *S. lalandi*, lack the cleavage site, identified in mammals, that is important for generation of the mature active molecule. In humans, the enzyme caspase-1, also known as the Interleukin-1 beta converting enzyme, cleaves IL-1β at aspartate-116 (Reis, do Vale, Pereira, Azevedo, & Dos Santos, 2012). However, in teleost species another potential cleavage site has been identified, which in *Dicentrarchus labrax* is at aspartate-116 which is highly conserved throughout all vertebrates (Reis et al., 2012) and also present in the predicted *S. lalandi* IL-1β

transcriptome sequence. This could be an alternative cleavage site for fish IL-1β, however, this will require further study as the cleavage site would then potentially be part of the first β-sheet, and cleavage at this point could destabilise the IL-1β (Reis et al., 2012).

4.5 Expression of Immune Genes during Larval Expression

Understanding how the immune system develops in fish is crucial for determining the most effective time to vaccinate, and in determining the effect the vaccine has had within the fish (Hirono et al., 2003). Key immune organs were observed in the New Zealand Groper (*Polyprion oxygeneios*) in order to understand the development of the immune system (Parker, La Flamme, & Salinas, 2012). It was found that the thymus, spleen, and head kidney could be first identified at 20 dph, 16 dph, and 6 dph, respectively. It was also found that IgM^+ B cells could be found in these tissues at 45 dph, 32 dph and 12 dph, respectively. In kelp grouper (*Epinephelus bruneus*) the development of the thymus, kidney and spleen were studied using histological analysis (Kato et al., 2004). The study found early indications of the kidney, spleen, and thymus at 1 dph, 6 dph, and 12 dph, respectively. These studies are consistent in the order of identifiable immune organs with kidney becoming detectable much earlier than spleen, and the thymus last. As an indication of adaptive immunocompetence in larval fish, immune related tissue has also been studied in Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) (Gradil et al., 2014). It was found that tissue, including spleen, thymus and meningeal myeloid tissue could not be identified in the fish before 33 dph. This suggests the adaptive immune response does not occur until later in life in this species, as demonstrated by the late development of the thymus (Gradil et al., 2014). These studies show the variability present in the development of the immune system and demonstrate the necessity of the characterisation of the immune system in each individual fish species.

One of the aims of this study was to build a profile of immune gene expression in the early stages of *S. lalandi* larval development. At the start of this project, two approaches were proposed, in situ hybridization and qPCR, to look at the expression of IL-1β IgD and RAG1. However, due to issues with obtaining good sections from larvae at different stages, which is discussed in detail later (See section 1.6), only qPCR was carried out. For this analysis, primers were

designed to the characterised *S. lalandi* IL-1β, IgD and RAG1, as well as the housekeeping genes β-actin and GAPDH, which were used as reference genes. The expression of the genes of interest was targeted at four age groups, hatch, 3 days post hatch (dph), 12 dph and 18 dph. Four replicates of each age group were collected, containing pools of 50 larvae. Initially each set of reference and immune gene primers had their amplification efficiency tested, to ensure they were suitable in this study. A qPCR was carried out on each sample and relative expression was determined using the comparative ΔCq test (S. Bird et al., 2005), with statistical analysis of the expression results achieved using ANOVA and the Independent Student's t-test, both available in Microsoft Excel. Expression data from the three immune genes showed they followed similar patterns, with expression of each gene seen at hatch followed by little expression seen at 3 dph. At 12 dph the expression seemed to increase for each gene and was followed by a decrease in expression at 18 dph. No statistical significance in expression change was found between any of the groups as none of the P values were under 0.05.

While the aim of the expression work was to obtain a preliminary overview of the gene expression of IL-1β, IgD and RAG1, a number of factors, may have limited the result and could have been improved upon. In particular, only a small number of time points were measured. In order to obtain a more accurate understanding of how expression changes over development, it would have been good to have used more of the samples collected. However, this had to be limited due to a lack of resources. Also, each pool of larvae consisted of 50 samples which could be raised to 100 samples per pool to increase accuracy. Another factor that should be taken into consideration was the use of housekeeping genes used to normalise the expression data. The expression of the housekeeping genes used for this investigation, β-actin and GAPDH, has not previously been determined for *S. lalandi*. Selection of the correct housekeeping gene for expression work has been discussed and debated (H. H. Chen et al., 2012; Dienz et al., 2012) and there are methods that can be used to screen for variability of housekeeping genes during development. Recently, a number of studies have attempted to determine the ideal reference gene to use for expression analysis in developing fish (Dang & Sun, 2011; De Santis, Smith-Keune, & Jerry, 2011; Fernandes, Mommens, Hagen, Babiak, & Solberg, 2008; Infante et al., 2008; Liu et al., 2014; Mitter et al., 2009; Mo, Zhao, Liu, Cao, & Jiang, 2014;

Overgard, Nerland, & Patel, 2010; C. G. Yang et al., 2013; Zhong et al., 2008). It appears that there is no universal set of reference genes, and that selection of genes could be dependent on the species of fish. It has also been shown that housekeeping genes may also have to be selected based on the type of tissue used. However, due to time constraints, further attempts at qPCR could not be achieved using more housekeeping genes to normalise the data, but is something for consideration in future studies.

The expression of the genes of interest used in this work, IgD, RAG1 and IL-1β have been characterised in a wide range of fish species. The expression of IgD is often excluded in favour of IgM, however a number of studies have shown the expression of IgD in teleosts. In zebrafish, the expression of the immunoglobulin genes IgM, IgD and IgZ were studied over development (Zimmerman et al., 2011). It was found that IgD had low expression levels in the embryonic and juvenile stages which increased over development. Compared to the other immunoglobulins, IgD had the lowest expression levels with IgM being the most common (Zimmerman et al., 2011). This was similar to a study in Atlantic salmon (Tadiso et al., 2011) which compared the expression of IgM, IgT and IgD in different tissues. The immunoglobulins were found to be the most highly expressed in the head kidney and spleen. It was also found that IgM was the most highly expressed immunoglobulin, again followed by IgT and then IgD with the lowest expression (Tadiso et al., 2011). A recent study (Alvarez-Pellitero, 2008) looked at the expression of a number of immune genes after infection with viral haemorrhagic septicaemia virus (VHSV) in the gill of rainbow trout. Although it was not seen that IgM or IgD were significantly modulated in response to the viral challenge, the immune gene MHC-II was upregulated. This indicates the role B cells may have had as antigen presenters (Alvarez-Pellitero, 2008). The expression of RAG1 and RAG2 during development has been studied in common carp (*Cyprinus carpio*) (Huttenhuis et al., 2005). It was found in this species that RAG1 was expressed at 4 dpf in the thymus, followed by 6 dpf in the head kidney and 1 wpf in the kidney. During this study, it was also found that the spleen lacked expression of the RAG1 and RAG2, which supported our findings from the spleen transcriptome. The use of *in situ* hybridisation techniques also revealed the rapid increase of B cells in the spleen after 6 wpf. This presence of B cells coupled with the lack of RAG1 and RAG2 expression is what has

substantiated the notion that the spleen may function as a secondary lymphoid organ in fish (Huttenhuis et al., 2005). The expression of RAG1 in larval striped trumpeter (*Latris lineata*) has also been analysed using qPCR in both unfertilised eggs and larval fish from 4 dph to 100 dph (Covello et al., 2013). It was found that RAG1 expression increased from 4 dph to 80 dph, at which point the expression decreased and was undetected at 100 dph. Lastly, in haddock (*Melanogrammus aeglefinus*) RAG1 expression was used to determine the point during development where the adaptive immune response was developing (Corripio-Miyar, Bird, Treasurer, et al., 2007). Using qPCR, it was found that expression of RAG1 occurred at 10 dph. This study also used whole-mount *in situ* hybridisation to identify the expression of RAG1 in the thymus of the fish.

Larval expression of IL-1β has been studied in a number of fish species under a number of conditions. Expression of immune related genes in larval grouper (*Epinephelus coioides*) was studied in response to oral or bath vaccination of nervous necrosis virus (NNV) (Kai, Wu, & Chi, 2014). It was found that four weeks post immunisation (wpi) that there was significant upregulation of Il-1β, among other immune genes, such as Mx, IgM and IgT. Similarly, the expression of IL-1β was observed in larval rainbow trout after infection of a skin parasite, *Ichthyophthirius multifiliis* (Heinecke & Buchmann, 2013). In this study 10 dph larval fish were infected with the parasite and sampled at 3, 6, 12, 24, 48 and 72 hours post infection. It was found that IL-1β was upregulated and was the first of the genes under scrutiny to be upregulated. The gene expression of Japanese amberjack immune genes (*S. quinqueradiata*) has also been studied after stimulation with LPS (Darawiroj et al., 2008). Kidney and spleen cells of the fish were removed and stimulated with concanavalin A (conA) and LPS and it was seen that expression of IL-1β was upregulated.

4.6 Histology

Along with qPCR-based expression of immune genes, attempts were made at developing *in situ* hybridization, to characterize their expression within histological sections. Larval fish of increasing age were prepared for sectioning, to demonstrate how the tissue changed over the development of the fish. Two methods were used for the sectioning of fish in this study, paraffin wax microtome and cryostat microtome. Most sections were produced using the wax microtome and included larvae of age; 9 dph, 14 dph, 21 dph, 25 dph, 30 dph, 35 dph, and 40 dph. Sections produced with the cryostat were 25 dph and 60 dph. As the paraffin wax protocol provided sections that were of higher quality than the cryostat sections, more time was allocated to producing higher quality paraffin sections. The majority of slides were stained with toluidine blue solution; however the 21 dph sagittal section was stained with H&E. The basic haematoxylin stained cell nuclei a blue-purple colour, and the acidic eosin counterstain dyes erythrocytes, collagen and cellular cytoplasm a red-pink (Franchi, Warner, Viani, & Nunez, 2009). However, this method was labour intensive and was not continued. Toluidine blue stains acidic components as it is a basic dye (Franchi et al., 2009) and was sufficient for most of the preliminary histological analysis. After a number of attempts, a protocol was developed that produced sections that were of good enough quality to be taken forward for expression analysis. A number of teleost species have been sectioned in order to further understand immune development. This includes the development of the thymus in *Siniperca chuatsi* (Rajendran et al., 2012), and the morphological study of the kidney in juvenile sturgeon species, *Huso huso* and *Acipenser persicus* (Ye, Kaattari, Ma, & Kaattari, 2013).

Using these sections, some identification of tissues was carried out, which relied upon histology atlases compiled from different species (Soanes, Figuereido, Richards, Mattatall, & Ewart, 2004). Labelling of structures on the sections presented in this work were mostly focused on easily identified structures, however future studies will employ *in situ* hybridisation, which will help in the characterisation of particular immune tissues and expression patterns of particular immune genes during development. This approach involves producing a labelled nucleotide probe that can bind to mRNA of the gene of interest and can be used to localise where the gene is being expressed (Alvarez-Pellitero, 2008). Due to the

time taken to achieve good sections, there was no possibility of attempting *in situ* hybridisation, as this method itself requires a fair amount of optimisation. Hurdles to overcome include the possibility of errors introduced during *in situ* hybridisation, with non-specific probe binding, loss of label signal after wash steps and poor hybridisation. All of which would require time to fix and optimise (Takeuchi & Akira, 2010). However, looking at the location of expression of the *S. lalandi* RAG1 gene could have been used for *in situ* hybridisation work, in an attempt to locate important immune tissue, including the thymus and head kidney. In a study on the immune system of haddock (Corripio-Miyar, Bird, Treasurer, et al., 2007) a RAG1 probe was used to identify where the gene was expressed and at what age it was first expressed. I*n situ* hybridisation was also performed in Atlantic cod (*Gadus morhua* L.) in an attempt to identify cells expressing surface IgM and IgD transcripts in head kidney and spleen (X. Yang et al., 2006)..

4.7 Problems and Limitations

There were a number of problems encountered during the course of this work that prevented the completion of all of the aims. Firstly, a number of primers were designed for amplification of the genes of interest and for 3' RACE. However, it was found that not all primers would amplify a product. In particular, this was seen when primers were designed to produce a large product. A lot of different amplification protocols were used, in an attempt to optimise the primers being used and in some cases this did help. However, an initial attempt at amplifying a large product, by increasing the extension time during thermocylcing, did not produce a desired product. The problem may have been helped by using a high fidelity DNA polymerase, such as *Pfu* polymerase (Zelensky & Gready, 2004). Also, it was found in this study, that designing primers to amplify a smaller product, at approximately 1 Kb or less helped overcome amplification problems.

Once amplification of genes by PCR produced consistent results experimental work was delayed due to problems with bacterial cloning. PCR products were ligated into the pLUG-Prime® TA-cloning vector and left overnight at 4°C. This was outlined in the protocol to allow for maximum efficiency. However, transformation of bacteria and subsequent plating on LB+ plates yielded no growth. Two methods of bacterial transformation were

attempted to remedy the lack of bacterial growth with neither method initially producing the desired bacterial colonies; however small amounts of growth were detected. A major factor in this could have been the age of the ligation kit that was used as the activity of the ligase enzyme is prone to diminishing over time. This was suspected as bacterial transformation succeeded using the *E. cloni*® cells with colonies growing on LB+ plates, once a new stock of pLUG-Prime® was ordered. Most colonies were white which indicated successful ligation and transformation.

Lastly, initial attempts at paraffin embedding proved very difficult, especially when embedding very small larval fish. The orientation of the fish was important as transverse sections were desired. Problems arose as paraffin wax would set quickly, this meant that the sample had to be orientated and placed before the wax set. The cryostat was thought to be a better alternative for preparing samples for sectioning. This was because the fish could be placed in the OCT and once oriented correctly could be frozen, providing more time for the sample to be oriented correctly. Although the preparation for the cryostat was easier, the sections that were produced were of lower quality than the paraffin sections. This was due to the quality decreasing with thinner sections, and no sections could be used that were under 10 µm. The cryostat did have the advantage of taking larger samples and the 60 dph larvae could only be sectioned well using this approach. However, eventually, the paraffin wax microtome was revisited, but samples were proving difficult to cut, using the microtome, with sections tearing and tissues missing. Interestingly, sections produced after a newer stock of xylene was purchased, allowed the production of high quality sections, with less tearing.

Although these issues prevented all of the aims of the project to be achieved, within the timeframe, this investigation has contributed toward future studies of the immune response within *S. lalandi*. Three genes have been partially characterised and primers designed that can be used in future expression studies using qPCR. In addition, high quality histological section can be produced which can go on to be used in *in situ* hybridization.

4.8 Future Recommendations

Modification of Methods

To further the understanding of the immune system of *S. lalandi* more genes related to the immune system could be characterised, using the same approach adopted in this investigation. Using the spleen transcriptome, many more innate genes could be searched for such as TLR22, Mx and MHC class II. In addition to these adaptive immune genes, such as TCR-α, TCR-β, and IgT could also be targeted for characterisation. TLR22 and Mx have been shown to have important roles in the immune response against viral infections (Matsuo et al., 2008; Robertsen, 2006). Given the role of MHC class II in antigen presentation, characterising this gene and determining its expression under normal conditions could be used to compare to fish after infection (L. Y. Zhu et al., 2013). The TCR-α and TCR-β chains could be suitable candidates for further understanding of the adaptive immune system as they are expressed on all αβ T-cells (Laing & Hansen, 2011). IgT was initially selected as suitable candidates for this study and was identified in the spleen transcriptome. This gene is therefore available for characterisation in future work.

A transcriptomic approach could be utilised for characterising the expression of a number of immune genes. A number of studies have been published on the use of RNA-seq transcriptomics to compare the expression of genes. This includes determining the effects of vaccines, understanding the immune system from an evolutionary perspective and in the selection of brood stock that are disease resistant (Qian, Ba, Zhuang, & Zhong, 2014). In zebrafish, a transcriptomic analysis of liver tissue was performed after a vaccination (D. Yang et al., 2012). Zebrafish were immunised using an *Edwardsiella tarda* vaccine and a sample of liver tissue was used to construct a transcriptomic library. It was found that the MHC-I pathway had been upregulated and the MHC-II pathway downregulated. Similarly, an analysis was performed on Atlantic salmon where a number of genes were found to have altered expression in macrophage and dendritic cell lines after treatment with INF-I (C. Xu, Evensen, & Munang'andu, 2015)

For the histological sectioning, an alternative to paraffin wax and cryostat sectioning exists, plastic embedding, that was considered during this investigation

and could be used in future work. This method could not be performed during this research due to insufficient time and resources. Plastic embedding has the advantage of producing very stable thin sections which would have more detail and higher resolution (Gertz et al., 2006). As previously mentioned, the purpose of optimising a sectioning protocol was for *in situ* hybridisation work. Probes can be designed for the genes characterised in this research, especially RAG1 which has been done in Haddock (Corripio-Miyar, Bird, Treasurer, et al., 2007). Similarly, *in situ* hybridisation could be performed using a probe IgD which has been done in Atlantic cod (X. Yang et al., 2006).

Application of the Results

The primary aim of this work was to characterise immune genes, to use in future studies of immune system development and function in *S. lalandi*. For example, cytokine expression profiles can be examined within farmed fish during the occurrence of diseses. In *Plecoglossus altivelis* (Ayu), an asian species where captive juveniles are raised in aquaculture, IL-1β has been characterised and fish subjected to intraperitoneal injections of *Listonella anguillarum* bacteria, shown to increase its production (Lu et al., 2013). In striped trumpeter (*Latris lineata*), a candidate for aquaculture in Australia, the parasite *Chondracanthus goldsmidi*, is found to infect farmed fish (Covello et al., 2009) and studies involving the use of pro-inflammatory cytokines, which includes IL-1β, have been carried out in an attempt to understand the fishes immune response.

Having a suite of immune genes as biomarkers will also prove invaluable for studies into ecotoxicological factors and their effects on a fish's health. Traditional assessment of ecotoxicological factors has been achieved by analysing blood serum for proteins, cortisol and salt concentrations (Marino, Di, Mandich, Finoia, & Cataudella, 2001). However, with the characterisation of immune genes, a fishes' health can easily be monitored by looking at expression of these immune genes, in both wild and farmed populations of fish, using molecular techniques. Recent examples have looked at stress-related genes in river pufferfish (*Takifugu obscurus*) (Kim, Dahms, & Han, 2013) and immune genes in Atlantic cod (*Gadus morhua*) (Yandell & Ence, 2012).

The understanding of the immune systems of any new fish species will aid their introduction into the aquaculture setting. Identification of immune genes and

their use as biomarkers to analyse the impact of stress or disease in farmed fish will be invaluable. Characterising immune genes within *S. lalandi*, will provide useful tools, for investigating production bottlenecks within the aquaculture industry.

4.9 Conclusion

The successful introduction of *S. lalandi* into the New Zealand aquaculture industry will be greatly enhanced by understanding the physiology and molecular characteristics of the fish. The aims of this research was to identify immune related genes within the transcriptomic library, sequence the complete mRNA sequence of the identified genes, and finally to characterise the expression of these genes as the fish developed from hatch to 60 dph. This investigation has demonstrated the successful use of a *S. lalandi* spleen transcriptomic library in the identification of immune genes. Because of this tools developed in this study exist that can follow the expression of immune genes as fish undergo changes in their environment or in response to disease. In addition, the development of a method, to obtain good quality wax embedded sections, will allow future investigations into the development of immunity within this species, using techniques such as *in* situ hybridisation. Overall, this investigation represents a preliminary step into understanding the immune system of *S. lalandi*, to enable the successful introduction of this species into aquaculture.

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Appendices

Appendix I

Recombination Activating Gene 1

Seriola dumerili (accession number: JQ938283.1). An alignment was performed that included the RAG1 amino acid sequences of the following: *Seriola dumerili, Niphon spinosus* (HQ731313.1), *Trachinus draco* (KF017119.1), *Serranus baldwini* (HQ731315.1), *Paralabrax clathratus* (KF017122.1).

```
S_baldwini        ATGGATGGGCTGAGAGAGAGTGGGATGGAAGACAGCGCCTGCACCTCAGGCTTCAGTGTCATGA
P_clathratus      ATGGAGGGGCTGAGAGAGAGTGGGATGGAAGACAGTGCTTGCACCTCAGGCTTCAGTGTTATGA
N_spinosus        ATGGAGGGGCTGAGAGAGAGTGGGATGGAAGACAGTGCTTGCACCTCAGGCTTCAGTGTCATGA
T_draco           ATGGAGGGGCTGAGAGAGAGTGGGATGGAAGACAGTGCTTGCACCTCAGGCTTCAGTGTCATGA
S_dumerili        ATGGAGGGGCTGAGAGAGAGCGGGATGGAAGACAGTGCTTGCACCTCAGGCTTTACTGTCATGA
                  ***** ************** ************** ** ************** * *** ****

S_baldwini        TCAAGGAGTCCTGTGACGGTATGGGCGATGTCAGCGAGAAGCACGGTGTAGGACCAGCTGTTCC
P_clathratus      TCAAGGAGTCCTGTGACGGTATGGGCGATGTCAGCGAGAAGCACGGTGGAGGACCAGCTGTTCC
N_spinosus        TCAAGGAATCCTGTGATGGCATGGGCGATGTCAGCGAGAAGCACGGTGGAGGACCAGCTGTTCC
T_draco           TCAAAGAATCCTGTGATGGCATGGGCGATGTCAGCGAGAAGCACGGCGGAGGACCGGCTGTTCC
S_dumerili        TCAAGGAATGCTGTGATGGCATGGGCGATGTCAGCGAGAAGCACGGCGGAGGACCAGCCATTCC
                  **** ** * ****** ** ************************** * ****** **  ****

S_baldwini        CGAGAAGGCTGTACGTTTCTCTTTCACTGTTATGTCTATCTCTGTCCTGGCGGATGAAGAGGAG
P_clathratus      TGAGAAGGCTGTACGTTTCTCTATCACTGTTATGTCTATCTCCGTCCTGGCGGATGAAGAGGAG
N_spinosus        TGAGAAGGCTGTACGTTTTTCTATCACTGTTATGTCTGTCTCTATCCTGGCAGACGAGGAGGAG
T_draco           CGAGAAGGCTGTACGTTTTTCTTTCACTGTTATGTCTGTCTCTGTCCTGGGCAGATGATGAGGAG
S_dumerili        CGAGAAGGCTGTACGTTTCTCTTTCACTGTTATGTCTGTCTCTGTCCGGGCAGAGGAGGAGGAG
                  ***************** *** ************** ****  *** *** ** ** ******

S_baldwini        AAAGAGGTTACTATCTTCACTGAGCCGAAGCCAAACTCAGAACTGTCCTGTAAGCCCCTTTGCC
P_clathratus      AAAGAGGTTACTATCTTCACTGAGCCGAAGCCAAACTCAGAACTGTCCTGTAAGCCCCTTTGCC
N_spinosus        GAAGAGGTTACCATCTTCACTGAGCCAAAGCCAAACTCAGAACTGTCCTGTAAGCCCCTTTGCC
T_draco           GAAGAGGTTACCATCTTCACTGAGCAAAAGCCAAACTCAGAACTGTCCTGTAAGCCCCTTTGCC
S_dumerili        GAGGACGTTACCATCTTCACCGAGCCAAGGCCGAACTCGGAGCTGTCCTGTAAGCCCCTTTGCT
                   * ** ***** ******** ****  * *** ***** ** ********************

S_baldwini        TGATGTTTGTGGATGAGTCAGACCATGAGACACTCACATCCGTCCTGGGGCCTATAGTTGCCGA
P_clathratus      TGATGTTTGTGGATGAGTCAGACCATGAGACACTCACAGCCGTCCTGGGGCCTATAGTTGCAGA
N_spinosus        TGATGTTTGTGGATGAGTCAGACCGTGAGACACTCACAGCCGTCCTGGGGCCTATAGTTGCAGA
T_draco           TGATGCTTGTGGATGAGTCCGACCACGAGACGCTCACAGCTCTCCTGGCGCCTGTAGTTGCAGA
S_dumerili        TGATGTTTGTGGATGAGTCAGACCACGAGACACTCACAGCCCTCCTGGGGCCTGTAGTTGCAGA
                  ***** ************** ****  ***** ****** *  ****** **** ******* **

S_baldwini        GCGTGGTGCTATGAAAGAGAGCAGGCTCATCCTCTCTGTAGGCGGACTCCCTCGTTCCTTCCGC
P_clathratus      GCGGGATGCTATGAAAGAGAGCAGGCTCATCCTCTCCGTGGGTGGGCTCCCTCGCTCCTTCCGC
N_spinosus        GCGTAATGCAATGAAAGAGAGCAGGCTCATCCTATCCTTGGGTGGACTACCTCGCTCCTTCCGC
T_draco           GCGAAATGCAATGAAAGAGAGCAGGCTCATTCTATCTGTGGGTGGACTACCTCGCTCCTTCCGC
S_dumerili        GCGTCATGCAATGAAAGAGAGCAGGCTCATCCTCTCCATCGGCGGCCTTCCTCGCTCCTTCCGC
                  ***   *** ******************** ** **  * ** ** ** ***** *********

S_baldwini        TTCCACTTCAGAGGCACAGGATATGATGAGAAGATGGTGCGTGAGATGGAAGGCCTCGAGGCCT
P_clathratus      TTCCACTTCAGAGGCACGGGATATGATGAGAAGATGGTGCGTGAGATRGAAGGCCTCGAGGCCT
N_spinosus        TTTCACTTCAGAGGCACGGGATACGATGAGAAGATGGTGCGTGAGATGGAGGGCCTCGAGGCCT
T_draco           TTTCACTTCAGAGGTACGGGATATGATGAGAAGATGGTGCGTGAGATGGAGGGCCTCGAGGCCT
S_dumerili        TTCCACTTCAGGGGCACGGGATACGATGAGAAGATGGTGCGGGAGATGGAAGGCCTGGAGTCCT
                  ** ******** ** ** ***** ***************** ***** ** ***** *** ***

S_baldwini        CGGGGTCCACCTATGTCTGCACTCTTTGTGACTCCACYCGGGCGGAGGCCTCTCAAAACATGGT
P_clathratus      CGGGGTCCACCTATGTCTGCACTCTTTGTGACTCCACTCGGGCGCAGGCCTCTCAAAACATGGT
N_spinosus        CAGGGTCCACCTATGTCTGCACTCTTTGTGACTCCACTCGGGCAGAGGCCTCTCAAAACATGGT
T_draco           CAGGGTCCACCTATGTCTGCACTCTTTGTGACTCCACTCGGGCAGAAGCCTCTCAAAACATGGT
S_dumerili        CGGGGTCCACGTATGTCTGCACTCTGTGTGACTCCAGCCGGGCGAACGCCTCACAAAACATGGT
                  * ******** ************** **********  *****  * ***** ***********

S_baldwini        GCTGCACTCAGTCACACGCAGTCATGAAGAGAACCTAGATCGCTACGAAATATGGAGAACCAAC
P_clathratus      GCTGCATTCCGTCACTCGCAATCATGACGAGAACCTAGATCGCTACGAAATATGGAGAACCAAC
N_spinosus        GCTACACTCCGTCACCCGCAGTCATGAAGAGAACCTAGATCGTTACGAAATATGGAGAACCAAC
T_draco           GCTGCACTCCATCACCCGCAGTCATGAAGAGAACCTAGATCGTTACGAAATATGGAGAACCAAC
S_dumerili        GCTACACTCCATCACCCGCGGCCATGAAGAGAACCTAGAACGTTATGAAATATGGAGAACCAAC
                  *** ** **  **** ***   ***** *********** ** ** ******************

S_baldwini        CCGTTTTCTGAGTCTGTGGATGAGCTGCGAGACAGAGTCAAAGGGATCTCAGCCAAGCCCTTCT
P_clathratus      CCCTTTTCTGAGTCTGTGGACGAGCTGCGAGACAGAGTCAAAGGGATCTCAGCCAAGCCCTTCT
N_spinosus        CCCTTTTCTGAGTCTGTGGATGAGCTGCGAGACAGAGTCAAAGGGGTCTCTGCCAAGCCCTTCA
T_draco           CCCTTTTCTGAGTCTGTAGATGAGTTGCGGGACAGAGTCAAAGGGGTCTCCGCCAAGCCTTTTA
S_dumerili        CCCTTCTCTGAGTCTGTGGACGAACTGCGAGACAGAGTCAAAGGTGTCTCTGCAAAGCCCTTCA
                  ** ** ********** ** ** **** ***************  **** ** ***** **
```

```
S_baldwini        TGGAGACCCACCCCACACTGGATGCATTACACTGTGACATTGGCAACGCAATTGAGTTCTACAA
P_clathratus      TGGAGACCCACCCCACGCTGGATGCATTACACTGTGACATTGGCAATGCCATTGAGTTCTACAA
N_spinosus        TGGAGACCCATCCCACGCTGGATGCATTACACTGTGACATTGGCAATGCCACTGAGTTCTACAA
T_draco           TGGAGACCCAGCCCACGATGGATGCATTACACTGTGACATTGGCAATGCCACCGAATTCTACAA
S_dumerili        TGGAGACCCACCCCACGCTAGATGCGTTGCACTGCGACATTGGCAATGCCACTGAGTTCTACAA
                  ********** *****  * ***** ** ***** ********** ** *  ** ********
S_baldwini        AATCTTCCAGGATGAGATTGGCGAGATGTACAAAAAGGTCAACCCCCGCCGGGAGGAGCGGCGC
P_clathratus      AATCTTCCAGGACGAGATCGGGGAGGTGTACACAAAGGTTAACCCCAGCCGGGAGGAACGGCGC
N_spinosus        AATCTTCCAGGACGAGATCGGGGAGGTGTACCAAAAGGTCAACCCCAGCCGGGAGGAACGGCGC
T_draco           AATCTTCCAGGATGAGATCGGCGAGGTGTACAAAAAGGTCAACCCCAGCCGGGAGGAGCGACGC
S_dumerili        AATCTTCCAGGACGAGATCGGGGAGGTGTATCAAAAGGTCAACCCCAGCCGGGAGGAAAGACGC
                  ************ ***** ** *** ****   ****** ****** ********** * ***
S_baldwini        AGCTGGAGGGCAGCCCTAGATAAACAGCTGAGGAAGAAGATGAAGCTAAAACCAGTGATGAGAA
P_clathratus      AGCTGGAGGGCAGCCCTAGATAAACAGCTGAGGAAGAAGATGAAGCTAAAACCAGTGATGAGAA
N_spinosus        AGCTGGAGGGCAGCCCTAGATAAACAGCTGAGGAAGAAGTTGAAGCTTAGACCGGTGATGAGGA
T_draco           AGCTGGAGGGCTGCCCTAGACAAACAGCTGAGGAAGAAGATGAAGCTAAAACCGGTGATGAGGA
S_dumerili        AGCTGGAGGGCCGCCCTAGATAAACAGCTGAGGAAGAAGATGAAGCTTAAACCGGTAATGAGGA
                  *********** ******** ****************** ******* * *** ** ***** *
S_baldwini        TGAATGGGAACTATGCCCGCAGGCTAATGACCCAGGAGACTGTGGAGGTGGTTTGTGAGCTGGT
P_clathratus      TGAATGGGAACTATGCCCGCAGGCTAATGACCCAGGAGACTGTGGAGGTGGTGTGTGAGCTGGT
N_spinosus        TGAATGGGAACTACGCCCGCAGGCTAATGACCCAGGAGGCTGTGGAGGTGGTGTGTGAGCTGGT
T_draco           TGAATGGGAACTATGCCCGCAGGCTAATGACCCAGGAGGCTGTAGAGGTGGTGTGTGAGTTGGT
S_dumerili        TGAATGGGAACTACGCCCGCAGGCTAATGACCCTGGAGACTGTGGAGGTGGTGTGTGAACTGGT
                  ************* ****************** **** **** ******** *****  ****
S_baldwini        ACCTTCAGAGGACAGAAGGGAGGCCCTGAGGGAGCTCATGAGGATCTACCTCCAGATGAAACCT
P_clathratus      ACCCACAGAGGACAGAAGGGAGGCCCTGAGGGAGCTCATGAGGATCTACCTCCAGATGAAGCCC
N_spinosus        GCCCTCAGAGGAGAGGAGGGAGGCCCTGAGGGAGCTTATAAGGATCTACCTCCAGATGAAGCCT
T_draco           CCCCTCAGAGGAGAGGAGGGAGGCCCTGAGGGAGCTTATACAGATCTACATCCAAATGAAGCCT
S_dumerili        GCCCTCAGAGGAGAGGAGAGAGGCCCTGAGGGAGCTCATGAGGCTCTACCTCCAGATGAGGCCT
                  ** **  ******* ** ** ***************** **   * ***** **** *******
S_baldwini        GTGTGGCGTGCCACCTGCCCCGCCAAGGAGTGCCCCGACCAGCTGTGCCGCTACAGCTTCAACT
P_clathratus      GTGTGGCGCGCAACCTGCCCTGCCAAGGAGTGCCCCGACCAGCTGTGCCGCTACAGCTTTAACT
N_spinosus        GTGTGGCGCGCCACCTGCCCTGCCAAGGAGTGCCCCGACCAGCTGTGCCGCTACAGCTTTAACT
T_draco           GTGTGGCGCGCCACCTGCCCTGCCAAGGAGTGCCCCGACCAGCTGTGCCGCTACAGCTTTAACT
S_dumerili        GTGTGGCGCGCCACCTGCCCAGCCAAGGAATGCCCAGACCAGCTGTGCCGCTACAGCTTTAACT
                  ******** ** ******** ******** ***** ********************** ****
S_baldwini        CCCAGCGCTTTGCCGACCTCCTCGCCTCTACCTTCAAATACCGCTACAACGGGAAGATAACCAA
P_clathratus      CCCAGCGCTTCGCCGACCTCCTCGCCTCTACCTTCAAATACAGGTACAACGGAAAGATAACCAA
N_spinosus        CTCAGCGCTTTGCCGACCTTCTCTCCTCTACCTTCAAATATAGGTACGATGGAAAGATAACCAA
T_draco           CCCAGCGCTTCGCCGACCTTCTCTCCTCTACCTTCAAATATAGGTACAACGGAAAGATAACCAA
S_dumerili        CGCAGCACTTTGCCGACCTCCTCTCCTCTACCTTCAAATACAGGTACAACGGAAAGATCACCAA
                  * **** *** ******** *** ***************  * *** * ** **********
S_baldwini        TTACCTGCACAAGACCCTGGCACATGTGCCTGAAATCATTGAGAAAGATGGATCCATAGGGGCC
P_clathratus      TTACCTGCACAAGACCCTGGCACATGTGCCTGAAATCATTGAGAAAGATGGATCCATAGGAGCC
N_spinosus        TTACCTGCACAAGACCCTGGCCCATGTGCCTGAAATCATAGAGAGAGATGGATCCATAGGAGCT
T_draco           TTACCTGCACAAGACCCTGGCCCAYGTTCCTGAAATCATCGAGAGAGAYGGATCCATAGGAGCA
S_dumerili        TTACCTGCACAAGACTCTGGCCCATGTGCCTGAAATCATAGAGAGAGATGGATCCATAGGAGCC
                  ************** ***** ** ** *********** **** *** *********** **
S_baldwini        TGGGCCAGCGAGGGGAACGAGTCAGCAAACAAGCTGTTCAGGCGTTTCCGGAAGATGAATGCAC
P_clathratus      TGGGCCAGCGAGGGGAACGAGTCGGCAAACAAGCTGTTCAGGCGTTTCCGGAAGATGAATGCAC
N_spinosus        TGGGCCAGCGAGGGGAACGAGTCGGCAAACAAACTGTTCAGGCGTTTCCGTAAGATGAATGCAC
T_draco           TGGGCCAGCGAGGGGAACGAGTCGGCAAACAAACTGTTCAGGCGCTTCCGTAAGATGAATGCAC
S_dumerili        TGGGCCAGTGAGGGGAACGAGTCGGCAAACAAACTGTTCAGGCGTTTCCGG-------------
                  ******** ************** ******** *********** *****
S_baldwini        GTCAGTCAAAGTTCTTTGAGCTG-------------------
P_clathratus      GTCAGTCAAAAATCTTTGAGCTGGAGGACGTGCTGAAACAC
N_spinosus        GTCAGTCAAAGGCCTTTG------------------------
T_draco           GTCAGTCAAAG-------------------------------
S_dumerili        ----------------------------------------
```

Immunoglobulin Theta

An Amino acid alignment of between *Epinephelus coioides* (ACZ54909.1) IgT sequence and the *S. lalandi* transcriptome IgT sequence.

```
S_lalandi       ------------------------------------------------------------
E_coioides      GGGGCGCAGATCACTGGGACAGTTTGATATATTTCTGAAACACTCAGATCACAAAGCACT

S_lalandi       -GGCCAAGGCGGTT-TAACATGATGGACTATAGGAC------AGGACTGCTGCTTKGM--
E_coioides      GAACTACAGTGTTTATCACAGAATGGAAAATACAGTTATCTGGAGTTTGTTATTTGTAGT
                   * *  * * ** * ***  *****  ***           *  ** *  **
S_lalandi       -MMASKCKGCTGGGCAGGTGTTGATGGTCAGACTCKGMCANARKCNGAACCAGCGGTTAA
E_coioides      TACTATTCATGGAGTTTGGAGTGA-GATCAAACTGGACCAG--TCTTCCTCTGAGGTGAA
                           * *   *   *** * *** ***    **    *     * * *** **
S_lalandi       AAGGCCTGGAGAATCCCACAGACTGACCTGTACAGCCTCTGGATTCACATTCAGCAATTA
E_coioides      AAGACCTGGAGAGACAGTGAAGATGTCATGTATCATTTCTGGTTTTGACATGACAGACTA
                *** ********  *    *   ** * ****     ***** **     * *   * **
S_lalandi       CTGGATGAACTGGGTCAGACAGGCTCCTGGAAAAGGACTGGAGTGGATTGCCTGGCATGA
E_coioides      CAATATTCACTGGATACGACAGAGGCCAGGGAATGCTCTGGAGTGGATTGGGTGGATGAA
                *   **  ***** *  *****   ** ** ** *  *************  ***    *
S_lalandi       TANNNCTGGTAGYRGCAAATMCTACTCTCAGTCAGTCCAAGGCCGGTTTACCATSKCCAG
E_coioides      CACAGGCACAAACTCTGCTAGCTATGGCAGTTCCTTTCAAAGTCGTTTCATCATGACT-G
                 *        *          ***       **  * *** * ** ** * ***  *  *
S_lalandi       ARGMYGYRKCAGRCAGCANGCTGTATCTGCAGATGAAASAGTCNTNGARGMCWGAAGACTT
E_coioides      AAGATGTGCCCAGCAGCACTCAGTACCTCGAGGTCAAGAGCCTGACAG--CAGAAGA--T
                * *  *   *   *****  * *** **  ** * ** ** *    *   * * *****  *
S_lalandi       GDBKYBTDTTR-SYRYSYRSRSGSRKSSKRKTWTTTCGACTACTGGGGTAAAGGGACTGA
E_coioides      GTTTACTTCTGTGCTCGGTCGGCTGGGCCAGCTTT-CGACTACTGGGGCCGGGGGACTGC
                *     *  *                          ** ************    *******
S_lalandi       TGTCACAGTANACAACAGCATCAACTGTGCCACCGACTGTGTTCCCTTTGATTCAGTGCA
E_coioides      GGTCACAGTATATT-CACAAACAACTGCGGCCCCGGCTCTGTTTCCTTTGGTTCAGTGTA
                 ********* *   **  * ****** * * *** ** **** ****** ******* *
S_lalandi       ACCCCGGGTCTGGAGATACAATCACTGTTGCTTGTCTTGCATACAACTTCTCCCCAAAGA
E_coioides      AGTCTGGGACTGCAGGTACAGTCACTGTTGGCTGTATTGCACAAGACTTCTTCCCAGAGA
                *  * *** *** ** **** *********  *** ***** *  ****** **** ***
S_lalandi       ATCTTGATTTCCAGTGGACTGATGTCAGTGGGGCCGTCCTGACTTCATTACAATATTCTC
E_coioides      GTCTCACTTTCCAGTGGACCGATGCCAGCGGGACCACGCAGACTTTTAAACAATATCCTA
                 ***   ************ **** *** *** **   * *****    ******* **
S_lalandi       CTGCTCAGAAAAACAACAAATATACAGCAGCCAGTATGATCCAAGTATCAAAATCTGACT
E_coioides      CGGTTATGAAAGACAACAAATATACAGGAGTCAGTGTGCTAGATGTGTCAAAGTCTGCCT
                * * *  **** *************** ** **** ** *  * ** ***** **** **
S_lalandi       ACGATTCAAGGAAGTCTTTTAAATGTTCAGTAACTCATGCCGGATCTGAACAATCCGTGC
E_coioides      GGGATTCAAGGAGGAGTTTTAGTTGCTCAGTGACTCACCCTGGAGGCTCCGAAAGTGTGA
                  ********** *  *****  ** ***** *****  * ***       **   ***
S_lalandi       AAGTGCAAAAGTTGGTTCCAGCA---AAAATTACTTTGCTATCGGTGCCAAATGAAGACA
E_coioides      CATTGCAAAAGCCTCCTCCACCTCCTAAGGTAACCTTGGTAGCAGTGCCAGCAGGAGACA
                 * ********     **** *    **  * ** *** ** * ******   * *****
S_lalandi       CCCAGGCCCTGGTCTGCACAATTGAGGCACAGTCGCAATGGAACATTTTGATTCATTTAA
E_coioides      CTCAGACCCTGGTGTGTACGATTGAGG---ATCTTCCCTCAAATCAATTG--TCAGTCAA
                * *** ******* ** ** *******   *    *  *  **    ***  *** * **
S_lalandi       ATGGAAAAAGAATGGCSMAGGCGTGAGAGACTACAGTCAATGTCTCCTCCATAAAACTGG
E_coioides      ATGGAAAAAGGATGATAACTCTGTTACTGGCTTCACTGACTGCCCCCCCCAACTCAATGG
                ********** ***        ** *  * ** ** * * ** * ** ***    * ***
S_lalandi       AAGTTTATATTCAGCGATCAGTGTCCTGAAAGTCAAGAACACAGACTGGGACAGCAATGA
E_coioides      AGGCGTATATACAGCTGTCAGTATCCTAAAGGTCACGAACTCAGAGTGGGACAGTAAAGC
                * *  ***** ****  ***** **** ** **** **** **** ******** ** *
S_lalandi       CGTTTACACCTGTGAGATAAAATACAATGGAAACCTGTATATAAGAGAGACCTCAAAAGC
E_coioides      TGTTTACACATGCGAGGTGACGAACCAAGGAACAACATATCCAAAAAAGGTCTCTAAAGT
                 ******** ** *** * *   ** * ****     ***  ** * **  *** ****
S_lalandi       TTTTATCACGGTGACACTGAAACAGCCAAGTGTTAAAGCAATTTTCAGCAACAACCAAGC
E_coioides      TCCTATCACAGTGACACTGACCCAATCAAGTCCCAAAGAAATATTCAGCAACAACCAGGC
                *  ****** **********  **  *****   **** *** ************** **
S_lalandi       AGAGCTGGAATGTGTCATCACTGGACAAGACAAAACCGTTGTCGGTGACACTGACATCAC
E_coioides      AAAGTTTGAGTGTGTCATTACTGGAACGGACCAGACCG--GACCA-GACTTTCAGATCAT
                * ** * ** ******** ******   *** * ****  * *   ***  * * ****
S_lalandi       AATTGACGGAAAAAATGTGACCAGCACCATTACTTCAACATCGTCTGGCGGCGCGCCGTA
E_coioides      AGTCGATGGACAAAATGTGACCGACAACATTGA---AACAAAACCTGGAAGCA-------
                * * ** *** ***********  ** ****     ****    ****  **
S_lalandi       CAGCNAAAACCAGCAAGATGACTCAGAATTACGAACAGTGGAAGACAGTCAACAAAGTAC
E_coioides      ---AGAAAATCAGCACGATGACTCGTGCTCACACTGATTGGCAGAGTATCAACAAAGTGC
                     **** ***** ********    * **    * *** ***   ********** *
S_lalandi       GCTGTTCTGCCATTAGAAAAGATATGACACCAGTTATTCAAGATCTGACTGTCCAAAAAG
E_coioides      GGTGTTCTGCCATAAGAGACAATATGACACCAGTTATTCAGGAACTGACCATCCAGAAAG
                * *********** *** *  ****************** ** *****  **** ****
S_lalandi       GAGATGGCAGTCAGCTAAAAGTGGCAGTCCACGTCCTTCCAGTGGAGGACATCGGCCAAG
E_coioides      GAGATGGGAGTGATCCAAAAGTGACAGTCCACATTCTCCCATTGGAGGACATCGACAAAG
                ******* *** * * ******* ******** * ** *** ************ * ***
```

```
S_lalandi        GAGACTCAG----CTAAGGTCACTCTGGTGTGTCTGATCTCCAGTCCTGTGCAGCAGGAT
E_coioides       CAG-CTCAGGGGTCAGAGGTCACTCTGGTGTGTCTGGTCTCCAGTCGTGCGCAGCAGGAT
                  ** *****    *  ******************** ********* ** **********
S_lalandi        TACTACATTGCGTGGTCAGAATATCATGGACAAAAACCCCCCATCTATACTGATGGCGTT
E_coioides       TACTACATCGCCTGGAGAGAAGATACTGGACAAAATACCGGCACCTATAGCGATGGCATT
                 ******** ** ***  **** **  *********  **  ** *****  ****** **
S_lalandi        AACTTC-------AGAGTCACCTGAGCGG-----TAATCGGGACTCTGAAGACGACCAAT
E_coioides       AACTTCCCCCCCACAGAAGATCCAAAATAGATACTTAGTTACAAGTATTTACATCACCACC
                 ******       ***    **  *   *     ** *    * * *  * *  ****
S_lalandi        GAGACGACGTTTACC---------------------------------------------
E_coioides       AAGGACAAGTGGAACACAACCAAGTTCTCATGCAACGTCTGGCCAGCTGGTGGGAACAAG
                  **   * **  * *
S_lalandi        ------------------------------------------------------------
E_coioides       ATCCCGAGACGTGTCTTACGCCATGAGTAACTCCGTTGAATGTAAGAAATAGCAGACTCA

S_lalandi        ------------------------------------------------------------
E_coioides       GCTTTAAATGTGGCACTCTGTGCTCATGTCTGTCTCTGTGTGTGCTCTCTGCCATGAGAG

S_lalandi        ------------------------------------------------------------
E_coioides       CCTGTCTTTGTGATTGTACAATTCACAGTCAACATGTCAGTTTACTTTGGTTTGTCATAA

S_lalandi        ------------------------------------------------------------
E_coioides       TGTTGTGTTTCAATCTGTCTTTGTCACACTGTCGAACAGCTTGAGAAGTGTGCTGTTTCT

S_lalandi        ----------------------------------------
E_coioides       AATAAATGTTGCATGGAGAAAAAAAAAAAAAAAAAAAAAA
```

Appendix II

Recipes for Molecular Work

70% Ethanol	
100% Ethanol	350 mL
ddH_2O	150 mL

50X Tris-acetate EDTA Buffer	
Tris Base	252 g
Glacial Acetic Acid	57.1 mL
0.5M EDTA	100 mL
mQH_2O	Make up to 1 L

1X Tris-acetate EDTA Buffer	
50X TAE Buffer	40 mL
mQH_2O	1960 mL

6X Loading Buffer	
Glycerol	6 mL
Bromophenol Blue	24 mg
Xylene Cyanol	20 µL
mQH_2O	Make up to 10 mL

0.1% DEPC H_2O	
DEPC	2 mL
mQH_2O	Make up to 2 L

Solution was mixed overnight at room temperature and the autoclaved.

1.5% Agarose Gel	
Agarose	0.525
1X TAE	35 mL
Ethidium Bromide	1 µL

Ampicillin	
Ampicillin	100 mg
mQH_2O	1 mL

Solution was the filter sterilised and stored in the freezer.

Lysogeny Broth Media	
Lysogeny Broth	12.5 g
mQH_2O	Make up to 500 mL

Solution was mix, autoclaved and stored at 4°C.

IPTG	
IPTG	200 mg
dH_2O	1 mL

Xgal

Xgal	20 mg
Dimethyl formamide	1 mL

LB+ Plates

Agar	7.5 g
Lysogeny Broth	12.5 g
mQH_2O	Make up to 500 mL

Solution was mix and autoclaved. Following the autoclave, the broth was incubated in a 60°C water bath for 1 hour. After this incubation 5 µL of 100nL/mL ampicillin was added to the broth and mixed by inversion. Agar was poured into plates which were left to set.

Recipes for Histology

1X Phosphate Buffered Saline

NaCl	8 g
KCl	0.25 g
KH_2PO_4	0.2 g
Na_2HPO_4	1.15 g
mQH_2O	Make up to 1 L

Toluidine blue solution (Paraffin)

Toluidine Blue Dye	0.1 g
H_2O	10 mL

Toluidine blue solution (Cryostat)

Toluidine Blue Dye	0.28 g
Urea	0.40 g
100% Ethanol	28 ml
H_2O	12 ml

Dissolve urea in H2O. Slowly mix in ethanol. Add toluidine blue and mix. Filter sterilise.

Haematoxylin solution

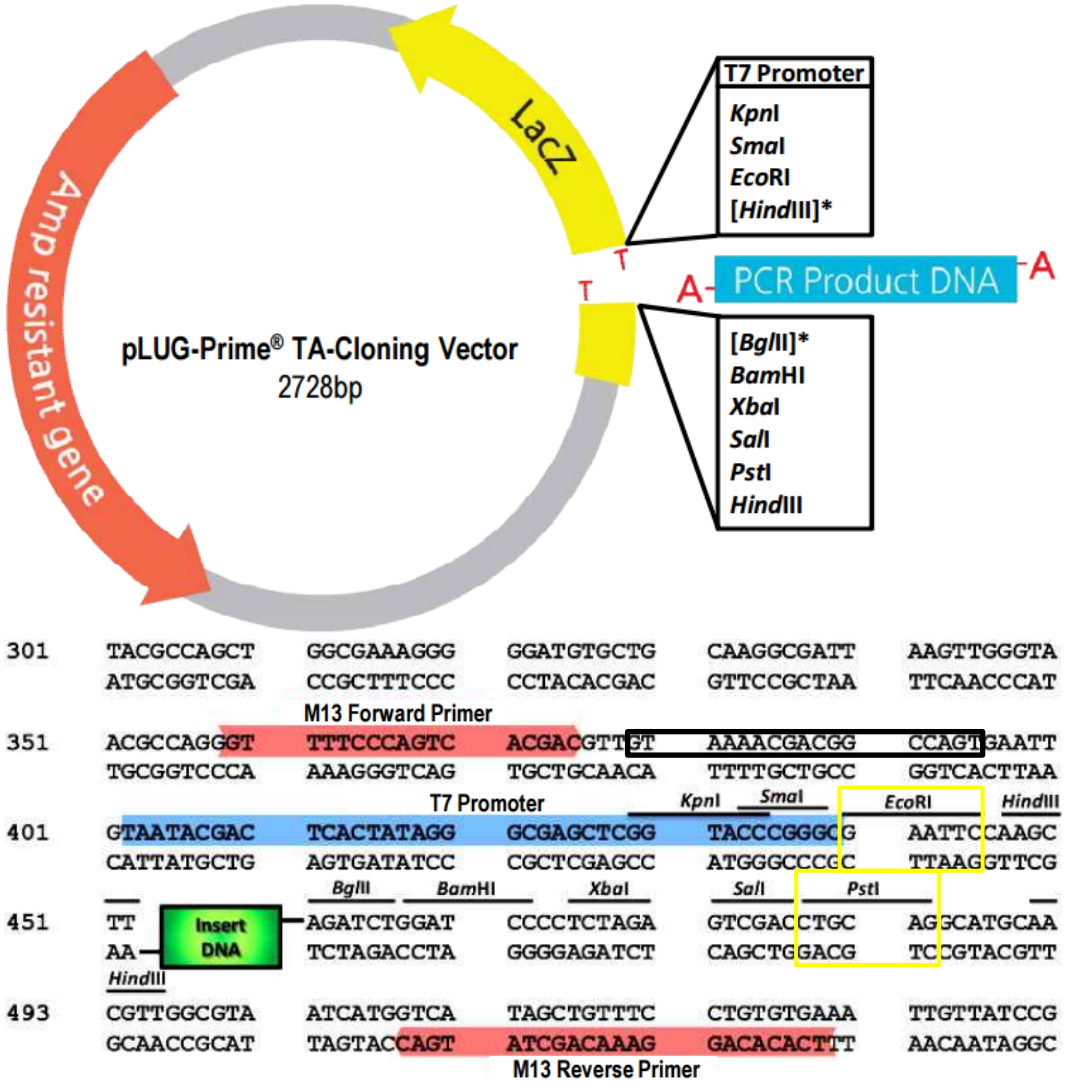
Alum	50 g
Haematoxylin Dye	1 g
$NaIO_3$	0.2 g
Glacial Acetic Acid	20 mL
H_2O	1 L

Alum firs dissolved in H_2O, followed by dissolving haematoxylin. Sodium iodate and glacial acetic acid added and solution boiled. The solution is cooled and filter sterilised

Eosin 1% Solution

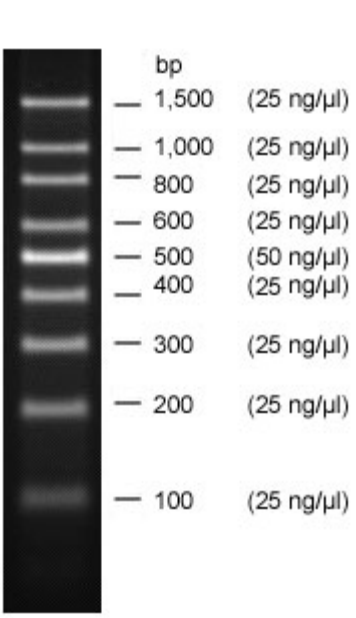
Eosin Y	1 g
H_2O	20 mL
Ethanol	80 mL

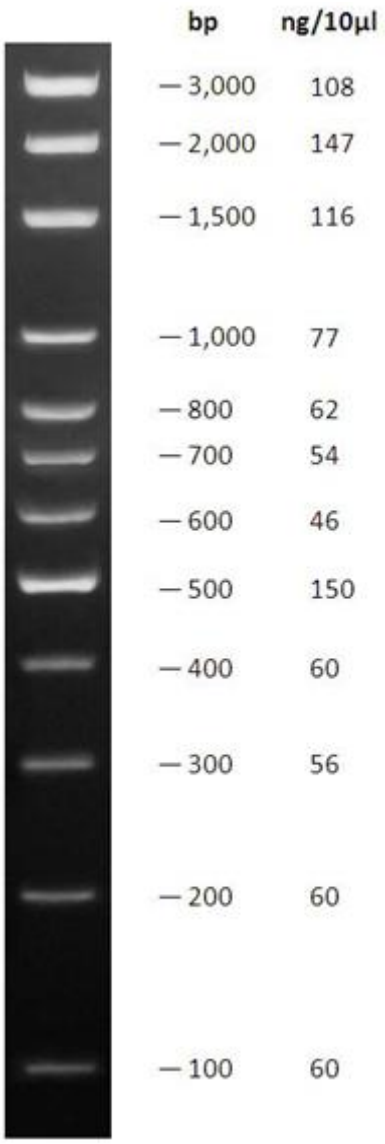
Appendix III



pLUG-Prime® cloning vector map used in this work for cloning and sequence around cloning site that shows the location of the T7 promoter, the M13 primer sites and the restriction sites. The cut sites used in this work for *Eco*RI and *Pst*I are shown in the yellow boxes. The black box represent the alternative M13 forward primer. Images were taken from the pLUG-Prime® protocol.

Restriction enzyme recognition sites for *Eco*RI and *Pst*I. Retrieved from https://www.neb.com/





100 bp ladders from GenScript (left) and Solis BioDyne (right). Retrieved from:

http://www.genscript.com/dna_ladders.html
https://www.sbd.ee/EN/products/dna_ladders/100_bp_dna_ladder_

Appendix IV

Accession Numbers of Species used for Phylogenetic Trees

Immunoglobulin Delta Tree

Homo sapiens (P01880.2), *Pan troglodytes* (ABB89455.1), *Bos taurus* (AAN03670.1), *Canis lupid familiaris* (ABB89467.1), *House Mouse* (AAB59653.1), *Rattus norvegicus* (AAO19643.1), *Ictalurus punctatus* (ADF56020.1), *Salmo Salar* (AAD43527.1), *Salmo trutta* (AAV28078.1), *Takifugu rubripes* (BAD34541.1), *Hippoglossus hippoglossus* (AAL79934.1), *Paralichthys olivaceus* (BAB41204.1), *Scophthalmus maximus* (AFQ38975.1),), *Siniperca chuatsi* (ACO88906.1), *Lutjanus sanguineus* (AIC33830.1), *Epinephelus coioides* (AFI33218.1), *Oreochromis niloticus* (AHY86392.1) and *Hydrolagus colliei* (AAC12881.1).

Recombination Activating Gene 1

Homo sapiens (NP_000439.1), *Pan troglodytes* (H2Q3F4), *Bos taurus* (F1MG60), *Mus musculus* (NP_033045.2), *Gallus gallus* (NP_001026359.1), *Seriola dumerili* (JQ938283.1), *Carassius auratus* (ABM46911.2), *Cyprinus carpio* (AAX16495.1), *Ctenopharyngodon idella* (ABM65103.1), *Danio rerio* (NP_571464.1), *Oncorhynchus mykiss* (NP_001118209.1), *Trachinus draco* (AHA61994.1), *Takifugu rubripes* (Q9W699), *Niphon spinosus* (AEK98033.1), *Lutjanus sanguineus* (AEH96360.1), *Epinephelus akaara* (AEK82141.1), *Hippoglossus hippoglossus* (AAR83678.1), *Paralichthys olivaceus* (AIK29461.1), *Serranus baldwini* (AEK98035.1), *Paralabrax clathratus* (AHA61997.1), *Carcharhinus leucas* (AAB17267.1).

Interleukin-1β

Homo sapiens (AAA74137.1), *Pan troglodytes* (JAA37246.1), *Mus musculus* (EDL28238.1), *Bos Taurus* (ABX72065.1), *Bubalus bubalis* (NP_001277827.1), *Gallus gallus* (NP_989855.1), *Xenopus laevis* (NP_001079074.1), *Oncorhynchus mykiss* (NP_001117819.1), *Salmo salar* (NP_001117054.1), *Siniperca chuatsi* (AAV65041.1), *Paralichthys olivaceus* (BAM66989.1), *Hippoglossus hippoglossus* (ACY54774.1), *Epinephelus coioides* (ABV02593.1), *Lutjanus sanguineus* (AIC33818.1), *Takifugu rubripes* (NP_001267019.1), *Sparus aurata* (CAD11603.1), *Dicentrarchus labrax* (CAC41006.1), *Scyliorhinus canicula* (CAC80866.1).

Appendix V

Hazardous Substances and New Organisms Declaration

The University of Waikato received HSNO approval (GMD101146) from the New Zealand Environmental Protection Authority (EPA) in 2011 to develop a range of genetically modified non-pathogenic microorganisms, cell lines and zebrafish carrying genes coding for proteins involved in causation of disease, in the evolution of protein stability and cellular functions. The research in this thesis meets these requirements by developing a genetically modified organism (GMO) containing the immune genes, IL-1β, IgD and RAG1 for work towards characterising the expression of these genes in *Seriola lalandi*. The location and nature of the development and the disposal of the approved genetically modified E. coli were in accordance with the APP201152 application submitted to the EPA, and controls listed in the GMD101146 approval. Details of this application can be found on the EPA website (Retrieved from http://www.epa.govt.nz/search-databases/Pages/applications-details.aspx?appID=APP201152, 2015).

Table: Raw data from generated from qPCR analysis of β-actin, GAPDH, IL-1β, IgD and RAG1.

B-actin	Name	Ct		GAPDH	Name	Ct		IL1	Name	Ct		IgD	Name	Ct		RAG	Name	Ct	
1	H1	**23.55**		1	H1	**33.65**		1	H1	**25.41**		1	H1	**30**		1	H1	**36.72**	
2	H1	**23.82**	23.685	2	H1	**34.37**	34.01	2	H1	**25.4**	25.405	2	H1	**29.97**	29.985	2	H1	**36.92**	36.82
3	H2	**35.82**		3	H2	**25.9**		3	H2	**32.16**		3	H2	**38.86**		3	H2	**32.34**	
4	H2	**34.46**	35.14	4	H2	**25.84**	25.87	4	H2	**31.51**	31.835	4	H2	**38**	38.43	4	H2	**33.76**	33.05
5	H3	**28.77**		5	H3	**32.73**		5	H3	**26.06**		5	H3	**35.12**		5	H3	**35**	
6	H3	**29.01**	28.89	6	H3	**32.61**	32.67	6	H3	**26.08**	26.07	6	H3	**36.38**	35.75	6	H3	**36.1**	35.55
7	H4	**32.49**		7	H4	**30.89**		7	H4	**23.47**		7	H4	**37.99**		7	H4	**35.52**	
8	H4	**32.25**	32.37	8	H4	**30.73**	30.81	8	H4	**23.6**	23.535	8	H4	**38.82**	38.405	8	H4	**36.8**	36.16
9	3.1	**23.15**		9	3.1	**23.02**		9	3.1	**24**		9	3.1	**32.09**		9	3.1	**33.17**	
10	3.1	**23.3**	23.225	10	3.1	**23.15**	23.085	10	3.1	**23.66**	23.83	10	3.1	**31.87**	31.98	10	3.1	**34.7**	33.935
11	3.2	**22.04**		11	3.2	**24.48**		11	3.2	**21.32**		11	3.2	**34.44**		11	3.2	**36.45**	
12	3.2	**22.07**	22.055	12	3.2	**24.46**	24.47	12	3.2	**21.03**	21.175	12	3.2	**33.47**	33.955	12	3.2	**35.15**	35.8
13	3.3	**22.59**		13	3.3	**23.46**		13	3.3	**21.08**		13	3.3	**31.65**		13	3.3	**32.7**	
14	3.3	**22.82**	22.705	14	3.3	**23.45**	23.455	14	3.3	**20.52**	20.8	14	3.3	**31.58**	31.615	14	3.3	**33.63**	33.165
15	3.4	**28.99**		15	3.4	**29.28**		15	3.4	**30.79**		15	3.4	**35.2**		15	3.4	**37.05**	
16	3.4	**28.94**	28.965	16	3.4	**29.39**	29.335	16	3.4	**30.96**	30.875	16	3.4	**35.62**	35.41	16	3.4	**37.3**	37.175
17	12.1	**33.28**		17	12.1	**32.54**		17	12.1	**22.34**		17	12.1	**35.4**		17	12.1	**36.45**	
18	12.1	**33.08**	33.18	18	12.1	**32.58**	32.56	18	12.1	**22.38**	22.36	18	12.1	**36.5**	35.95	18	12.1	**34.46**	35.455
19	12.2	**34.42**		19	12.2	**32.04**		19	12.2	**25.18**		19	12.2	**35.33**		19	12.2	**32.34**	
20	12.2	**34.85**	34.635	20	12.2	**34.11**	33.075	20	12.2	**25.17**	25.175	20	12.2	**34.36**	34.845	20	12.2	**35.64**	33.99
21	12.3	**35.56**		21	12.3	**26.7**		21	12.3	**25.16**		21	12.3	**33.62**		21	12.3	**35.93**	
22	12.3	**35.09**	35.325	22	12.3	**26.82**	26.76	22	12.3	**25.05**	25.105	22	12.3	**32.65**	33.135	22	12.3	**33.59**	34.76
23	12.4	**27.65**		23	12.4	**25.52**		23	12.4	**23.41**		23	12.4	**34.84**		23	12.4	**32.3**	
24	12.4	**27.84**	27.745	24	12.4	**25.53**	25.525	24	12.4	**23.51**	23.46	24	12.4	**36.73**	35.785	24	12.4	**34**	33.15
25	18.1	**17.8**		25	18.1	**21.76**		25	18.1	**24.33**		25	18.1	**30.72**		25	18.1	**32.02**	
26	18.1	**17.96**	17.88	26	18.1	**21.56**	21.66	26	18.1	**24.32**	24.325	26	18.1	**31.23**	30.975	26	18.1	**30.18**	31.1
27	18.2	**15.94**		27	18.2	**20.36**		27	18.2	**26.87**		27	18.2	**30.28**		27	18.2	**29.69**	
28	18.2	**16.12**	16.03	28	18.2	**20.23**	20.295	28	18.2	**27**	26.935	28	18.2	**29.7**	29.99	28	18.2	**29.76**	29.725
29	18.3	**16.26**		29	18.3	**19.75**		29	18.3	**22.51**		29	18.3	**29.46**		29	18.3	**29.42**	
30	18.3	**16.02**	16.14	30	18.3	**19.72**	19.735	30	18.3	**22.16**	22.335	30	18.3	**29.26**	29.36	30	18.3	**30.13**	29.775
31	18.4	**16.1**		31	18.4	**20.06**		31	18.4	**26.05**		31	18.4	**29.8**		31	18.4	**30.51**	
32	18.4	**16.19**	16.145	32	18.4	**20.2**	20.455	32	18.4	**25.86**	24.8875	32	18.4	**29.83**	29.815	32	18.4	**30.45**	30.48

Table: Summary of averages obtained from above table

	b-actin	gapdh	G. Mean	IL1	IgD	RAG
H1	23.685	34.01	28.3818	25.405	29.985	36.82
H2	35.14	25.87	30.15082	31.835	38.43	33.05
H3	28.89	32.67	30.72192	26.07	35.75	35.55
H4	32.37	30.81	31.58037	23.535	38.405	36.16
3.1	23.225	23.085	23.15489	23.83	31.98	33.935
3.2	22.055	24.47	23.23114	21.175	33.955	35.8
3.3	22.705	23.455	23.07695	20.8	31.615	33.165
3.4	28.965	29.335	29.14941	30.875	35.41	37.175
12.1	33.18	32.56	32.86854	22.36	35.95	35.455
12.2	34.635	33.075	33.84601	25.175	34.845	33.99
12.3	35.325	26.76	30.74568	25.105	33.135	34.76
12.4	27.745	25.525	26.61186	23.46	35.785	33.15
18.1	17.88	21.66	19.67945	24.325	30.975	31.1
18.2	16.03	20.295	18.03687	26.935	29.99	29.725
18.3	16.14	19.735	17.84721	22.335	29.36	29.775
18.4	16.145	20.455	18.17267	24.8875	29.815	30.48

Table: ΔCq values obtained using values from table above

	IL1	IgD	RAG
H1	7.872408	0.329147	0.002883
H2	0.311179	0.003219	0.134048
H3	25.14011	0.030648	0.035205
H4	264.1784	0.008822	0.041821
Average	**74.37553**	**0.092959**	**0.053489**
StdError	**63.48062**	**0.078951**	**0.028168**
3.1	0.626286	0.002205	0.000569
3.2	4.15872	0.000591	0.000165
3.3	4.846534	0.00269	0.000919
3.4	0.302375	0.013043	0.003838
Average	**2.483479**	**0.004632**	**0.001372**
StdError	**1.17604**	**0.002839**	**0.000836**
12.1	1456.751	0.118137	0.166494
12.2	407.6009	0.500351	0.905015
12.3	49.89013	0.190873	0.061883
12.4	8.888011	0.001732	0.01076
Average	**480.7824**	**0.202773**	**0.286038**
StdError	**337.4194**	**0.106566**	**0.208855**
18.1	0.039953	0.000398	0.000365
18.2	0.002096	0.000252	0.000303
18.3	0.04457	0.000342	0.000257
18.4	0.00952	0.000313	0.000197
Average	**0.024035**	**0.000326**	**0.00028**
StdError	**0.010673**	**3.03E-05**	**3.55E-05**

ANOVA

IL1	H	Day 3	Day 12	Day 18
	7.872408	0.626286	1456.751	0.039953
	0.311179	4.15872	407.6009	0.002096
	25.14011	4.846534	49.89013	0.04457
	264.1784	0.302375	8.888011	0.00952

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
H	4	297.5021	74.37553	16119.15
Day 3	4	9.933916	2.483479	5.53228
Day 12	4	1923.13	480.7824	455407.3
Day 18	4	0.096139	0.024035	0.000456

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	635767.7	3	211922.6	1.797736	**0.201172**	3.490295
Within Groups	1414596	12	117883			
Total	2050364	15				

IgD	H	Day 3	Day 12	Day 18
	0.329147	0.002205	0.118137	0.000398
	0.003219	0.000591	0.500351	0.000252
	0.030648	0.00269	0.190873	0.000342
	0.008822	0.013043	0.001732	0.000313

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
H	4	0.371836	0.092959	0.024933
Day 3	4	0.018529	0.004632	3.22E-05
Day 12	4	0.811094	0.202773	0.045425
Day 18	4	0.001305	0.000326	3.68E-09

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.108705	3	0.036235	2.059082	**0.159303**	3.490295
Within Groups	0.211172	12	0.017598			
Total	0.319877	15				

RAG	H	Day 3	Day 12	Day 18
	0.002883	0.000569	0.166494	0.000365
	0.134048	0.000165	0.905015	0.000303
	0.035205	0.000919	0.061883	0.000257
	0.041821	0.003838	0.01076	0.000197

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
H	4	0.213956	0.053489	0.003174
Day 3	4	0.00549	0.001372	2.8E-06
Day 12	4	1.144152	0.286038	0.174482
Day 18	4	0.001122	0.00028	5.04E-09

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.222319	3	0.074106	1.668511	**0.226345**	3.490295
Within Groups	0.532976	12	0.044415			
Total	0.755295	15				