Antimicrobial Peptides in Jawed and Jawless Vertebrates

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Abstract

Antimicrobial peptides (AMPs) are a major part of the innate immune defence system which shows a broad spectrum of activity, defending the host against invading microbes. The aim of this work was to identify the AMPs present in yellowtail kingfish (*Seriola lalandi*) and pouched lamprey (*Geotria australis*) and use molecular techniques to fully sequence their cDNA and quantify their expression in adult individuals.

Using bioinformatic approaches candidate AMP genes were ascertained from available *S. lalandi* and *G. australis* RNA-seq transcriptomic databases, obtained from various tissues. Selected AMPs were chosen to have their full cDNA sequence amplified using RACE-PCR, which were then cloned and sequenced. Complete cDNA sequences were obtained for *S. lalandi* hepcidin and moronecidin, whereas attempts to complete the *G. australis* defensin-like cDNA were unsuccessful. Comparison of the *S. lalandi* hepcidin and moronecidin protein sequences with proteins already characterised in other fish showed good homology and conservation of important features. In addition, specific primers were designed to examine the expression levels of *S. lalandi* hepcidin and moronecidin in gill, liver or spleens of three fish. Analysis showed hepcidin expression to be highest in liver tissues, whereas moronecidin expression was highest in the gills and spleens.

This study provides a comprehensive overview of the AMP genes present in *S. lalandi* and *G. australis* and some initial characterisation of *S. lalandi* hepcidin and moronecidin, which will permit the development of future research applications. Overall, characterising AMP genes in jawed and jawless vertebrates is vital for economical and successful fish farming, while also providing possible therapeutic benefits associated with AMP research in biomedicine and disease in wild fish stocks.

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List of Abbreviations

α	Alpha
β	Beta
θ	Theta
˚C	Degrees Celsius
AAP	Abridged Anchor Primer
ACTH	Adrenocorticotrophic Hormone
AMP	Antimicrobial Peptide
apoA	Apolipoprotein-A
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
cDNA	Complementary DNA
CIP	Calf Intestine Alkaline Phosphatase
CLR	C-Type Lectin Receptor
Cq	Quantification Cycle
Ct	Threshold Cyle
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide Triphosphates
E	Efficiencies
EGC	Eosinophilic Granule Cell
EST	Expressed Sequence Tags
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
gDNA	Genomic Deoxyribose Nucleic Acid
HD1	Human Defensin 1
HDL	High Density Lipoprotein
HSDF-1	Histone Derived Fragment 1
HSNO	Hazardous Substances and New Organisms
IFN	Interferon
IgM	Immunoglobulin M
IL	Interleukin
IPNV	Infectious Pancreatic Necrosis Virus
IPTG	Isopropyl β-D-1-Thiogalctopyranoside
LB	Lysogeny Broth
LCRP	Lamprey Corticostatin Related Peptide
LEAP	Liver Expressed Antimicrobial Peptide
LPS	Lipopolysaccharides
LRS	Lamprey Reddening Syndrome
MAC	Membrane Attacking Complex
MHC	Major Histocompatibility Complex
M-MLV	Maxima M-MuLV
mRNA	Messenger RNA
MRSA	Methicillin Resistant *Pseudomonas aeruginosa*
NCC	Non Specific Cytotoxic Cell
NGS	Next Generation Sequencing
NK	Natural Killer

NLR	Nucleotide Binding Oligomerisation Domain Like Receptors
NNV	Nervous Necrosis Virus
PAMPs	Pathogen Associated Molecular Patterns
PBLs	Peripheral Blood Lymphocytes
PCR	Polymerase Chain Reaction
PID	Primary Immunodeficiency
PRRs	Pattern Recognition Receptors
RACE	Rapid Amplification of cDNA Ends
RBCs	Red Blood Cells
RLM	RNA Ligase Mediated
RLR	Rig-1-Like Receptor
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNA-seq	RNA Sequencing
RSIV	Red Sea Bream Iridovirus
RT-PCR	Real-Time Polymerase Chain Reaction
SVCV	Spring Viraema of Carp Virus
TAE	Tris-Acetate Ethylenediaminetetraacetic Acid Buffer
TAP	Tobacco Acid Pyrophosphatase
Taq	*Thermus aquaticus* DNA Polymerase
TLRs	Toll-Like Receptors
TNF	Tumour Necrosis Factor
UAP	Abridged Universal Amplification Primer
UTR	Untranslated Region
VHSV	Viral Haemorrhagic Septicaemia Virus
VRE	Vancomycin Resistant *Enterococcus faecalis*
Xgal	5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside

Chapter One: Introduction and Literature Review

1.1 Vertebrate Immunity

The immune system is an organisation of biological structures that processes within an organism protecting them against disease such as renegade body cells (cancer cells) and invading pathogens such as viruses, bacteria, fungi, and parasites (Buchmann, 2014). For a pathogen, the vertebrate body is an ideal habitat offering nutrients and a protected environment for growth, reproduction, and transportation to new hosts (Campbell *et al.*, 2009). Therefore, vertebrates have evolved a specialised means of searching out and destroying foreign cells (Pough *et al.*, 2009). In all vertebrate species including humans, the immune system can be classified into two subsystems, the innate immune system and the adaptive immune system (Zhu, *et al.*, 2013). The innate immune system is a non-specific line of defence and considered a primitive form of immunity as aspects of it can also be found in plants and invertebrates (Buchmann, 2014). The innate immune system is active immediately upon infection, where the response is the same each time regardless of whether the pathogen has been encountered before (Campbell, *et al.*, 2009). In contrast, the adaptive immune system provides specific responses to infection where each response is tailored to a particular invader taking time to develop following infection (Pough, *et al.*, 2009). This system is restricted to vertebrates and composed of highly specialised cells with immunological memory (Rauta *et al.*, 2012). Jawed vertebrates are the only phylum to have an adaptive immune system where each response is based on the coordinated activities of specialised cells, T and B lymphocytes (Chaplin, 2010). These cell types carry out two major types of adaptive immunity, cell mediated immunity and antibody mediated immunity (Martini & Nath, 2009). Cell mediated immunity is T cell activated which provides defence against abnormal cell types and intracellular pathogens (Pough, *et al.*, 2009). Whereas, antibody mediated immunity is B cell activated and works extracellularly (does not enter cells), defending against antigens and pathogens in the body fluids (Martini &

Nath, 2009). Jawless vertebrates also possess an adaptive immune response, although unique cells and effector molecules are involved (Zhu, *et al.*, 2013). Understanding vertebrate immunology provides a fascinating insight into the interaction between animals, and the organisms that infect them (Buonocore, 2011). Overall, vertebrate immunity is a complex network of systems integral to the survival and success of vertebrate organisms (Chaplin, 2010; Buchmann, 2014).

1.2 Why Study Non-Mammalian Immunology?

A large amount of research has been conducted over the last 20 years to characterise the immune responses that organisms outside of mammals are capable of. As well as providing a fascinating insight into the interactions between animals, and the organisms that infect them (Buonocore, 2011), there are a number of useful applications, which are outlined below.

1.2.1 Understanding Immune System Evolution

The innate immune system has origins in ancient evolutionary history reaching as far back as early metazoan (proifera) over 1 billion years ago (Litman & Cooper, 2007). Whereas, the origin of the adaptive immune system can be traced back to jawed vertebrates 450 million years ago with the appearance of RAG genes (Magnadóttir, 2006). The vital role played by the innate immune system is evident by the efficient immune defence found in invertebrates, as they are dependent exclusively on their innate immune system to protect them from a wide range of pathogens in diverse environmental conditions (Magnadóttir, 2006). Increased diversity, maturation and memory has enhanced the evolution of the adaptive immune system as seen in mammals (Chaplin, 2010). Non classical animal models are useful in defining the evolutionary history of immune systems and provide the basis for discovery of unknown molecules and biochemical pathways in mammalian immunity (Sunyer, 2013). There is significant overlap in the complex immune systems of amphibians, reptiles, aves and mammals (Table 1). Thus, knowledge of overlapping immune parameters between multicellular organisms highlights functionally interconnected immune systems with shared ancestry and co-evolution (Litman & Cooper, 2007). Overall, understanding

descent is indicative of the evolutionary forces which underlie diversification of immune parameters (Boehm, 2012).

Table 1: Comparative overview of amphibian, reptilian, avian and mammalian immune systems.Table re-created from information retrieved from Zimmerman *et al.* (2010).

	Amphibians	Reptiles	Aves	Mammals
Innate				
Antimicrobial peptides	✓	✓	✓	✓
Complement	✓	✓	✓	✓
Non-specific leukocytes				
Macrophages	✓	✓	✓	✓
Heterophils/neutrophils	✓	✓	✓	✓
Basophils	✓	✓	✓	✓
Eosinophils	✓	✓	✓	✓
Inflammation				
Fever	✓ (behavioural)	✓ (behavioural)	✓	✓
Adaptive				
Cell-mediated				
MHC (Class I, II)	✓	✓	✓	✓
T-cell receptor	✓	✓	✓	✓
Humoral				
Antibody heavy chain isotypes	IgM, IgX, IgY, IgD, IgF	IgM, IgY, IgA?, IgD?	IgM, IgY, IgA, IgD	IgM, IgG, IgA, IgD, IgE
Light chain isotypes	λ, κ, σ	λ, κ	λ	λ, κ
Phagocytic B cells	✓	✓	?	×
RAG	✓	✓	✓	✓
Class switching	✓	✓	✓	✓
Affinity maturation	×	×	Poor	✓
Somatic hypermutation	✓	✓	✓	✓

1.2.2 Discovering Novel Mechanisms of Immunity

The use of non-mammalian models to uncover novel mechanisms of immunity has improved with the recent advances in genetic technologies (Sunyer,

2013). Using these technologies researchers are able to use more accessible and less expensive non-mammalian models to gain insight into the vertebrate immune system. This has allowed similarities in immune parameters between mammalian vertebrates and their non-mammalian counterparts to be elucidated (Boehm, 2012). Elie Metchnikoff contributed to the birth of immunology in 1882 with the discovery of phagocytosis by amoebocytes found in echinoderms (Sunyer, 2013). From Metchinkoff's discovery self versus non-self immune cell recognition was proposed, thus highlighting the importance of non-mammalian models as far back as the 1800's (Litman & Cooper, 2007). Toll receptors are another example, which were first discovered in the fruit fly *Drosophila melanogaster* from which toll-like genes were consequently discovered in vertebrates (Magnadóttir, 2006). These receptors which are a part of the innate immune system have been found to be crucial mediators in immune responses (Litman & Cooper, 2007). A recent example of non-mammalian immune discoveries is highlighted by the finding that teleost B cells are professional phagocytes (Sunyer, 2013). This finding in teleost B cells led to the discovery that mammalian B cells are also phagocytic. Overall, non-mammalian immune research has greatly expanded the knowledge of the diverse vertebrate immune system through the discovery of novel immune mechanisms.

1.2.3 Developing Alternative Models for Immune Research

Using non-mammalian models for scientific research is preferable as they have faster reproduction rates than mammals, they are inexpensive to house, and typically they are genetically simpler (Tobia *et al.*, 2011; Kounatidis & Ligoxygakis, 2012). Therefore, when studying aspects of mammalian disease using other vertebrate species as animal models may be preferential. As an example, from a human perspective, zebrafish (*Danio rerio*) have been used to study primary immunodeficiency (PID) which is characterised by a functionally abnormal immune system (Iwanami, 2014). Zebrafish posses several immune parameters including T and B lymphocytes which resemble their mammalian counterparts and, therefore, genetic manipulation can be used to mimic these PID disorders (Trede *et al.*, 2004). Using this model, drug therapies can be tested, one such being haematopoietic stem cell transplantation which has emerged as a

treatment option for PID patients (Iwanami, 2014). Mutagenesis screenings can also be applied to specific developmental pathways to understand disease pathology and identify immune aspects as potential therapeutic agents (Litman & Cooper, 2007). For example, human short and long QT syndrome can be mimicked via mutation in the zebrafish homolog hERG potassium channel gene (Staudt & Stainier, 2012). Thus, various drug treatment schemes can be carried out on these zebrafish models to determine their effects on the mimicked short or long QT syndrome (Staudt & Stainier, 2012).

1.2.4 Understanding Immunity in Commercial Species

In the commercial world, farming of non-mammalian organisms is mainly dominated by poultry farming and aquaculture (Chemineau *et al.*, 2007). Aquaculture is the farming of fresh and salt water aquatic species for human consumption, including organisms such as molluscs, crustaceans, fish and aquatic plants (Naylor *et al.*, 2001). Approximately 600 aquatic species are farmed worldwide, with three species currently farmed in New Zealand, King salmon (*Oncorhynchus tshawytscha*), green lipped mussel (*Perna canaliculi*) and Pacific oyster (*Crassostrea gigas*) (Brudeseth *et al.*, 2013). Aquaculture is becoming increasingly necessary in the developed world because of both rising population sizes and decreasing wild fish stocks from recreational and commercial fishing (Dimitroglou *et al.*, 2011). Aquaculture is a promising field for global food production but growth in this industry has been constrained by high mortality rate in young fish, abnormalities during development and adult disease (Miller *et al.*, 2011). Therefore, it is vital to develop tools that will increase survival and quality of these fish under farmed conditions (Stewart Fielder, 2013). Greater success of species in aquaculture will be possible through the understanding the immune system of farmed species which will give insights into their development, health, growth and reproduction (Agawa *et al.*, 1991). An example of a method currently being used to improve aquaculture success is vaccination (Brudeseth, *et al.*, 2013). A number of countries in the world have implemented vaccine administration in aquaculture (Figure 1). In Norway all Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) are vaccinated against the three major bacterial diseases, vibriosis, cold water vibriosis, and furunculosis (Hastein *et al.*, 2005).

Vaccination of fish has reduced the use of antibiotics in aquaculture, which has been highlighted in Norway within the salmon industry (Brudeseth, *et al.*, 2013). In 1987, 50,000kg of antibiotics were being used, however, the introduction of a water-in-oil emulsion vaccine drastically reduced antibiotic use to 1000kg by 1997 (Brudeseth, *et al.*, 2013). During this same period salmon fish production increased from 50,000 tonnes to 350,000 tonnes. Despite vaccines improving the survival rate of farmed fish they still need a significant amount of improvement which will be achieved by further understanding the immune system of farmed fish species (Van Muiswinkel, 2008).

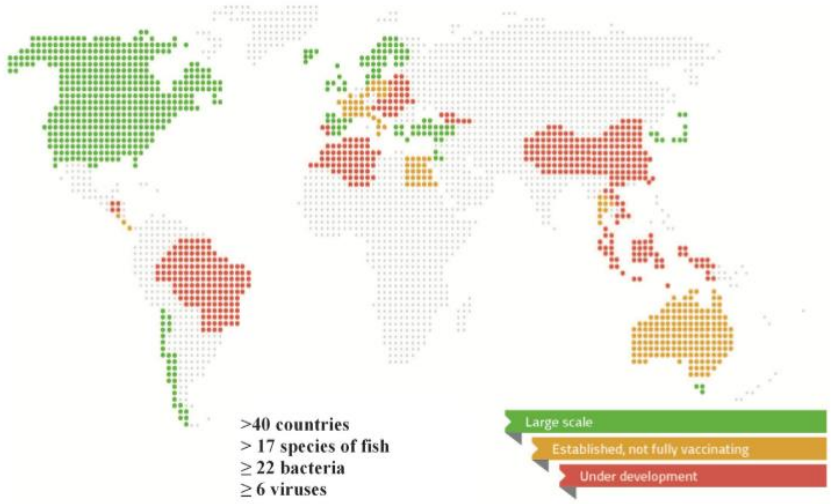

Figure 1: Countries that have implemented vaccination in aquaculture categorised by colour. Green shows common use of vaccines, orange highlights countries that use vaccines although are not fully implemented, and red shows countries where vaccination is under development. Retrieved from Brudeseth, *et al.* (2013).

1.2.5 Why fish?

Fish immunology is of particular interest because fish are the earliest known vertebrate class with the presence of both innate and adaptive immunity (Magnadóttir, 2006). It is assumed 10^{29} prokaryotic organisms exist in the ocean environment (Tort *et al.*, 2003) and, therefore, a fishes body is continuously subjected to contact with micro-organisms, making them crucial in the search for potential pharmaceuticals (Cantisani *et al.*, 2014). Studying immunity in fish provides us with insights into immune system evolution as fish are significantly more primitive than popular mammalian research models (Zhu, *et al.*, 2013). In particular innate immune parameters, where fish share aspects with both

invertebrates and higher vertebrates such as mammals (Falco *et al.*, 2012). Fish also represent 43% of extant vertebrates and investigating the immune system of a range of fish species will provide insight into how they maintain such diversity within a huge range of environments (deep sea, lakes, rivers) (Tort, *et al.*, 2003; Scapigliati, 2013). Studying fish innate immunity will also allow for improvements to aquaculture systems around the world, as understanding the immune system of a fish will allow the management of diseases and pathogens that are prevalent within current aquaculture systems (Alvarez-Pellitero, 2008). Overall, understanding fish immune parameters is of significant importance in many areas including biomedical therapeutics, evolutionary research, and commercial successes in aquaculture.

1.3 Immunity in Fish

The innate immune system in fish is a fundamental defence mechanism as it precedes adaptive immunity, determines the nature of the adaptive immune response, and is vital for the maintenance of homeostasis (Magnadóttir, 2006). Fishes depend heavily on their innate immune system considerably more throughout their lives than mammals, which is primarily due to their evolutionary status and poikilothermic nature (Ravichandran *et al.*, 2010; Uribe *et al.*, 2011). The innate immune system in fish plays a central role in the response to infections as fish become free living at an early embryonic stage, therefore, relying on their innate immune defences for extended periods of time (Uribe, *et al.*, 2011). The adaptive immune system in fish is inactive until they are completely developed and even at that point their adaptive immune system only displays rudimentary immunological memory, where memory is significantly more developed in higher vertebrates (Buonocore, 2011). This is due to the evolutionary status of fish, as they mainly exhibit immunoglobulin-M (IgM) like responses (produced by B cells), in contrast to the complex response seen in mammals (Falco, *et al.*, 2012). In addition, the resulting adaptive immune system has restricted memory, and slow lymphocyte proliferation (Magnadóttir, 2006). The adaptive immune response in fish is, therefore, more sluggish, sometimes taking up to 12 weeks to develop, compared to the relatively instant and temperature independent innate immune system (Magnadóttir, 2006). Therefore, understanding the innate immune

system and how it functions in fish will provide vital information on the evolution, working mechanisms and various components of immune responses applicable to humans (Falco, *et al.*, 2012).

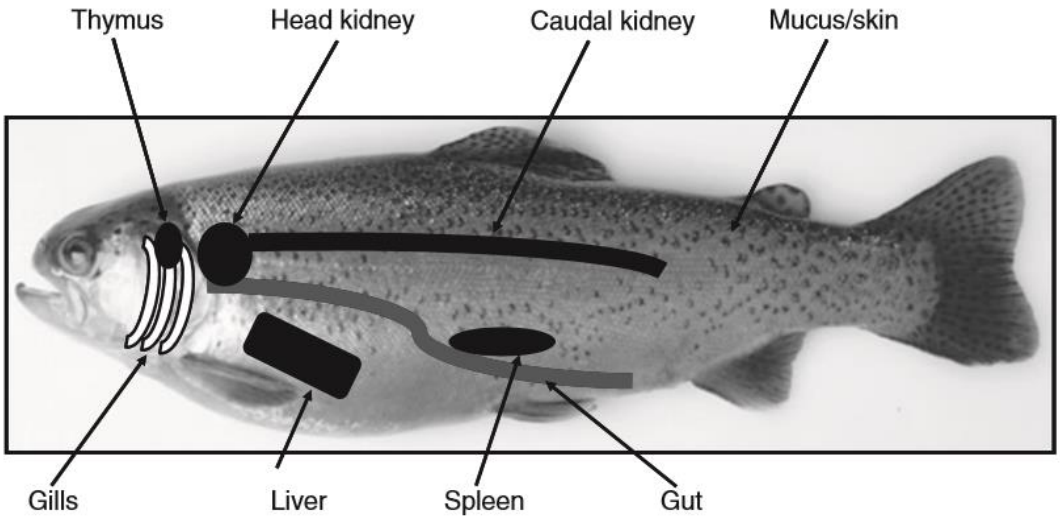

Figure 2: Major immune organs in teleosts with approximate sites overlaid onto a rainbow trout (*Oncorhynchus mykiss*). Taken from Secombes and Wang (2012).

Within fish the immune system is made up of several lymphoid tissues (Figure 2), with the largest being the thymus, kidney (anterior and middle) and the spleen (Uribe, *et al.*, 2011). The kidney is a Y-shaped organ that is dispersed along the body axis where the lower part of the kidney (tail end) works as a renal system and the head-kidney is the active immune part of the organ (Tort, *et al.*, 2003). The head-kidney assumes haematopoietic functions similar to that seen in the bone marrow of mammals producing erythrocytes, macrophages and plasma cells (Zapata *et al.*, 2006). The head-kidney is also the principle immune organ responsible for phagocytosis, antigen processing and immune memory (Uribe, *et al.*, 2011). The thymus located near the opercula cavity in fish is generally considered an aggregation of macrophages and this is where T and B lymphocytes are produced (Tort, *et al.*, 2003). Myeloid cells and eosinophilic granular cells are also found here, although functionally the thymus cannot be universally generalised as the thymic structure is highly variable throughout different fish species (Uribe, *et al.*, 2011). The spleen in fish is similar in structure and function as a large mammalian lymph node, where they principally operate as a filter for circulating blood (Zapata, *et al.*, 2006). Red blood cells, lymphocytes and

macrophages are also developed and housed here (Iwanami, 2014). Agnatha are the only vertebrates that do not contain a spleen and instead contain haematopoietic tissue within the gut wall which is assumed to have homologous functions to the vertebrate spleen (Tort, *et al.*, 2003). Interestingly, fish are a heterogeneous group where immune organs can vary between species, although overall there is some similar homology to immune organs found within mammals.

1.4 Innate Immunity in Fish

The innate immune system is the front line of defence, playing a major role in pathogen resistance (Secombes & Wang, 2012). Innate immunity begins with discrimination of self from non-self cells followed by an immune response which can be broadly divided into three groups; physical barriers, cellular components and humoral parameters (Figure 3).

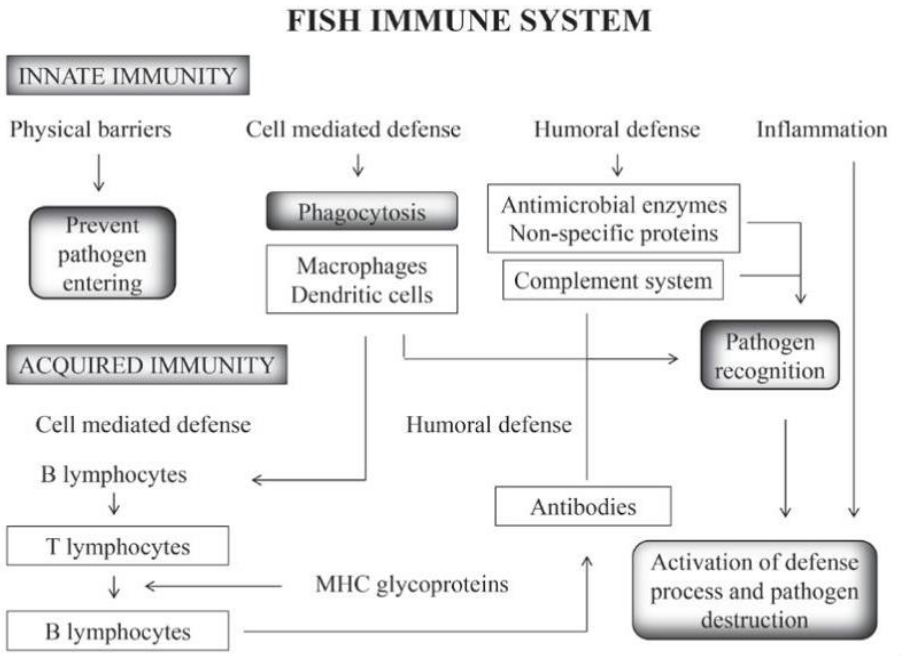

Figure 3: Overview of the immune system in fish. Taken from Biller-Takahashi and Urbinati (2014).

1.4.1 Discrimination of Self from Non-Self

In order for a successful immune response, an organism must first distinguish self from non-self (Buchmann, 2014). The innate immune system

distinguishes self and non-self cells through germ line encoded pattern recognition receptors (PRRs) that recognise specific molecular patterns characteristic of microbes, referred to as pathogen associated molecular patterns (PAMPs) (Magnadóttir, 2006). PAMPs are ideal structures for PRRs to detect, as they are essential for the survival of a pathogen. PAMPs are molecules that are highly conserved within the prokaryotic phylum and are not normally found in eukaryotic cells, for example, lipopolysaccharides (LPS), double stranded viral RNA, peptidoglycan, bacterial DNA, and polysaccharides (Magnadóttir, 2006). Once PRRs are activated recognition molecules can signal for destruction or phagocytosis of the pathogen (Buchmann, 2014). There are four main families of PRRs that have been described in vertebrates, including Toll-like receptors (TLRs), retinoic acid inducible gene 1-like receptors (RLRs), nucleotide-binding oligomerisation domain-like receptors (NLRs) and C-type lectin receptors (CLRs) (Zhu, *et al.*, 2013). TLRs were the first type of PRRs described and are consequently the most understood. TLRs are type-1 transmembrane proteins that are typically expressed on sentinel cells which recognise the structurally conserved PAMPs expressed on microbes (Alvarez-Pellitero, 2008). TLRs are composed of an intracellular complex and extracellular domain where receptor ligand binding initiates a complex series of steps, resulting in the activation of an immune response consequently inactivating or eliminating an invader (Buchmann, 2014). Various TLR types can be found within vertebrates where each receptor shows individual affinities for a range of PAMPs (Alvarez-Pellitero, 2008). Recent research has established that fish TLRs have distinct features and greater diversity in comparison to mammals (Zhu, *et al.*, 2013). At least eighteen TLRs have been found in teleosts and seven in agnatha, in comparison to thirteen found in mammals (Chaplin, 2010). TLRs have also been linked to some of the most primitive multicellular organisms such as sponges, molluscs (snails and octopus) and oligochaetes (earthworms) (Alvarez-Pellitero, 2008; Chaplin, 2010; Zhu, *et al.*, 2013; Buchmann, 2014). Fish have been shown to contain a unique family of TLRs which likely arose through gene duplication, although the functionality of these TLRs are largely unknown (Palti, 2011). Future research into fish specific TLRs and immunity will aid in developing the understanding of disease resistance in fish (Palti, 2011). Overall, PRRs are vital for vertebrates to distinguish self

from non-self cells in order to initiate an immune response to destroy or inactivate an invader.

1.4.2 Physical Barriers

The first line of defence a pathogen must evade before attempting to colonise the host is barrier defences, also known as the integumentary system. The integumentary system in fish is made up of scales, skin and mucosal surfaces of the gills and epidermis (Tort, *et al.*, 2003; Magnadóttir, 2006). Each aspect of the integumentary system acts as a physical obstacle for invading pathogens (Martini & Nath, 2009). This form of simple defence is one of the most primitive but has been shown to be surprisingly successful. Experimentally, it has been shown that salmon immersed in water inoculated with a potent fish pathogen, *Aeromonas salmonidica*, can resist microbial invasion into the body simply through the presence of physical barriers (Tort, *et al.*, 2003; Magnadóttir, 2006). In addition to skin, mucus is also an important physical barrier in fish (Anbuchezhian *et al.*, 2011). Mucus is a viscous fluid that acts by trapping microbes and other particles while also providing substrate for antibacterial mechanisms to act (Campbell, *et al.*, 2009). It has been shown that mucus contains several immune products, (Cantisani, *et al.*, 2014) such as, lysozyme, pentraxins, antimicrobial peptides (AMPs), and complement proteins, which can act directly on invading pathogens and kill them (Magnadóttir, 2006). Mucus is produced by mucosal membranes where it is secreted to cover most external surfaces including skin and within the mouth (Tort, *et al.*, 2003; Alvarez-Pellitero, 2008). In addition, microbes that attempt to enter the body through ingested food and water have to contend with the acidity of the stomach, which kills most microbes before they can move further through the digestive tract (Campbell, *et al.*, 2009). Overall, physical barriers are the first line of defence in fish where all orifices show some form of defence aiding in the prevention of pathogen colonisation (Martini & Nath, 2009). However, some pathogens are able to evade this form of defence, which is where the cellular and humoral aspects of the innate immune system then come into play.

1.4.3 Cellular Components

Cell mediated immunity is a protective function associated with cells, where specialised cells such as macrophages directly destroy pathogens or secrete various cytokines and proteins resulting in pathogen death. The key cells in the innate immune response of fish are non-specific cytotoxic cells (NCC), monocytes, macrophages, dendritic cells, and other phagocytic cells such as granulocytes (Figure 4).

Fish NCCs are thought to be an evolutionary precursor to mammalian natural killer (NK) cells which are large granular lymphocytes (Alvarez-Pellitero, 2008). NCCs are seen as morphologically distinct from mammalian NK cells although they are thought to be functionally similar (Uribe, *et al.*, 2011). In fish NCCs are localised in lymphoid tissues such as the anterior kidney and spleen (Shen *et al.*, 2002) where their role is to target and kill invading parasitic organisms, such as protozoan parasites, virally transformed cells and tumour cells (Secombes & Wang, 2012). NCCs have been found in an array of fish species including, tilapia (*Oreochromis mossambicus*) (Faisal *et al.*, 1989), crucian carp (*Carassius auratus langsdorfii*) (Somamoto *et al.*, 2013) and rainbow trout (*Oncorhynchus mykiss*) (Greenlee *et al.*, 1991).

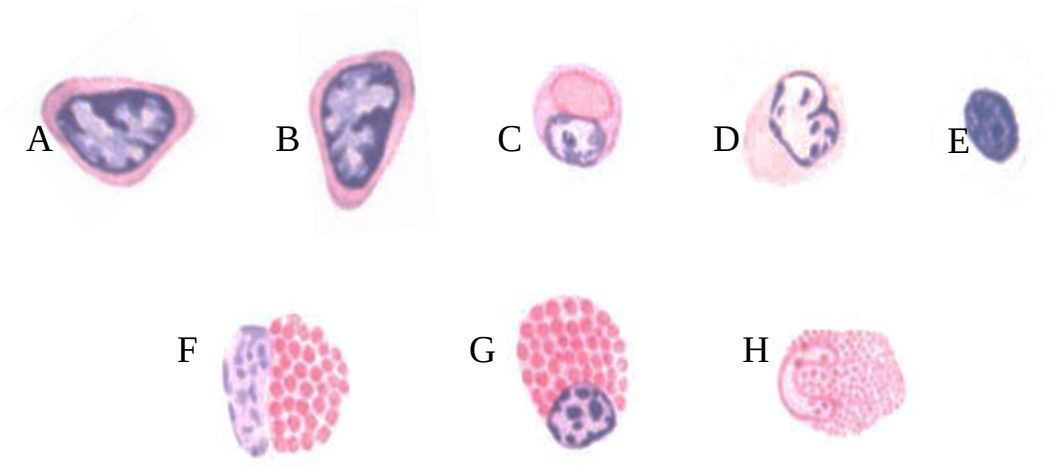

Figure 4: Lymphocytes of fish. From the blood of a skate (*Raja ocellata*), large lymphocytes (A&B), neutrophil granulocytes (D&E) and an eosinophilic granulocyte F&G). From the kidney cells of a goldfish (*Carassius auratus*) a macrophage containing a haemoglobin globule (C). Lastly, from the kidney cells of a buffalo fish (*Ictiobus bubalis*) a pseudo-eosinophilic granulocyte (H). Taken from Jordan and Speidel (1924).

Monocytes are the largest of all leukocytes, where high numbers are found to be stored in the spleen of a fish (Uribe, *et al.*, 2011). They are phagocytic cells which circulate throughout the body of a fish with the ability to differentiate into macrophages or dendritic cells, both of which are antigen presenting cells (Secombes & Wang, 2012). Initial detection of pathogens is typically carried out by tissue resident macrophages that ubiquitously express PRRs (Secombes & Wang, 2012). Macrophages are antigen presenting cells that are able to bind to invading pathogens and directly phagocytose them (Neumann *et al.*, 2001), while also releasing cytokines to further increase the immune response (Secombes & Wang, 2012). Macrophages also show remarkable plasticity and even when not fighting infection can play an important role in homeostasis where they clear cellular debris and foreign waste products (Buchmann, 2014). Dendritic cells are typically expressed in tissues that are in contact with the external environment (Bassity & Clark, 2012), they are also phagocytic antigen presenting cells, acting as a messenger between innate and adaptive immunity (Shao *et al.*, 2015).

Another group of immune cells are granulocytes which includes neutrophils, eosinophils, basophils and mast cells. Neutrophils are the first immune cells recruited during a pathogen invasion and migrate to the site of infection. They have been shown to kill pathogens via phagocytosis and the release of AMPs, degenerative enzymes and toxic metabolites at the site of infection (Neumann, *et al.*, 2001). The main purpose of neutrophils is to destroy pathogens at the site of infection and recruit other immune cells (Uribe, *et al.*, 2011). Neutrophils have been found in several fish species including, zebrafish (Keightley *et al.*, 2014), Atlantic cod (Øverland *et al.*, 2010), and channel catfish (Bly *et al.*, 1990). Eosinophils are granular leukocytes that are a vital part of mammalian immunity maintaining antimicrobial and ribonuclease properties, while also playing a major role in allergic reactions (Ainsworth, 1992). Eosinophils have been implicated in many aspects of fish immunity including parasite killing and attraction of leukocytes to the area (Ainsworth, 1992), however, their precise function remains enigmatic (Alvarez-Pellitero, 2008). Eosinophilic-like cells have been observed in zebrafish (*Danio rerio*) (Balla *et al.*, 2010), sturgeon (*Acipenser brevirostrum*) (Hine, 1992), striped bass (*Morone saxatilis*) (Bodammer, 1986) and many other species of fish (Ainsworth, 1992).

Basophils found in mammals are utilised in helminth (parasitic worm) infection, and also release histamine in response to an allergic reaction (Ainsworth, 1992). They are similar in appearance to mast cells, although differ due to their location, as mast cells are tissue resident, whereas, basophils are found in blood (Hine, 1992). In fish basophil existence has previously been debated, attributed to difficulty in defining cells based on various parameters, such as function, morphology, and/or ontogeny. However, basophil-like cells have been identified in several marine and freshwater fish species which include, zebrafish (*Danio rerio*) (Balla, *et al.*, 2010), carp (*Cyprinus carpio*) (Hamers, 1995), pond loach (*Misgurnus anguillicaudatus*) (Ainsworth, 1992) and sturgeon (*Acipenser brevirostrum*) (Hine & Wain, 1988). It is important to note that functional aspects of fish basophils have not yet been explored, due to studies focusing mainly on basophilic morphology and because only limited amounts of basophils can be isolated from fish, restricting the possibility of functional assays (Ainsworth, 1992).

Mammalian mast cells originate in bone marrow and mature in peripheral tissues where they play a role in inflammation, defence against invading pathogens, and in allergic reactions as they release histamine (Secombes & Wang, 2012). Mast cells are important functionally in immunity and are active against a wide range of pathogens due to the expression of biologically active proteins such as lysozyme, AMPs and alkaline and acid phosphatases (Silphaduang & Noga, 2001). The presence of mammalian-like mast cells in fish has strongly been debated due to differences in tissue distribution and histochemical staining (Sfacteria *et al.*, 2015). It has now been widely accepted that fish contain analogous mast cells to mammals as the main functional roles exploited by the immune system are similar (Reite & Evensen, 2006). Fish mast cells are sometimes referred to as eosinophilic granule cells (EGC), as historical studies have shown the presence of an eosinophilic domain through histochemical staining (Reite & Evensen, 2006; Dezfuli *et al.*, 2012). In fish, mast cells have been found in the intestine, gills, skin, respiratory system and reproductive systems (Secombes & Wang, 2012), their presence has also been associated with the hypothalamus and pancreas (Sfacteria, *et al.*, 2015). Investigations into mast

cells propose there are two main populations found in fish, tissue resident mast cells and highly motile circulating mast cells (Dezfuli, *et al.*, 2012). It has been suggested that the range and distribution that mast cells exhibit is likely explained by the large number of fish living in a wide range of environments (Sfacteria, *et al.*, 2015). Fish mast cell properties are highly associated with other cells of the innate immune system including eosinophils and basophils as they are all granular leukocytes (Secombes & Wang, 2012).

In summary, cell mediated innate immunity is made up of a vast array of cell types, where structure and function differ from mammals to fish, and even significantly within fish themselves. Research into each aspect of cell mediated immunity in fish still has a long way to go and this knowledge will improve with investigation and exploration into their vast array of unique immune parameters.

1.4.4 Humoral Components

Humoral immunity refers to non-cellular immune components found in blood and other tissues. It is a non-specific line of defence that aids in the prevention of pathogen invasion within the body. These mechanisms are found in most domains of life and although are considered primitive they still offer extremely effective defence against invading pathogens. This type of immunity is mediated by macromolecules found in extracellular fluids (body fluid), which is made up of secreted compounds such as lysozyme, complement proteins, cytokines and AMPs. Lysozyme is a bacteriolytic enzyme which acts on the peptidoglycan layer of a bacterial cell wall causing cell lysis (Alvarez-Pellitero, 2008). It is a non-specific primitive defence mechanism found in most animals with a wide distribution throughout the body (Tort, *et al.*, 2003). Fish lysozyme is secreted by leukocytes, predominantly monocytes, macrophages and neutrophils where potency is dependent on the species and tissue affected (Uribe, *et al.*, 2011).

The complement system is a major component of innate defences found in fish and higher vertebrates, composed of a complex protein cascade. In fish the complement system is made up of over 35 plasma proteins which play a role in immune effector functions such as, inflammation, promotion of pathogen

phagocytosis, chemotaxis, clearance of apoptotic cells and modulation of the adaptive immune response (Tort, *et al.*, 2003; Uribe, *et al.*, 2011; Secombes & Wang, 2012; Zhu, *et al.*, 2013). Proteins belonging to the complement system can be divided into three biochemical pathways, the classical pathway, alternative pathway, and the lectin pathway. The classical pathway is activated by antigen-antibody interactions, the alternative pathway is initiated directly by foreign micro-organisms, and the lectin pathway is triggered by a complex made up of mannose/mannan binding to mannose residues on the pathogen cell surface (Uribe, *et al.*, 2011). Although each pathway is initiated by different elements they all produce the same anti-infectious effects (Zhu, *et al.*, 2013). Firstly, opsonisation, which is the recruitment of phagocytic cells to sites of inflammation and promotion of their activity. Secondly, the formation of a membrane attacking complex (MAC) causing pathogen cell lysis. Lastly, anaphylatoxic leukocyte stimulation producing local inflammation which further enhances the immune response (Tort, *et al.*, 2003; Alvarez-Pellitero, 2008; Secombes & Wang, 2012; Zhu, *et al.*, 2013).

Cytokines are low molecular weight proteins that act in a network as signalling molecules to control and coordinate innate and adaptive immune responses (Magnadóttir, 2006). Cytokines are secreted by cells of the immune system including granulocytes, dendritic cells and macrophages (Figure 5) which bind to specific cellular receptors through autocrine and paracrine mechanisms (Secombes *et al.*, 2011). Fish cytokines can be broadly divided into three groups, interleukins (ILs), interferons (IFNs) and tumour necrosis factors (TNFs) (Tort, *et al.*, 2003). The function of ILs includes cell differentiation, growth and motility, and are also vital in the regulation of inflammation where different ILs may have pro- or anti-inflammatory effects (Uribe, *et al.*, 2011). IL families found in mammals predominantly have had direct homologues identified in fish, although some novel proteins do exist, likely due to gene duplication events (Secombes, *et al.*, 2011). TNFs identified in fish have experimentally been shown to directly enhance neutrophil migration, induce apoptosis and heighten macrophage activity (Tort, *et al.*, 2003). Nineteen mammalian TNF members have been described, with only several currently identified in fish, TNF-α and β being the most prominent (Uribe, *et al.*, 2011; Zhu, *et al.*, 2013). IFNs identified in fish and

mammals play a vital role in defence against viral infections, upon recognition of viral nucleic acids IFNs are secreted by vertebrate host cells (Zhu, *et al.*, 2013). IFNs act to protect other cells by altering the regulation of mRNA transcription to produce an antiviral state in a cell. This includes induction of genes that encode antiviral proteins such as protein MX1 (Uribe, *et al.*, 2011).

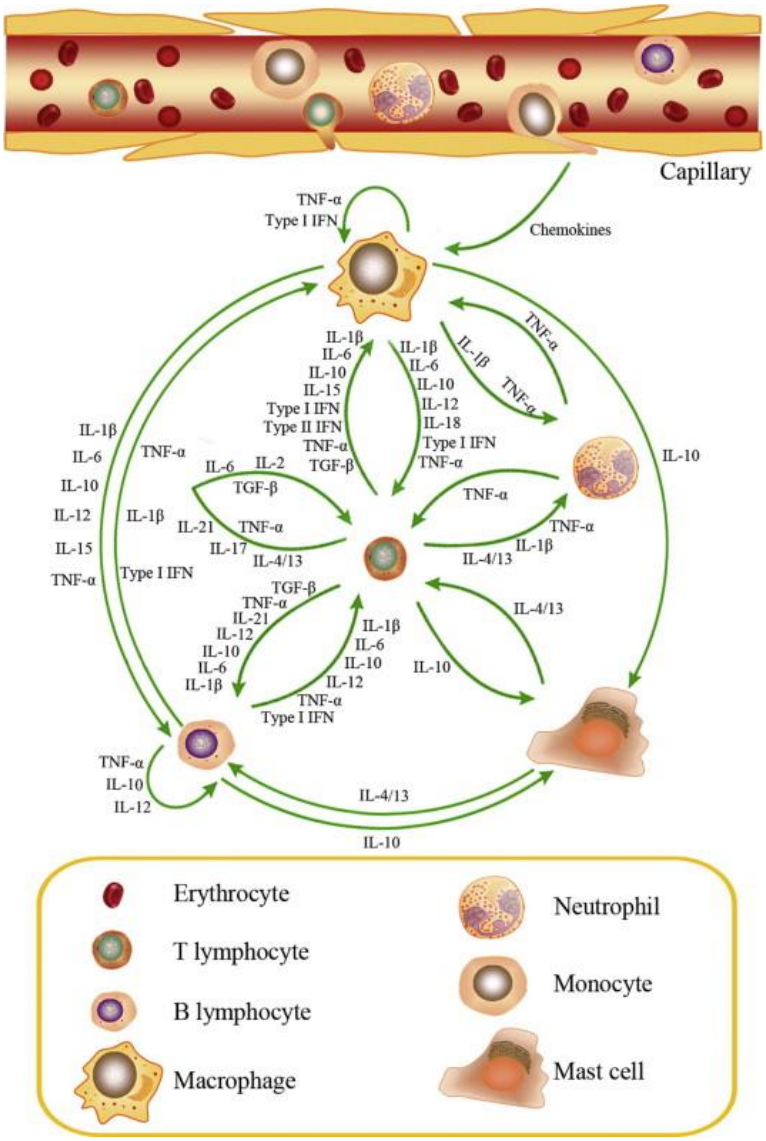

Figure 5: Known cytokines found in fish that play a role in regulating inflammatory cell functions, including differentiation, apoptosis, cell proliferation, and gene expression (Zhu *et al.*, 2013).

AMPs are an ancient mechanism of immunity, found in microorganisms, plants, invertebrates and vertebrates. They show a wide range of activity against viruses, fungi, bacteria, parasites and in some cases also antitumor activity. In depth discussion of these peptides will be explored further in the next section.

1.5 Antimicrobial Peptides

AMP's are naturally occurring low molecular weight polypeptides that play a major role in host immunity (Buonocore, 2011; Masso-Silva & Diamond, 2014). AMPs are highly conserved throughout nature where they show a broad spectrum of activity defending their host against invading bacteria, viruses and fungi (Uribe, *et al.*, 2011; Zhang & Gallo, 2016). In some cases antitumor activity has also been shown toward carcinogenic cells (Perez Espitia *et al.*, 2012). These peptides are considered a primitive mechanism of immunity as they are found in all domains of life (Wiesner 2010). Two physical features are common for AMPs which are thought to be important in how they target certain pathogens, which are: **1) Being cationic** (positively charged) which attracts them to negatively charged pathogenic membranes, they are not attracted to mammalian cells which have a zwitterionic charge. **2) Containing a significant proportion of hydrophobic residues** facilitating interactions between fatty acyl chains (Izadpanah & Gallo, 2005; Guaní-Guerra *et al.*, 2010; Wiesner & Vilcinskas, 2010; Nguyen, *et al.*, 2011; Noga *et al.*, 2011; Rakers *et al.*, 2013; Valero *et al.*, 2013; Masso-Silva & Diamond, 2014). AMPs are typically expressed onto primary physical barriers such as mucosal epithelia and skin of an organism to prevent pathogen colonisation of host tissues (Guaní-Guerra, *et al.*, 2010). These peptides can also be stored in cells of the immune system such as macrophages and granulocytes which assist in micro-organism destruction (Kościuczuk *et al.*, 2012).

1.5.1 Mode of Action

The mode of action of AMPs can vary dependent on the invading micro-organism, displaying antibacterial, antiviral and antifungal properties (Perez Espitia, *et al.*, 2012). The antibacterial effects of AMPs have been shown to destroy gram positive and gram negative bacteria through two main mechanisms (Zhang & Gallo, 2016), firstly targeting bacterial membranes, and secondly penetrating the membrane where they interfere with important cellular components or processes such as DNA and protein synthesis (Nguyen, *et al.*, 2011). After initial attachment of AMPs to the bacterial cell wall, several models have been proposed to explain AMP action on bacterial cell membranes (Figure 6).

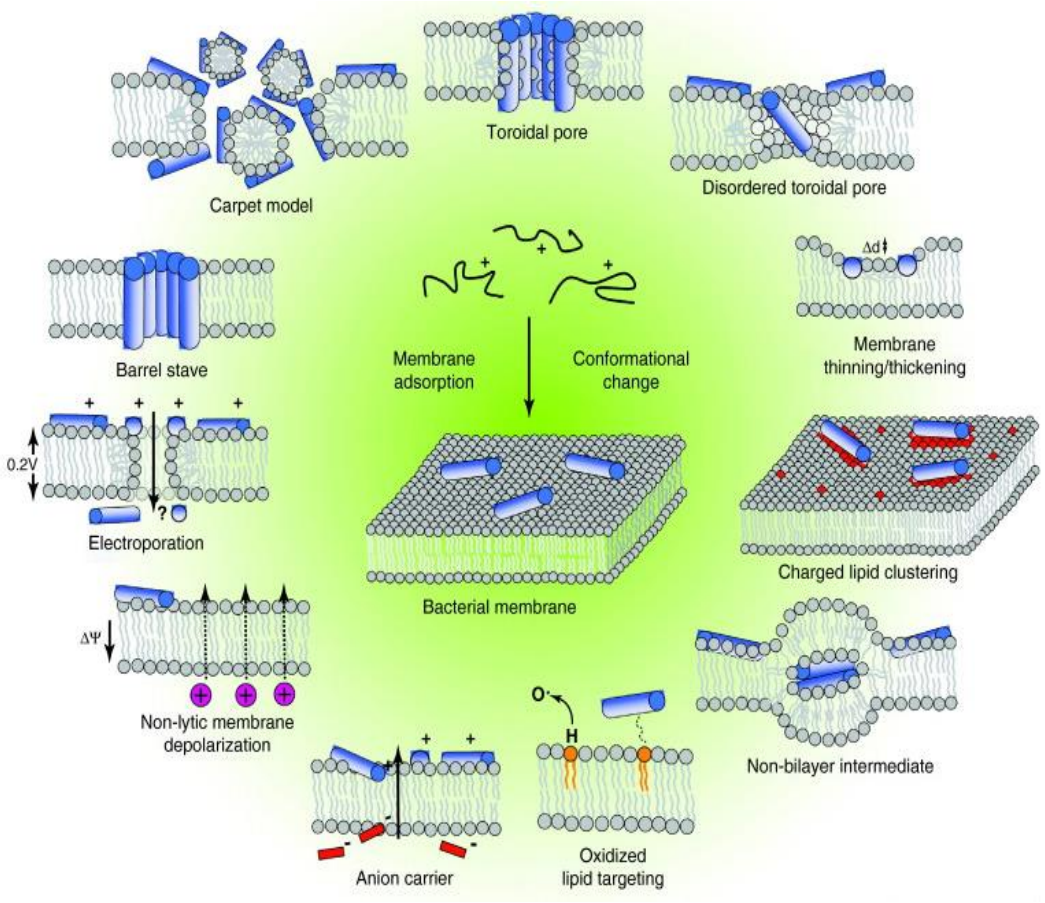

Figure 6: Examples of AMP action against bacterial membranes. Taken from Nguyen *et al.* (2011).

The exact mechanism of AMP action is not fully understood, although the most widely accepted and popular theories are the barrel stave model, carpet model and the toroidal pore model (Wiesner & Vilcinskas, 2010). The barrel stave model describes the formation of a pore or transmembrane channel (Pasupuleti *et al.*, 2012). This is where the hydrophobic side of the peptide binds to the lipid core of the bacterial membrane while the hydrophilic side is orientated to the inside (Nguyen, *et al.*, 2011). The recruitment of several peptides results in the formation of aqueous pores causing loss of cell contents and the inability to maintain a proton motive force resulting in cell death (Perez Espitia, *et al.*, 2012). The carpet model is characterised by peptides covering the membrane surface in a carpet like manner by remaining parallel in orientation (Nguyen, *et al.*, 2011). At a critical concentration, the peptides disrupt the membrane surface where the hydrophilic surface is in contact with phospholipids causing reorientation of these peptides, resulting in the formations of small peptide-lipid aggregates with a

hydrophobic core (Wiesner & Vilcinskas, 2010). This disintegration of the bacterial cell membrane results in cell death (Pasupuleti, *et al.*, 2012). The toroidal pore model is similar to the barrel stave model, although it differs in that AMPs are always associated with the phospholipid head groups and perpendicularly inserted, causing the membrane to be bent inward, therefore, resulting in a pore that is lined with both lipid head groups and AMPs (Wiesner & Vilcinskas, 2010). The ultimate result, similar to that of the barrel stave model, is the generated pore disrupts the cellular contents of the pathogen causing depletion of the proton motive force and, therefore, no ATP synthesis eventually killing the cell (Pasupuleti, *et al.*, 2012). In contrast, it has been shown that some AMPs have no activity on the cell membrane but instead are able to translocate through the pathogenic membrane (Rajanbabu & Chen, 2011). The translocated AMPs accumulate inside the bacterial cell where they can disrupt essential cellular processes resulting in cell death (Pasupuleti, *et al.*, 2012). These AMPs can target processes such as DNA synthesis, enzyme activity and proteins involved in cell wall amalgamation (Podda *et al.*, 2006). It has also been hypothesised that these AMPs can interact with transport systems within the bacterial membrane in contrast to non-specific membrane integrity disruption (Carnicelli *et al.*, 2013). An example of this type of peptide is Bac7 a proline rich mammalian AMP which through TEM localisation assays was shown to accumulate in the cytoplasm of *Escherichia coli* and *Salmonella enterica*, where changes in cellular morphology were observed (Podda, *et al.*, 2006). Similar to antibacterial AMPs, antifungal peptides work in much the same way through interaction with the cell wall or action on an intracellular target (Bahar & Ren, 2013).

In addition to targeting bacterial and fungal cells, AMPs have proven dynamic in their activity showing various mechanisms of action against viruses. Antiviral AMPs are peptides that have been shown to have direct action against invading viruses, with greater efficiency against enveloped viruses (Falco, *et al.*, 2012). Experimental analysis has concluded that AMPs are able to target viruses via two main mechanisms, firstly by assimilating into viral envelopes causing membrane instability, and secondly by reducing the binding efficiency of viruses to the host cell by integrating into the host cell membranes (Bahar & Ren, 2013). An example of an AMP blocking viral entry through interaction with the host cell

rather than inhibition of viral replication is lactoferrin, a common AMP found in the milk of mammals (Perez Espitia, *et al.*, 2012). Lactoferrins mode of action is through interaction with heparin sulphate a linear polysaccharide found in animal tissue (van der Strate *et al.*, 2001). Heparin sulphate is utilised by a range of viruses to gain access to mammalian cells. It is proposed lactoferrin interacts with heparin inhibiting viral infection (van der Strate, *et al.*, 2001; Perez Espitia, *et al.*, 2012). In contrast, an example of an AMP that interacts with a virus to prevent entry into vertebrate cells is α-lactalbumin which has been shown to inhibit infection of herpes simplex virus (Oevermann *et al.*, 2003). The antiviral activity of this peptide is based on direct interaction with viral glycoproteins which are responsible for penetration and absorption into host cells. Therefore, AMPs that interact with these glycoproteins prevent viral activity (Perez Espitia, *et al.*, 2012).

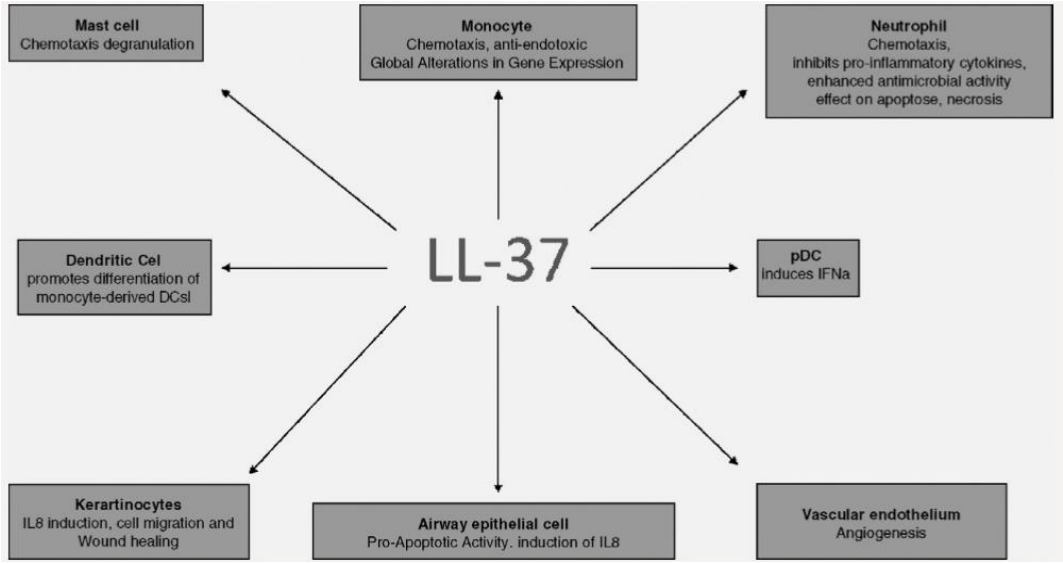

Figure 7: Human LL-37 immunomodulatory functions Taken from Steinstraesser *et al.* (2011).

Apart from their direct antibacterial, antifungal and antiviral activity AMPs also have immunomodulatory functions which are considered an equally important immune function as direct antimicrobial destruction (Falco, *et al.*, 2012). Immunomodulation is the adjustment of an immune response to a desired level, leading to immunosuppression and/or immunoenhancement (Bowdish *et al.*, 2005). AMPs are able to modulate immune responses through induction of cytokines, chemokines, alteration of gene expression and inhibition of proinflammatory responses (Falco, *et al.*, 2012). In addition, AMPs have been

shown to recruit neutrophil and fibroblasts, enhance activity of phagocytic cells and promote mast cell degranulation (Secombes & Wang, 2012). An example of an immunomodulatory AMP is the human cathelicidin LL-37 (Figure 7), which is secreted in sweat and airway surfaces, and is upregulated in response to injury and/or cutaneous infection (Bowdish, *et al.*, 2005; Steinstraesser, *et al.*, 2011).

1.5.2 Antimicrobial Peptides in Mammals and Other Organisms

The first reported animal AMP was in 1956 where a defensin was isolated from rabbit leukocytes (Hirsch, 1956). In the following years, the presence of lactoferrin in cows milk was shown, bombinin was isolated from frog epithelia, and it was proven that the lysozyme found in human leucocytes contains various AMPs (Bahar & Ren, 2013). It is now understood that the range of AMPs found in various organisms is extensive (Figure 8).

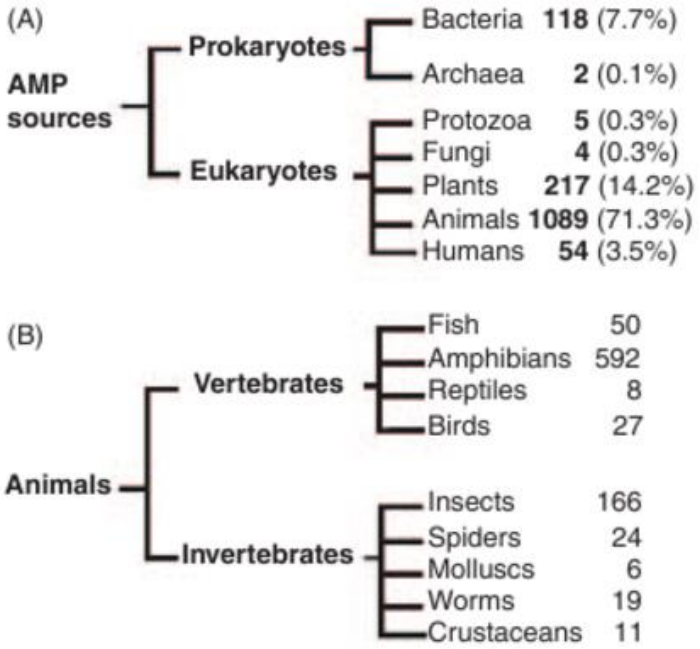

Figure 8: Summary of, (A) the number of AMPs isolated from a variety of kingdoms and (B) the number of AMPs identified from a range of animal families. Taken from Wang *et al.* (2010).

AMP classification is difficult and can be attributed to their considerable diversity in sequence, structure and mode of action. AMPs can loosely be divided into categories based on their size, conformational structure and amino acid

composition (Guaní-Guerra, *et al.*, 2010). The range of structure, distribution and activity of various AMPs are outlined in Table 2 (Ganz, 2003). One of the most prominent reasons for investigating vertebrate AMPs is antibiotic resistance. This is a significant and growing issue in contemporary medicine, highlighted by the rise in hospital acquired infections caused by microbes displaying resistance to antibiotics (Mazel & Davies, 1999). It is estimated that routine operations will become deadly within the next 20 years as the ability to fight conventional infections is lost due to the rise in antibiotic resistant microbes. Therefore, much research has been focused on discovery and isolation of alternative compounds to supercede traditional antibiotics (Nguyen, *et al.*, 2011). AMPs have been identified as a promising candidate for biomedical uses in order to combat the issue of antibiotic resistance (Wiesner 2010, Buonocore 2011).

Table 2: Structures, distributions and activities of AMPs found in a wide range of organisms. Table recreated from (Ganz, 2003)

Structure	Peptides	Species and Tissue	Reported Antimicrobial Activity
4-disulfide α-helix + β-sheet	Plant defensins	Plants	Fungi
	Drosomycin	Arthropod haemolymph	
3-disulfide β-sheet rich	α-defensins	Vertebrate neutrophils	Bacteria, fungi, and enveloped viruses
	β-defensins	Mammalian epithelia	
3-disulfide α-helix + β-sheet	Insect defensins	Arthropod haemolymph molluscs	Gram positive bacteria
3-disulfide 2α-helicies + β-sheet	γ-thionins	Plants	Bacteria, fungi and mammalian cells
2-disulfide β-sheet	Protegrins	Pig neutrophils	Bacteria, fungi and enveloped viruses
	Tachyplesins and polyphemusins	Horseshoe crab haemocytes	
1-disulfide cyclic	Bactenecin-1 and cyclic dodecapeptide	Ruminant leukocytes	Bacteria
	Ranalexin and brevinin	Amphibian skin	
α-helix	Cecropins	Insect Haemolymph	Bacteria
	Maganin and PGLa	Amphibian skin	
	LL-37	Mammalian leukocytes	
Linear with repeating motifs	Bactenecins 5 and 7, PR-39, and indolicidin	Mammalian leukocytes	Bacteria
	Diptericin and apidaecin	Insect haemolymph	

1.6 Antimicrobial Peptides in Fish

The ocean contains significant organism diversity and is, therefore, an important source of novel AMP discovery (Cantisani, *et al.*, 2014). Fish like other organisms secrete various kinds of AMPs. Although, unlike other vertebrates the antimicrobial activity of fish AMPs was not discovered until 1996 (Masso-Silva & Diamond, 2014). Fish AMPs exhibit many, if not all of the same characteristics as higher vertebrates showing a broad spectrum of antimicrobial activity and immunomodulatory functions. In fish AMPs have been divided into a number of groups that include; linear amphipathic α-helical peptides (piscidins & cathelicidins), peptides derived from larger proteins (histone derived & haemoglobin derived), peptides with cysteine's that form intramolecular bonding (defensins & hepcidins), and cationic peptides with diverse structures (high density lipoproteins & NK-lysin) (Valero, *et al.*, 2013; Masso-Silva & Diamond, 2014). A number of these AMPs will be discussed below and their role in host defence highlighted.

1.6.1 Piscidins

Piscidins are a family of AMPs containing a range of fish proteins, which includes piscidin (Valero, *et al.*, 2013), pleurocidin (Cole, *et al.*, 2000), moronecidin (Lauth *et al.*, 2002), dicentracin (Salerno *et al.*, 2007), paradaxin (Oren & Shai, 1996), gaduscidin (Browne *et al.*, 2011) and epinecidin (Yin *et al.*, 2006). Each protein belonging to the piscidin family are characterised by similarities in secondary peptide structure, as well as high gene sequence homology (Valero, *et al.*, 2013). Piscidins and pleurocidins are two of most extensively studied AMP sequences within this family and, therefore, will be discussed further.

Piscidin is a linear, amphipathic, α-helical peptide antibiotic that is found in a variety fish species (Figure 9), showing an evolutionarily related relationship to AMPs found in amphibian skin, and insects (Rakers, *et al.*, 2013; Masso-Silva & Diamond, 2014). Piscidin has been isolated from Atlantic cod (*Gadus morhua*) (Fernandes, *et al.*, 2010), hybrid striped bass (*Morone chrysops x Morone saxatilis*) (Salger *et al.*, 2011), mandarin fish (*Synchiropus splendidus*) (Sun *et al.*,

2007) and many other fish taxa (Valero, *et al.*, 2013; Masso-Silva & Diamond, 2014). Piscidin expression has been linked specifically to mast cells, skin, gills and the gastrointestinal tract of fish, although some traces of piscidin have also been found in the kidney, head and spleen (Ruangsri *et al.*, 2012; Rakers, *et al.*, 2013).

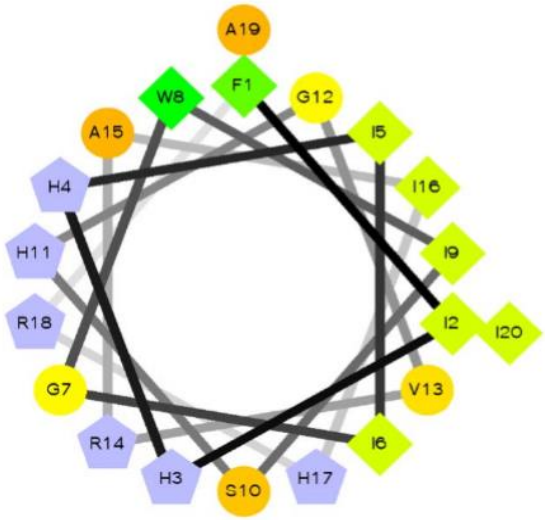

Figure 9: The amphipathic α-helical structure of cod piscidin. Hydrophilic residues are highlighted by blue pentagons and very hydrophobic residues are represented by green diamonds. Taken from Fernandes *et al.* (2010).

The gene structure of piscidin is made of four exons and three introns which encode a signal peptide, mature peptide, and carboxyl-terminal pro-domain (Masso-Silva & Diamond, 2014). The mature piscidin peptide is 18-26 amino acids long and is defined by it's N-terminus which is rich in histidine and phenylalanine (Falco, *et al.*, 2012). Piscidin shows potent antimicrobial activity against a range of microbes including fungi (Niu *et al.*, 2013), viruses (Park *et al.*, 2011), and parasites (Niu, *et al.*, 2013), although it is especially significant against gram positive and gram negative bacterial species (Sun, *et al.*, 2007), with the most potent antimicrobial activity recorded against *Streptococcus, Pseudomonas, Vibrio,* and *Bacillus* species (Masso-Silva & Diamond, 2014). Piscidin is hypothesised to kill microbial cells through membrane disruption via toroidal pore formation where membrane lipids are inserted between the α-helicies causing material efflux (Valero, *et al.*, 2013; Masso-Silva & Diamond, 2014).

Pleurocidin is a cationic, amphipathic peptide, exhibiting an α-helical structure, and was one of the first AMP sequences to be sequestered from a teleost (Cole *et al.*, 1997). Initial isolation of pleurocidin was from mucous cells in flounder skin where bioactivity assays highlighted this peptide to have a broad spectrum of antimicrobial activity (Cole, *et al.*, 1997; Anbuchezhian, *et al.*, 2011). Pleurocidin has subsequently been isolated from several fish species including, Atlantic halibut (*Hippoglossus hippoglossus*) (Patrzykat *et al.*, 2003), common dab (*Limanda limanda*) (Brocal *et al.*, 2006), and American plaice (*Hippoglossoides platessoides*) (Falco, *et al.*, 2012). Pleurocidin expression in fish tissues has been described in the goblet cells of the small intestine, epidermal mucus, skin epithelium and eosinophils of the gills (Cole, *et al.*, 2000; Falco, *et al.*, 2012). It has been shown that pleurocidin plays a significant role in mucosal defence where immunohistochemical studies localised high pleurocidin expression in skin mucous cells (Cole, *et al.*, 2000).

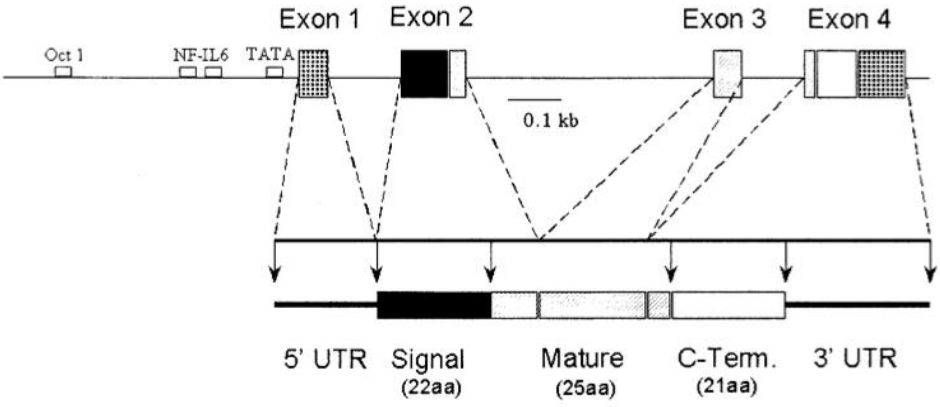

Figure 10: Pleurocidin gene structure and the putative pleurocidin precursor (Cole *et al.*, 2000).

Pleurocidin gene structure consists of four exons and three introns (Figure 10) encoding a signal peptide, mature peptide and pro-peptide (Falco, *et al.*, 2012). Most AMPs have an anionic pro-peptide attached to the NH_2-end of the mature protein, which is involved in charge neutralisation and cellular trafficking (Cole, *et al.*, 2000). However, pleurocidin is predicted to have anionic properties attached to the COOH terminal end of the mature peptide (Falco, *et al.*, 2012). This is a very unusual configuration but works in a similar way to pro-peptide attachment to the NH_2-end where post-translational enzymatic cleavage releases the mature peptide, activating it (Cole, *et al.*, 2000). Although pleurocidin does

not express the highly conserved N-terminus characteristic of a piscidin, it shows significant sequence homology at the genomic level and, therefore, suggests it is a members of the piscidin family (Sun, *et al.*, 2007). The mature pleurocidin peptide is 25 amino acids long which exerts its function by forming pores in bacterial cell membranes (Rakers, *et al.*, 2013). The mode of action of pleurocidin due to its positive charge is predicted to disrupt outer bacterial membranes forming transmembrane channels resulting in permeability of the phospholipid bilayer (Sun, *et al.*, 2007). It has been shown that pleurocidin likely acts via the toroidal pore model through determination of its single channel characteristics, and by measuring its pore forming activity (Saint *et al.*, 2002). Pleurocidin shows a wide range of bacteriostatic and bacteriocidal activity with strong action against *Bacillus subtilis* and *Escherichia coli* (Cole, et al., 1997). Pleurocidin has also shown weaker antibacterial activity against, *Leucothrix mucor, Pseudomonas aeruginosa* and *Serratia marcescens* (Cole, *et al.*, 1997). Further to this pleurocidin has shown significant antifungal (Jung *et al.*, 2007) and anticancer activity (Morash *et al.*, 2011). Overall, the piscidin family are an evolutionarily conserved group of AMPs specifically found in fish, although show a broad homology to classes of AMPs found in other vertebrate species.

1.6.2 Defensins

Defensins are one of the most well studied AMP families and are found in fungi, plants, invertebrates and vertebrates (Zhu & Gao, 2013). Vertebrate defensins are classified into three main groups, α-defensins, β-defensins and θ-defensins based on structure, location and distribution (Falco, *et al.*, 2012; Zhu & Gao, 2013). To date, α-defensins and θ-defensins have only been described in mammals, with θ-defensins limited to primates (Falco, *et al.*, 2012). As β-defensins are the only universal defensin group, found in more primitive vertebrates and invertebrates (Zhu & Gao, 2013), it is hypothesised that the other defensin groups evolved from an ancestral β-defensin (Falco, *et al.*, 2012; Masso-Silva & Diamond, 2014). In fish, β-defensins have been isolated from a variety of species including, pufferfish (*Tetraodon nigroviridis*), rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) (Falco, *et al.*, 2012), where it is found to be expressed in the pituitary, testis, skin epithelium, head-kidney and

spleen (Rakers, *et al.*, 2013). Constitutive expression of β-defensin in fish has been shown to begin early in development likely due to the vulnerability of juvenile fish where they rely heavily on their innate immune system (Masso-Silva & Diamond, 2014). Genomic analysis of β-defensin in a range of fish species has highlighted a similar gene organisation between species, with three exons divided by two introns encoding a pre-pro-peptide (signal peptide, pro-peptide, and mature peptide). The pre-pro-peptide is comprised of 60 to 77 amino acids, with the mature peptide ranging in size from 38 to 45 amino acids in length (Falco, *et al.*, 2012). As in mammals, fish β-defensins are characterised by their cationic charge, presence of six cysteine rich residues and exhibition of stabilised β-sheet structure (Valero, *et al.*, 2013). Apart from the conserved six cysteine residues (Figure 11), there is very little amino acid conservation in the mature peptides (Zhu & Gao, 2013).

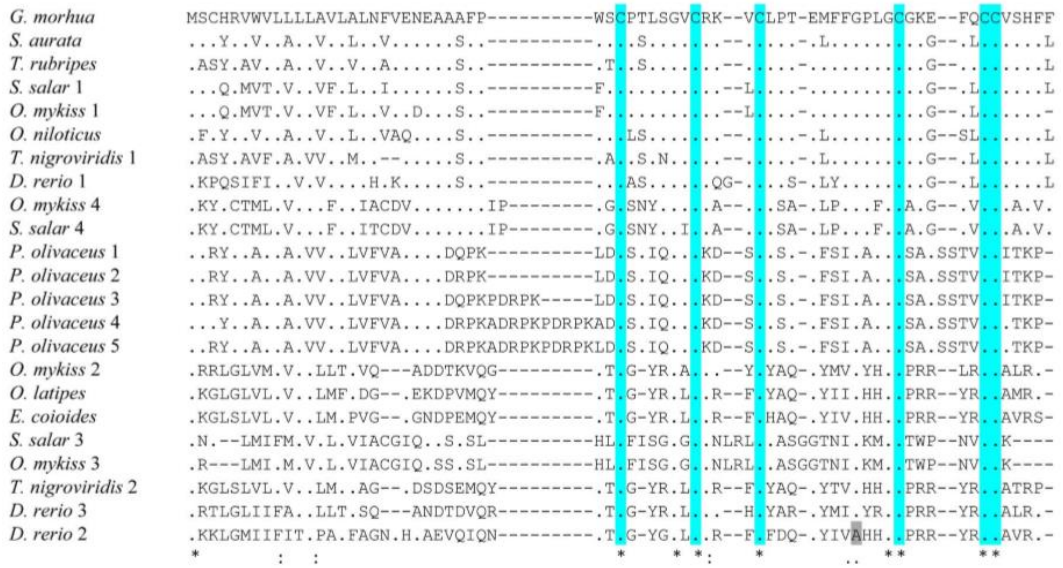

Figure 11: Alignment of β-defensin proteins from teleosts comparing them to an Atlantic cod β-defensin. The dots (.) represent amino acids that are identical to the cod, the six conserved cysteine residues are highlighted in blue. Taken from Ruangsri *et al.* (2013).

β-Defensins have been shown to kill both gram positive and gram negative bacteria, but more potent antimicrobial activity is found towards gram negative species such as *Escherichia coli, Vibrio fluvialis, Bacillus cereus* and *Pseudomonas aeruginosa* (Jin et al., 2010). In addition to antibacterial activity, β-defensin also shows antimicrobial activity against fish specific viruses including

nervous necrosis virus (NNV) and *haemorrhagic septicaemia virus* (VHSV) (Valero, *et al.*, 2013; Masso-Silva & Diamond, 2014). So far no studies have been published about the antiparasitic nature of fish β-defensin (Masso-Silva & Diamond, 2014), although these types of studies have been carried out using human defensin 1 (HD1). HD1 was successfully shown to kill the parasite *Trypanosoma cruzi* via pore formation and DNA fragmentation (Madison *et al.*, 2007). β-Defensin has also been shown to have immunomodulatory properties which was highlighted in a study looking at a defensin isolated from a gilthead seabream (*Sparus aurata*) (Cuesta *et al.*, 2011). The isolated defensin was shown to have chemotactic activity where it was able to attract head-kidney leukocytes (Cuesta, *et al.*, 2011; Masso-Silva & Diamond, 2014). Similarly, a β-defensin isolated from Atlantic cod (*Gadus morhua*) was experimentally shown to have chemotactic action while also stimulating phagocytic activity of head kidney leukocytes (Ruangsri, *et al.*, 2013). Overall, fish β-defensins demonstrate structural and functional similarities to mammalian defensins, thus, highlighting that β-defensins are an ancient and highly conserved mechanism of host defence throughout eukaryotic organisms.

1.6.3 Hepcidins

Hepcidin or liver expressed antimicrobial peptide (LEAP), was first discovered in humans but has been consecutively identified in many other vertebrates including, reptiles, fish and amphibians (Valero, *et al.*, 2013). Hepcidin in humans exists as a pre-pro-peptide (84 amino acids), a pro-peptide (60 amino acids) and a mature peptide (25 amino acids) (Hunter *et al.*, 2002). Fish hepcidin was first isolated from the hybrid striped bass (Shike, *et al.*, 2002) and has subsequently has been identified in at least 37 other fish species including medaka (*Oryzias latipes*), Japanese flounder (*Paralichthys olivaceus*) and zebrafish (*Danio rerio*) (Noga, *et al.*, 2011; Falco, *et al.*, 2012). Like pleurocidins, hepcidin is highly similar between species suggesting a relatively recent duplication of the ancestral gene (Douglas *et al.*, 2003). The general structure of fish hepcidins is a β-sheet hairpin shape, with conserved cysteine residues forming disulphide bridges (Douglas, *et al.*, 2003). Within fish tissues hepcidin is constitutively expressed in peritoneal leukocytes, head, kidney, liver, skin

epithelium, gills, heart and gonads (Rakers, *et al.*, 2013; Valero, *et al.*, 2013). Hepcidin expression has also been detected in turbot (*Scophthalamus maximus*) embryonic cell lines as early as 2 hours after fertilisation with increasing expression throughout embryonic development (Chen *et al.*, 2007). Thus, highlighting the importance of AMPs in innate immunity even before hatching (Valero, *et al.*, 2013). The gene structure of hepcidin is composed of three exons and two introns (Figure 12), and the pre-pro-peptide ranging in size from 81 to 96 amino acids (Falco, *et al.*, 2012), with a mature peptide that is 19 to 31 amino acids in length (Masso-Silva & Diamond, 2014).

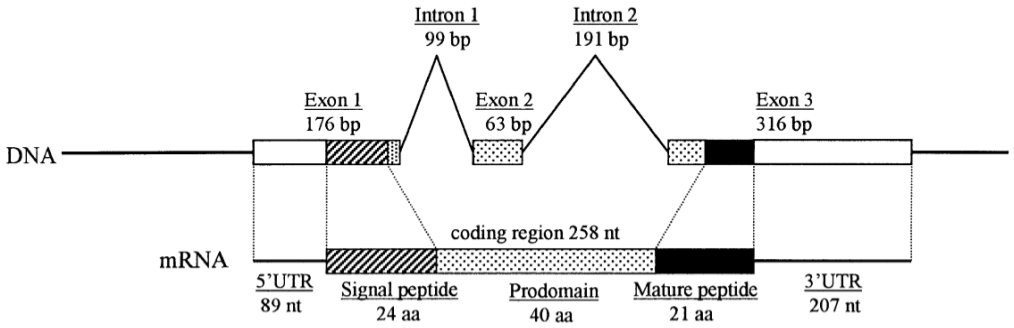

Figure 12: White bass (*Morone chrysops*) hepcidin gene organisation and peptide composition. Taken from Shike *et al.* (2002).

Fish hepcidin has experimentally been shown to have antibacterial activity against both gram negative (*Escherichia coli* and *Pasteurella damelae*) and gram positive (*Lactococcus garvieae* and *Staphylococcus aureus*) bacterium (Hirono *et al.*, 2005). Unlike most other AMPs the mode of action of hepcidin is non-membranolytic (Cai *et al.*, 2012) and, therefore, is hypothesised to work intracellularly (Masso-Silva & Diamond, 2014). The human mature peptide (hepcidin 25) has been shown via a retardation assay to bind to DNA therefore causing disruption of cellular processes (Masso-Silva & Diamond, 2014), however, this mode of action still needs to be confirmed with fish hepcidin. Antiviral activity of hepcidin has also been recorded where the presence of a tilapia (*Oreochromis mossambicus*) hepcidin increased survival of cells exposed to infectious pancreatic necrosis virus (IPNV) (Valero, *et al.*, 2013). Other functional characteristics of hepcidin include immunomodulatory aspects and regulation of intestinal iron absorption (Douglas, *et al.*, 2003; Wiesner & Vilcinskas, 2010; Falco, *et al.*, 2012; Valero, *et al.*, 2013). Overall, hepcidin is a

multifactorial AMP playing a role in many aspects of the vertebrate system including innate immunity, immunomodulation and iron regulation.

1.6.4 Cathelicidins

Cathelicidins are small linear amphipathic α-helical AMPs found in a variety of vertebrate species including humans, fish, horses and chickens (Kościuczuk, *et al.*, 2012). This family of AMPs was named cathelicidins due to the presence of a conserved cathelin domain present in the precursor peptide (Masso-Silva & Diamond, 2014). Fish cathelicidins were first identified in rainbow trout (*Oncorhynchus mykiss*) and have since been isolated from salmonids (Scocchi *et al.*, 2009), hagfish (*Myxine glutinosa*) (Uzzell *et al.*, 2003), Atlantic cod (*Gadus morhua*) (Noga, *et al.*, 2011) and ayu (*Plecoglossus altivelis*) (Valero, *et al.*, 2013). Cathelicidin expression has been recorded in a vast array of tissues including kidney, spleen, stomach, gonads and brain (Falco, *et al.*, 2012). Not all cathelicidins are constitutively expressed in tissues, some are inducible. For example, expression of asCATH1 in the skin of healthy Atlantic salmon was not detected, but asCATH1 expression was detected in the skin and gills after the fish had been exposed to a bacterial challenge (Bridle *et al.*, 2011). The fish cathelicidin genes are organised in the same way to mammals (Falco, *et al.*, 2012), made up of four exons and three introns encoding a signal peptide, pro-peptide, and mature peptide (Figure 13). Even with a similar gene organisation, the mature cathelicidin AMPs can vary in size (12 to 100 amino acids long), structure (α-helical or β-hairpin) and amino acid sequence (Scocchi, et al., 2009; Kościuczuk, et al., 2012).

Cathelicidin activity has been recorded against a large range of gram positive (*Enterococcus facieum*, *Staphylococcus aureus* and *Clostridium perfringens*) and gram negative (*Klebsiella pneumonia*, *Escherichia coli*, and *Pseudomonas aeruginosa*) bacteria (Uzzell, *et al.*, 2003). Antifungal activity against *Candida albicans* has also been recorded (Uzzell, *et al.*, 2003). The mode of action of fish cathelicidins has not been well explored due to the variable nature of the mature peptide sequences and structures between species (Scocchi, *et al.*, 2009). However, it has been elucidated that the amphipathic structure with net

positive charge allows interaction with bacterial membranes highlighting cathelicidin bactericidal activity (Kościuczuk, *et al.*, 2012). Mammalian cathelicidins have been shown to play various roles throughout the body, both immune and non-immune associated, although research into fish cathelicidins is so far limited (Masso-Silva & Diamond, 2014). One such study has emerged showing that two Atlantic salmon cathelicidins were able to induce IL-8 expression in blood leukocytes (Bridle, *et al.*, 2011). This suggests fish cathelicidins can play an immunomodulatory role as is seen in mammals and may be an evolutionarily conserved component of the vertebrate immune system (Bridle, *et al.*, 2011).

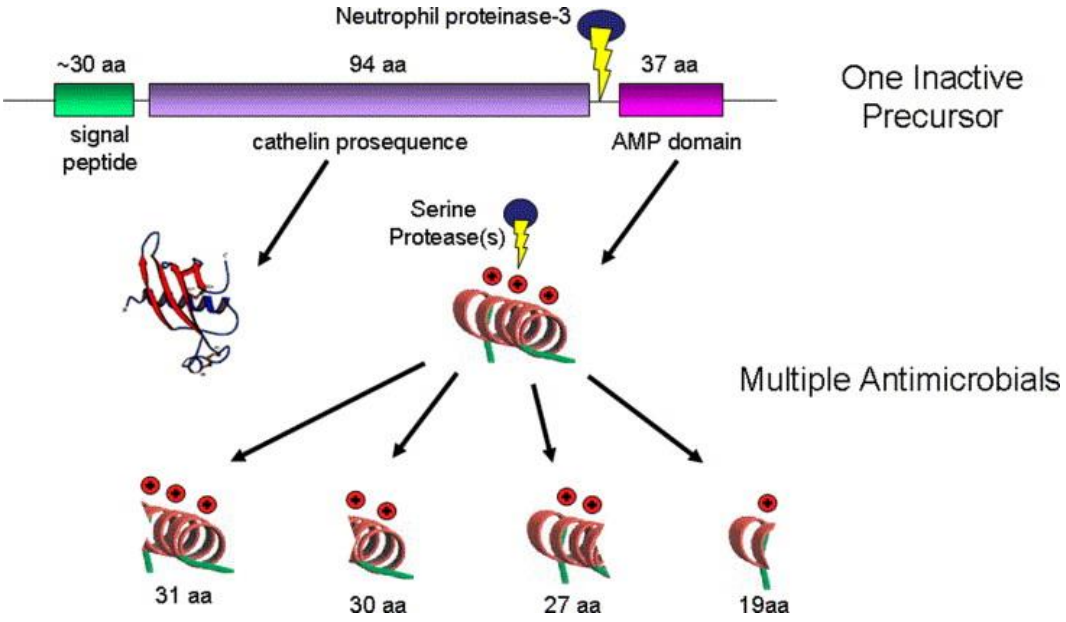

Figure 13: Gene product of cathelicidins. They are first transcribed as large inactive proteins where serine proteases such as proteinase 3 cleave the large product activating the cathelin domain and AMP region. Further processing of the AMP region leads to multiple active peptides with different activities. Taken from Izadpanah and Gallo (2005).

1.6.5 Histone Derived

Histones are proteins found within eukaryotic cells and are best recognised for their role in packaging DNA into structural units called nucleosomes (Parseghian & Luhrs, 2006). Histones are known for their rich cationic charge and have recently been identified as precursors for a range of histone derived AMPs (Chaithanya *et al.*, 2013). The first indication of antimicrobial activity in histones was described from calf thymus histone B where bactericidal action was shown

against a wide range of bacteria (Hirsch, 1958). Consequently, histone derived AMPs have now been identified in a range of organisms including invertebrates, amphibians, fish, birds and mammals (Valero, *et al.*, 2013). There are two main types of histones, core histones (H2A, H2B, H3, H4) and linker histones (H1), where each has been associated as an antimicrobial peptide precursor (Parseghian & Luhrs, 2006). Structurally there is a huge diversity in histone derived AMPs, from α-helical, to β-sheet, and even random coil domains resulting in an amphipathic structure (Valero, *et al.*, 2013). Histones are high in the cationic amino acids arginine and/or lysine resulting in a positive charge which is a prime feature of AMPs (Chaithanya, *et al.*, 2013). This allows them to bind to the anionic surface of a pathogen and causes permeabilisation of the target cell membrane (Valero, *et al.*, 2013). It has been shown these peptides destabilise bacterial membranes but do not form stable pores. Therefore, it is hypothesised they use a carpet method to enter invading cells where they then act intracellularly (Noga, *et al.*, 2011). In aquatic environments histone AMP activity has been recorded against many microorganisms including, water molds, fish pathogens and parasitic dinoflagellates (Masso-Silva & Diamond, 2014).

Histone derived AMPs were first isolated in fish from the skin of channel catfish (*Ictalurus punctatus*), where the isolated proteins showed high sequence similarity to histone H2B (Robinette *et al.*, 1998). To date, many other histone derived proteins have been discovered in fish, including Oncorhyncin II, SAMP H1, histone derived fragment 1 (HSDF-1) and hipposin. Oncorhyncin II was initially isolated from the skin of rainbow trout (*Oncorhynchus mykiss*) and shown to be a cleavage product from histone H1 (Fernandes *et al.*, 2004). SAMP H1 was isolated from the skin of Atlantic salmon (*Salmo* salar) and is derived from histone H1 (Narvaez *et al.*, 2010). HSDF-1 is also derived from histone H1 and has been isolated from the skin of coho salmon (*Oncorhynchus kisutch*) (Patrzykat *et al.*, 2001). Lastly, hipposin is derived from histone H2A and was initially isolated from the skin of Atlantic halibut (*Hippoglossus hippoglossus*) (Birkemo *et al.*, 2003). Hipposin is of particular interest because the full primary sequence contains the sequences of other histone derived AMPs (Figure 14), such as, parasin isolated from the skin mucus of Asian catfish (*Parasilurus asotus*) (Park *et al.*, 1998), and buforin isolated from the stomach of an Asian toad (*Bufo bufo*

garagrizans) (Park *et al.*, 2000). In all fish species studied to date, histone derived AMP expression has also been described in the gills, spleen and gut (Masso-Silva & Diamond, 2014). These findings highlight histone derived AMPs as a primitive form of innate immune defence as they are likely present in many if not all teleosts. Also, the similarity observed in histone H2A sequences between fish, amphibians, birds and mammals infers that histone proteins remain relatively similar throughout the course of evolution (Chaithanya, *et al.*, 2013).

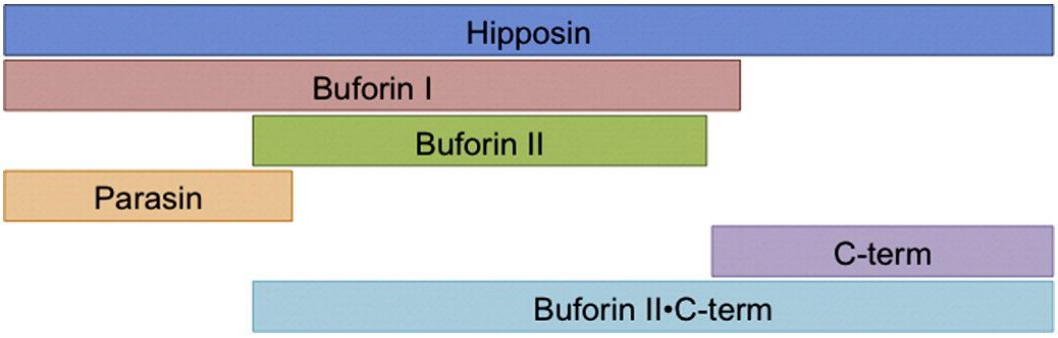

Figure 14: Schematic highlighting the primary sequence overlap between the naturally occurring histone fragments buforin I, buforin II and parasin to hipposin. Taken from Bustillo *et al.* (2014).

1.6.6 High Density Lipoproteins

High density lipoproteins (HDLs) are involved in extracellular transport of fats (cholesterol, phospholipids, and triglycerides) around the body, they move fat away from cells, tissues and artery walls to organs such as the liver or reproductive organs (Concha *et al.*, 2003). HDL fats delivered to the liver are converted into bile which is involved in digestion, whereas HDL fats delivered to reproductive organs are used in the synthesis of steroid hormones (Johnston *et al.*, 2008). Other interesting functions have also been attributed to HDL's including their role in immune defence where it has been shown they perform as AMPs (Valero, *et al.*, 2013). There are two main types of HDLs within vertebrates showing antimicrobial activity, apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II). The structure of apolipoproteins vary, apoA-I has an α-helical structure whereas, apoA-II has a crystal structure which is organised due to the presence of disulphide bonds (Valero, *et al.*, 2013). The primary structure of apoA-I shows very little conservation between species, but the secondary and

tertiary structures are highly similar, therefore, indicating the α-helical structure as an important aspect of this peptides antimicrobial activity (Concha *et al.*, 2004). The exact mode of action of apolipoprotein functionality is not yet known, although it has been hypothesised they form ion channels in bacterial membranes (Agawa, *et al.*, 1991), where their antimicrobial activity may solely be due to their amphipathic nature and α-helical structure (Johnston, *et al.*, 2008). Other studies from human apoA-I have suggested they form pores in lysosomal membranes where the pore causes an influx of calcium ions from the cytoplasm, the lysosome then swells compromising the physical integrity of the infecting microbe, resulting in lysis (Pérez-Morga *et al.*, 2005).

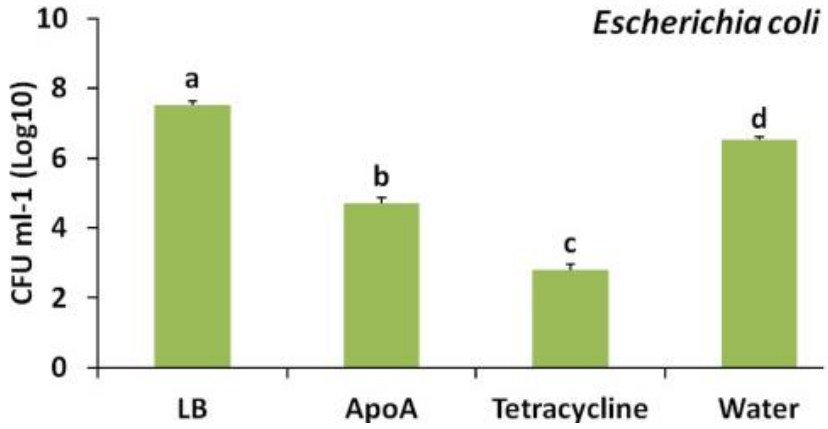

Figure 15: Rainbow trout apoA antimicrobial activity against *E. coli* JM109 in four different conditions, LB medium, isolated apoA, the antibiotic tetracycline and water. The letters indicated statistically significant (P<0.05) differences between treatments. Taken from Dietrich *et al.* (2015).

The antimicrobial activity of apoA-I and apoA-II in fish is less well understood in comparison to other AMPs, due to limited published studies and only recent discovery of the antimicrobial activity of HDLs (Valero, *et al.*, 2013). The antimicrobial activity of apoA-I and apoA-II in fish was first analysed in common carp (*Cyprinus carpio*) where these two peptides were isolated from the skin and epidermal mucus (Concha, *et al.*, 2003). Studies showed both apoA-I and apoA-II isolated from carp skin exhibited antimicrobial activity against gram positive (*Plancoccus citreus*) and gram negative (*Yersinia ruckeri*, *Escherichia coli* and *Pseudomonas sp.*) bacteria (Concha, *et al.*, 2004). In addition, apolipoproteins have been isolated and their antimicrobial activity studied in rainbow trout, (Figure 15) (Villarroel *et al.*, 2007), channel catfish (Pridgeon & Klesius, 2013) and striped bass (Johnston, *et al.*, 2008). Further evidence needs to

address the antimicrobial mechanism of apolipoproteins isolated from a range of different organisms acting against a range of microbes (viruses, parasites and bacteria).

1.6.7 NK-Lysins

Cytotoxic T lymphocytes and NK cells contain secretory lysozymes that contain the lytic proteins granzymes, perforin and granulysin (also known as NK-lysin) (Andersson *et al.*, 1996; Zhang *et al.*, 2013). In fish granulysins have been named NK-lysin which are a part of the saposin-like protein family involved in sphingolipid catabolism (Valero, *et al.*, 2013) and has been discovered to play a key role in immunity (Pereiro, *et al.*, 2015). The antimicrobial activity of NK-lysin was first described from the small intestine of pigs (Andersson, *et al.*, 1996), but has subsequently been found in various vertebrate species including mice, horses, fish and cows. NK-lysin has also been discovered within *Entamoeba histolytica*, a parasitic protozoan, which highlights their role as an ancient but evolutionary conserved antimicrobial defence mechanism (Pereiro, *et al.*, 2015). In fish the NK-lysin peptide has been isolated from the Japanese flounder (*Paralichthys olivaceus*) (Hirono *et al.*, 2007), tongue fish (*Cynoglossus semilaevia*) (Zhang, *et al.*, 2013), zebrafish (*Danio rerio*) (Pereiro, *et al.*, 2015) and channel catfish (*Ictalurus punctatus*) (Wang *et al.*, 2006). A comparison of genome locations for NK-lysins in selected fish show good conservation and synteny (Figure 16), however, no synteny is found between fish and humans (Pereiro, *et al.*, 2015). NK-lysin expression has been recorded in a wide range of fish tissues, with strong concentrations found in tissues that harbour high numbers of lymphocytes (Valero, *et al.*, 2013).

The teleost NK-lysin gene is composed of five exons and four introns with a cationic protein composed of 78 residues containing six conserved cysteine residues with a predicted three dimensional structure made up of five α-helices (Falco, *et al.*, 2012; Zhang, *et al.*, 2013; Zhang *et al.*, 2014). In mammals, the AMP activity of NK-lysin plays a significant role in defence against bacteria, fungi, viruses, parasites and tumour cells (Pereiro, *et al.*, 2015). In fish, strong antibacterial activity of NK-lysin has been shown against *Escherichia coli* and

Bacillus megaterium (Zhang, et al., 2013). The ability of NK-lysin to act on and eliminate intracellular pathogens has also been studied (Pereiro, *et al.*, 2015) and it has been shown to kill *Mycobacterium tuberculosis* in combination with perforin, another saposin-like family member. Perforin forms pores in the cellular membranes of infected cells, allowing NK-lysin to enter and lyse intracellular *Mycobacterium tuberculosis.* The mode of action of NK-lysin against viruses is poorly understood, but it has been shown that NK-lysin expression is up regulated in fish exposed to viruses, for example, zebrafish exposed to spring viraemia of carp virus (SVCV) infection (Pereiro, *et al.*, 2015).

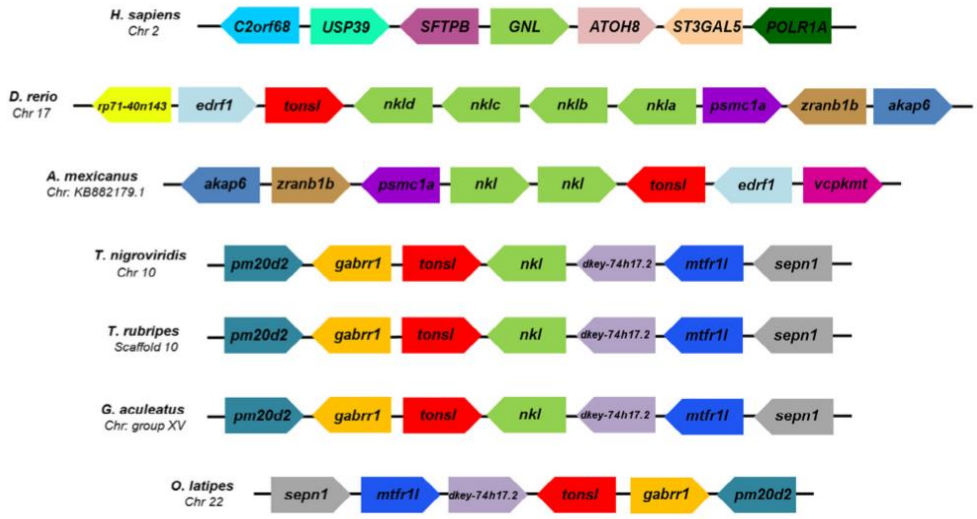

Figure 16: Comparative gene location and basic structure of human granulysin and NK-lysin from several teleost species. Taken from Pereiro *et al.* (2015).

Overall fish AMPs are a promising area of investigation where they are critical in the first line of immune defence. Additionally, apart from the direct antimicrobial activity AMPs show, they also play an important role in innate immune modulation, inflammation and adaptive immune responses.

1.7 Molecular Approaches to Understand Immunity

The transcriptome is defined as the complete set of RNA transcripts (mRNA and non-coding RNA) present within an organism (McGettigan, 2013). The transcriptome highlights an important link between encoding DNA and the resultant phenotype (Valdés *et al.*, 2013). Transcriptomics, also known as expression profiling, examines the expression levels of the transcriptome within a

selected cell population determining the functional components of the genome (Valdés, *et al.*, 2013). For example, testing if a particular gene is up-regulated or down-regulated in response to a specific external cue (Wang *et al.*, 2009).

Early transcriptomic work was carried out using microarray analysis, where oligonucleotides (probes) are bound to a surface which is designed to target specific RNA sequences derived from cDNA (target) (Karakach *et al.*, 2010). When the cDNA is reverse transcribed it becomes fluorescently labelled, consequently allowing detection when probe-target binding occurs (Malone & Oliver, 2011). Laser and computer systems are used to recognise and quantify fluorescent intensities in order to determine the relative abundance of RNA sequences in the target tissue (Karakach, *et al.*, 2010). However, microarray methods do have some limitations, one of which is the need for a transcriptome to be available before the microarray can be designed (Valdés, *et al.*, 2013). Due to these downfalls next generation sequencing (NGS) methods have become preferential research techniques (McGettigan, 2013). One such NGS method is RNA-seq, where total or mRNA from selected tissues is reverse transcribed into cDNA and then sequenced (Valdés, *et al.*, 2013). The result is a transcriptomic library made up of short RNA reads (Garber *et al.*, 2011) providing a glimpse of the gene expression present within a select tissue at a particular moment in time (Wang, *et al.*, 2009). The generated transcriptomic library can also be used for gene mapping, however, this is limited to species that have an existing reference genome (Zhao *et al.*, 2011). Another useful aspect of RNA-seq transcriptomics is the ability to search for genes of interest which can be done by using specific genetic attributes to identify candidate genes (Garber, *et al.*, 2011). With the emergence of NGS, RNA-seq has become a favourable option to identify immune related genes in fish which was previously carried out using techniques such as, subtractive hybridisation (Tsoi *et al.*, 2004), homology cloning (Albert, 2001), and expressed sequence tags (Altmann *et al.*, 2003), which were difficult to use and optimise. Overall, NGS can be used to provide comprehensive information about the immune system and selected immune genes of fish in a very short time frame.

1.8 Aims

AMP's are an important component of the innate immune response of all living organisms that have antimicrobial activity against a range microbes. A number of studies have characterised AMP's within non-mammalian vertebrates and started to determine their importance within the immune system. This investigation aims to characterise AMP's in jawed and jawless vertebrate fish species, where currently nothing is known. This will be achieved by:

1. Using RNA-seq transcriptome databases to identify AMP genes. Transcriptome libraries already exist for selected tissues from *S. lalandi* and *G. australis* and bioinformatic approaches will be used to aid in identifying candidate AMP genes.

2. Using Rapid Amplification of cDNA Ends (RACE)-PCR to obtain the full cDNA sequence of selected *S. lalandi* and *G. australis* AMP genes. Selected amplified products will be cloned and sequenced.

3. Determining the expression of the selected AMP genes within healthy adult tissues of *S. lalandi* using Real-Time (RT)-PCR.

Chapter Two: Materials and Methods

All work was carried out in the University of Waikato C.2.03 Molecular Biology Laboratory. The work was completed on designated laboratory benches cleaned with 70% ethanol (EMSURE®), unless otherwise stated. Waste was collected in containers filled with virkon disinfectant (DuPont), followed by a sterilisation process where the waste was autoclaved. Animal ethics was also considered (see Appendix I) and permission to use genetically modified organisms for cloning and sequencing was covered under the HSNO (see Appendix II).

2.1 Bioinformatics

A range of AMP genes in fish species were identified in published literature where the entire sequence of each gene was recorded and then aligned using ClustalX 2.1 (Jeanmougin *et al.*, 1998). The tree file that was created was bootstrapped 1000 times and then opened on Tree View 1.6.6 (Page, 1996), giving a phylogenetic tree of all recorded AMP sequences. The tree showed clear groupings of AMPs, where two to three representative sequences from each group were selected to search the transcriptomic libraries. Geneious v.7.1.7 (Biomatters Ltd) software was used to identify AMP genes in the yellowtail kingfish (*Seriola lalandi*) and lamprey (*Geotria australis*) transcriptomic databases. The transcriptomic libraries were established by Dr Steve Bird from *S. lalandi* spleen, ovary, testis and pituitary tissues, and *G. australis* kidney, liver, skin and gill tissues. Construction of the library was carried out using RNA-seq on the Ion PGM™ System (Life Technologies) for NGS. Searching the libraries was achieved using Geneious by taking a selected sequence from the phylogenetic tree and using tblastn, which allows a search of the translated nucleotide transcriptomic libraries, using a protein sequence (Gertz *et al.*, 2006). The results identified nucleotide sequences that match to a particular AMP in the library, these were downloaded and underwent de novo assembly to construct a consensus sequence. These consensus sequences were then put into the ExPASy translate tool (http://web.expasy.org/translate/) to obtain the amino acid sequence. These

sequences were then BLAST searched to confirm that they were a match to the original AMP used to search the library, corroborating that the correct sequence had been identified.

2.2 Primer Design

Primers to isolate the 5’ and 3’ ends of the *S. lalandi* and *G. australis* AMP sequences were designed using Primer3 (Untergasser *et al.*, 2012). The sequences chosen for further investigation were used and two forward primers for 3’ RACE-PCR and two reverse primers for 5’ RACE-PCR were designed (Figure 17). Primers were designed using a set of parameters (Table 3), however, in some instances due to sequence quality not all specifications for primer design were met, but were as close as possible.

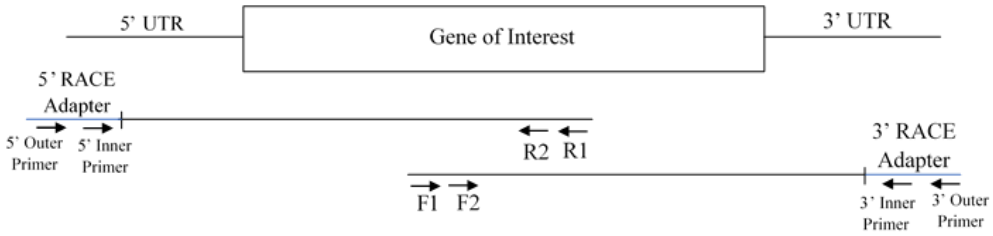

Figure 17: Schematic highlighting the position of forward and reverse primers used in 3' and 5' RACE.

Table 3: Parameter levels used in primer design for RACE-PCR

Parameter	Required Level
Size (bp)	18-30
Melting Temperature (˚C)	50-60
GC Content (%)	40-60
Max Self Complementarity	4
Max 3’ Self Complementarity	4
GC Clamp	1

2.3 RNA Extraction

RNA extraction was carried out using R&A BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology). This kit was chosen due to its economical, quick and efficient isolation of total RNA from animal tissue. The tissue selected for RNA extraction were added to 2.0mL RNase/DNase free conical base screw cap tubes (Neptune) containing 1mL of R&A BLUE™ solution (iNtRON Biotechnology). Also added to the screw cap tube was 0.2mL of 0.1mm zirconia/silica beads (BioSpec Productions) and 0.3mL of 0.5mm glass beads (BioSpec Productions). The tube was then placed in a bead beater (Alphatech Systems Limited) in order to homogenise the tissue at a rate of 4800 oscillations per min for 20sec. The tissue was then visually analysed for complete homogenisation, and this step was repeated if there was still large aggregates of tissue. After homogenisation, 200µL of chloroform (Ajax Finechem) was added and the tube was shaken vigorously for 15sec followed by centrifugation (Eppendorf Centrifuge 5424R) at 13,000rpm for 10min at 4˚C. Centrifugation precipitated out three layers within the tube and was removed carefully from centrifuge so as not to disturb the layers. The upper aqueous phase was extracted cautiously from the tube using a pipette and transferred to a new labelled 2.0mL RNase/DNase free conical base screw cap tube (Neptune). Hyper vigilance was used in removing the aqueous phase so as not to disturb any of the subsequent layers which could lead to contamination by genomic DNA, proteins, chloroform or other organic compounds. Next, 400µL of isopropanol (Ajax Finechem) was added to the new tube containing the aqueous phase in order to precipitate out the RNA and the tube was inverted five times. Caution was used at this step as rapid shaking could sheer the RNA. The tube was then centrifuged at 13,000rpm for 10min at 4˚C, resulting in a small whitish RNA pellet at the bottom of the tube. The supernatant was removed using a pipette, taking care not to disturb the pellet and 1mL of 75% ethanol (EMSURE®) was then added and the tube flicked several times in order to resuspend the pellet. Ethanol is used to wash the RNA pellet removing any salts. The tube was then centrifuged (Heraeus Biofuge Pico) at 13,000rpm for 1min at room temperature. The supernatant was then removed so as not to disturb the pellet. The ethanol wash step was then repeated and finally centrifuged again at 13,000rpm for 1min at room temperature. After completing the ethanol wash twice the supernatant was carefully removed using a pipette and

the tubes left open for the pellet to dry allowing the remaining ethanol to evaporate off, which took approximately 5min. Once the RNA pellet was dry it was resuspended in 30µL of diethylpyrocarbonate (DEPC) (Sigma-Aldrich) treated water.

Quality and concentration of extracted RNA was then determined using the NanoDrop 2000™ (Thermo Scientific). NanoDrop 2000 uses a range of absorbance measures to determine the concentration of RNA (260nm), contaminants such as phenol (230nm) and proteins (280nm). From these absorbance measures RNA quality can be inferred from 230/260 ratio and 260/280 ratio. To begin 2µL of DEPC water was used to blank the NanoDrop 2000. The NanoDrop was then wiped clean and 2µL of RNA extract was loaded. The machine then gave an output of absorbance levels at 230, 260, 280nm, from which the quality and quantity of extracted RNA was determined. A 260/280 ratio of 1.8-2.2 and a 260/230 ratio of 2.0-2.2 is generally accepted as a pure sample, whereas ratios outside of these indicate the presence of contaminants. While not in use RNA samples were kept in -20˚C freezer (Fisher & Paykel).

2.4 Rapid Amplification of cDNA Ends (RACE)-PCR

The FirstChoice® RLM RACE Kit (Ambion) was used to generate the 3’ and 5’ libraries for RACE-PCR.

2.4.1 3’ RACE Library Generation

3’ RACE is used to amplify the 3’ end of an mRNA sequence. The cDNA is generated using a primer sequence complementary to the polyA tail of mRNA, to which an adapter sequence is attached. Nested PCR can then be used to amplify the 3’ region using primers specific to the adapter sequence (Table 4). To generate the 3’ RACE cDNA, 2µL of total RNA isolated from selected tissues were transferred to a nuclease free 0.2mL DNase/RNase free PCR tube (Neptune Scientific). To this tube the following was added, 4µL of dNTPs, 2µL 3’ RACE adapter, 2µL 10X RT buffer, 1µL RNase inhibitor, 1µL M-MLV reverse transcriptase and 8µL of nuclease free water. Tubes were mixed gently and pulse spun in the centrifuge (Heraeus Biofuge Pico) to bring all liquid to the bottom of

the tube. Tubes were then incubated in a thermocycler (Eppendorf) at 42˚C for one hour. The generated 3' RACE libraries were then stored at -20˚C until required for nested PCR.

2.4.2 5' RACE Library Generation

5' RACE is more complex than 3' RACE as an adapter needs to be attached to the 5' end of the mRNA sequence as it does not have a characteristic sequence as is seen at the 3' end which has a polyA tail. Therefore, the 5' end is treated before attachment of the 5' RACE adapter. Nested PCR can then be used to amplify the 5' region using primers specific to the adapter sequence (Table 4). To generate the 5' RACE cDNA, between 1 - 10µg of RNA isolated from tissues was added to a nuclease free 0.2mL DNase/RNase free PCR tube (Neptune Scientific), followed by 2µL Calf Intestine Alkaline Phosphotase (CIP), 2µL CIP buffer, and nuclease free water to make the volume in the tube up to 20µL. Tubes were mixed gently and pulse spun in the centrifuge (Heraeus Biofuge Pico) to bring all liquid to the bottom of the tube. Tubes were then incubated in a thermocycler (Eppendorf) at 37˚C for one hour to allow CIP treatment to remove the 5 free phosphatases from the 5' end of the RNA. The CIP treated RNA was then purified using the Quick-RNA™ Mini Prep (Zymo Research). To the CIP treated RNA, 4 volumes of RNA lysis buffer was added (4:1). Next, 1 volume of 100% ethanol (EMSURE®) was added to the sample in RNA lysis buffer at a ratio of 1:1. The sample was mixed well and transferred to a Zymo-Spin IIICG Column in a collection tube. The tubes were then centrifuged at 13,000rpm for 30sec, this allowed the RNA to attach to the column. The precipitate in the bottom of the tube was discarded and 400µL of RNA Prep Buffer was added to the column, and the tubes centrifuged for another 30sec at 13,000 rpm. The flow through was again discarded and 700 µL of RNA wash buffer was added to the column and the tubes centrifuged again at 13,000 rpm for 30sec. The flow through was discarded and 400µL of RNA wash buffer was again added to the column and centrifuged for 2min at 13,000rpm, to ensure complete removal of wash buffer and any remaining contaminants. Next the lid was cut off a 1.5mL RNase/DNase free snap seal microfuge tube (Scientific Specialties Inc) and the column placed inside, the old collection tube was discarded. Changing the collection tube ensures no

contaminants remain. To the column matrix 30µL of nuclease free water (heated to 95˚C) was added and the tube centrifuged for 30sec at 13,000rpm to elute the purified RNA. The flow through was collected and placed on the matrix again and the centrifugation step repeated. Repeating the RNA elution step and heating the nuclease free water maximises the RNA yield. Next, the CIP treated purified RNA was treated with Tobacco Acid Pyrophosphatase (TAP) which removes the 5' cap structure found at the end of mRNA. In an RNAse free PCR tube (Neptune™) the following components were assembled, 5µL CIP treated purified RNA, 1µL 10x TAP buffer, 2µL TAP, and 2µL nuclease free water. The tubes were mixed gently and pulse spun and incubated at 37˚C for one hour before being taken on to 5' RACE adapter ligation, which attaches a known adapter sequence to the 5' end of the RNA. In new PCR tubes the following components were assembled 2µL CIP/TAP treated RNA, 1µL 5' RACE adapter, 1µL 10x RNA ligase buffer, 2µL T4 RNA ligase, and 4µL nuclease free water. Tubes were gently mixed, pulse spun and incubated at 37˚C for one hour. Lastly, the modified RNA was reverse transcribed. In new PCR tubes, the following was assembled 2µL ligated RNA, 4µL dNTP mix, 2µL random decamers, 2µL 10x RT buffer, 1µL RNase inhibitor, 1µL M-MLV reverse transcriptase, and 8µL nuclease free water (making it up to 20µL). The tubes were gently mixed, pulse spun and incubated at 42˚C for one hour. The generated 5' RACE libraries were then stored at -20˚C until required for nested PCR.

Table 4: Primer and adapter sequences for 5' and 3' RACE

Primer/Adapter	Sequence (5' – 3')
3' RACE Adapter	GCGAGCACAGAATTAATACGACTCACTATAGGT12VN
3' RACE Outer Primer	GCGAGCACAGAATTAATACGACT
3' RACE Inner Primer	CGCGGATCCGAATTAATACGACTCACTATAGG
5' RACE Adapter	GCUGAUGGCGAUGAAUGAACACUGCGCGUUUGCUGGCUUUGAUGAAA
5' RACE Outer Primer	GCTGATGGCGATGAATGAACACTG
5' RACE Inner Primer	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG

2.4.3 Nested PCR for 5' and 3' RACE

Nested PCR is a modified traditional PCR method which is used to overcome non-specific amplification that may occur due to unanticipated primer binding. Nested PCR requires two sets of primers that are used in two sequential runs of PCR, with the second set of primers designed to the target inside of the product amplified from the first round. The first primer set F1 or R1 were used in the first round of nested PCR and designed to amplify the 3' or 5' end of the selected sequence. The second set of primers F2 or R2 were designed to anneal slightly inside of where the first primer set bound, sometimes with primers overlapping.

For 3' RACE the first set of primers used in round 1 nested PCR are the reverse 3' RACE outer primer which corresponds to part of the adapter sequence (Table 4) and a forward primer specific to the gene of interest (F1). The second set of primers for 3' RACE for round 2 nested PCR are the reverse 3' RACE inner primer which also corresponds to part of the adapter sequence (Table 4) and another gene specific primer (F2). For 5' RACE the first set of primers used in nested PCR are the forward 5' RACE outer primer which corresponds to part of the adapter sequence (Table 4) and a reverse primer specific to the gene of interest (R1). The second set of primers for 5' RACE for round 2 nested PCR are the forward 5' RACE inner primer which also corresponds to part of the adapter sequence (Table 4) and another gene specific reverse primer (R2). Similar to 3' RACE the primers used in round 2 nested PCR select inside the primers used in the first round of nested PCR.

For each PCR, ZymoTaq™ DNA Polymerase Kit (Zymo Research) was used. For the first round of nested PCR a master mix was made up in a 1.5mL RNase/DNase free snap seal microfuge tube (Scientific Specialties Inc). The master mix contained 12.5µL Reaction Buffer (Zymo Research), 1µL dNTPs (Solis BioDyne), 0.2µL ZymoTaq DNA Polymerase, and 7.3µL of DEPC water per sample. Each ingredient was multiplied by how many reactions that were being run plus an additional sample to ensure enough master mix for each sample. For example, if four samples were being synthesised into cDNA, master mix for five samples was made up. The master mix solution was pipetted up and down

several times to ensure adequate mixing. In a 0.2mL DNase/RNase free PCR tube (Neptune Scientific) the following was added, 2µL of template DNA (from 5' RACE), 1µL of forward primer, 1µL of reverse primer and 21µL of master mix, which was then mixed thoroughly and pulse spun. The PCR mixes were then placed in a thermocycler (Bio-Rad T100) and run using a selection of parameters (Table 5).

Table 5: Nested PCR thermocycler parameters

	Temperature ˚C	**Time**	**Cycles**
Initial Denaturation	95	10min	1
Denaturation	95	30sec	40
Annealing	50-58	30sec	
Elongation	72	30sec	
Final Elongation	72	10min	1
Holding	4	∞	1

The amplified PCR products were run on a 1.5% TAE agarose gel (see section 2.5) to confirm the presence of an amplified gene of the right size. For the second round of nested PCR all volumes remained the same, the only difference being the template DNA was taken from the first round of nested PCR and the primers used were F2 and R2 fo 3' and 5' RACE respectively. PCR tubes were mixed, pulse spun and then placed in the thermocycler on the same programme as round 1 (Table 5). Again PCR products were run on a 1.5% TAE agarose gel (see section 2.5) to identify if a PCR product of the same size as the gene of interest had been amplified, which were then taken on to purification.

2.5 Gel Electrophoresis

PCR products were run on a 1.5% TAE agarose gel to analyse reaction quality and yield. The gel was made by dissolving 0.75g of agarose powder (dnature) in 50mL of TAE buffer (Sigma-Aldrich). The solution was mixed in a conical flask and microwaved (BRIO) for 2min or until no crystals were visible. The solution was left to cool for 3min after which 1µL of 10mg/ml ethidium bromide (Sigma-Aldrich) was added and mixed in by swirling the conical flask.

The cooled solution was then carefully poured into a gel mould to prevent the introduction of any bubbles. A 12 well comb mould was then placed in the gel. The gel was left for 20min to set after which the well moulds were removed and the gel placed into an electrophoresis mini gel system (OWL Separation System). 1% TAE buffer was poured into the tank covering the gel and 2µL of gel loading buffer (30% glycerol, 0.25% bromophenol blue) was placed onto parafilm (Bemis). 10µL of sample was then mixed with each 2µL dot by pipetting up and down. The mixed sample was then loaded into a well in the gel, and was repeated for each sample, 5µL of 100bp ladder (GenScript) was also loaded into one of the wells. Once the gel was loaded the electrodes and cover were connected to the gel tank and the voltage was set at 90V and run for 32min. After the set time the electrodes were disconnected and the gel removed from the tank and placed onto an Omega Lum G imager (Aplegen) in order to visualise the gel. A picture was taken using the camera and printed (Mitsubishi). The gel was then discarded and the PCR products were stored in a -20˚C freezer until required.

2.6 PCR Product Purification

In order to maximise the efficiency of downstream applications PCR products containing amplified bands of interest were purified using DNA Clean & Concentrator Kit™ (Zymo Reasearch). In a 1.5mL DNase/RNase free tube (Scientific Specialties Inc) 10µL of PCR product was added, along with 50µL of DNA binding buffer in, a ratio of 5:1. Tubes were well mixed and pulse spun. The entire mixture was then transferred to a labelled Zymo Spin Column in a collection tube. The tubes were centrifuged at 13,000rpm for 30sec allowing DNA to bind to the columns matrix. The flow through was then discarded and 200µL of DNA wash buffer was added directly into the column and the centrifugation step was repeated and flow through again discarded. This wash step was repeated to guarantee a high purity of DNA. Columns were then transferred to a new collection tube to ensure no contamination by wash buffer and 10µL of DEPC water was added directly to the column matrix and tubes incubated at room temperature for 1min. The tubes were then centrifuged for 30sec at 13,000rpm to elute DNA. This ultra-pure DNA was then immediately taken on to a ligation reaction.

2.7 Ligation and Bacterial Transformation

2.7.1 Ligation of PCR product into TA Cloning Vectors

The pLUG-Prime TA Cloning Vector Kit (iNtRON Biotechnology, Inc) was used to ligate PCR products into a TA-Cloning Vector. The TA-Cloning Vector is 2728bp in length and includes an ampicillin resistance gene and LacZ gene (Figure 18). The PCR product is inserted within the LacZ gene disrupting its transcription.

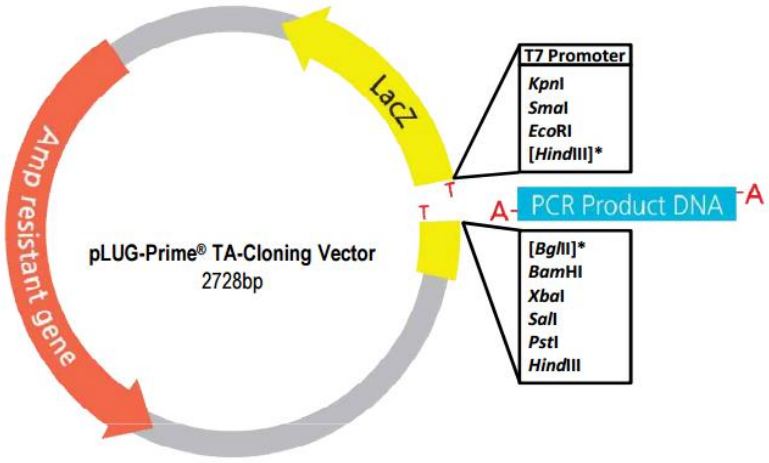

Figure 18: Map of the pLUG-Prime TA-Cloning Vector including sequence reference points

To a 0.2mL DNase/RNase free PCR tube (Neptune Scientific) the ligation reaction was set up (Table 6). The contents of the tubes were vortexed and pulse spun. Tubes were then incubated at 4˚C in the fridge overnight in order to maximise the efficiency of the T4 DNA ligase.

Table 6: Reaction reagents and volumes for ligation of purified PCR products into TA-Cloning Vectors

Reagent	Volume
10X Ligation Buffer A	1µL
10X Ligation Buffer B	1µL
TA-Cloning Vector	2µL
T4 DNA Ligase	1µL
Purified PCR Product	5µL
Total	10µL

2.7.2 Lysogeny Broth (LB) Agar Plates

In order to grow transformed bacteria agar plates containing lysogeny broth (LB) + ampicillin + isopropyl β-D-thiogalactopyranoside (IPTG) + 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) were prepared. To make up 250mL of agar, 6.25g LB powder (USB Corporation), 3.75g agar (Coast Biologicals Ltd), and 250mL of deionised water were added to a 250mL glass Schott bottle (Duran). The bottle was then autoclaved (Astell) on the pre-set media cycle. As the autoclave was running, a water bath (Life Technologies) was set to 50˚C and once the autoclave was completed, the bottle was put into the pre-warmed water bath and left for one hour to ensure cooling. This prevented the agar from setting and maintained the agar at a temperature that ampicillin could be added. After the set time period 125µL of 50µg/mL Ampicillin (Calbiochem), 250µL of 0.1M IPTG (Biotech), and 500µL of 20mg/mL Xgal (Progen) was added and the contents was thoroughly mixed by gently inverting the bottle. Over a flame, the agar was poured into 90mm petri dishes (Global Science). The plates were then left to set at room temperature, after which they were wrapped in aluminium foil (Gilmours) as light degrades ampicillin and Xgal. Plates were then stored in the fridge (FRIGOREX) at 4˚C up to a maximum of 4 weeks.

2.7.3 Transforming *E. cloni* 10G Chemically Competent Cells

Before beginning the transformation, the ligation reaction had to be terminated by placing the PCR tube containing the ligation mix in the thermocycler (Bio-Rad T100) at 70˚C for 15min. As the thermocycler was running tubes containing 40µL of *E. cloni* subcloning grade cells (Lucigen) were removed from long term storage in the -80˚C freezer (Thermo Electron Corporation) and placed on ice to thaw for 10min. Once cells had thawed, 4µL of heat inactivated ligation reaction was added to the 40µL of *E. cloni* cells and tubes were given a gentle mix, and incubated on ice for 30min. This incubation allowed the ligation vector to attach to the cell wall of the *E. cloni* cells. After incubation, cells were then heat shocked by placing tubes at 42˚C for 45sec. The heat shock causes the *E. cloni* cell membrane to increase in fluidity allowing uptake of the plasmids. Cells were then returned to ice for 2min after which 160µL of room temperature recovery media (Lucigen) was added to the cells. Tubes were then

placed in a shaking incubator (Alphatech) at 300rpm for 2 hours at 37˚C, this incubation allowed for expression of the ampicillin resistant gene. Pre-made LB + ampicillin+ IPTG + Xgal plates were placed in a 37˚C incubator for at least 20min to allow the plates to dry. Once incubation of the plates and transformed cells was completed 50µL of the transformed cells was pipetted onto plates and spread over the surface of the plate using a glass hockey stick spreader. Before and between samples the hockey stick was decontaminated by being dipped into 100% ethanol and waved over a flaming Bunsen burner. Plates were then incubated upside down at 37˚C (Sanyo) overnight.

2.7.4 Screening Transformed Bacterium

The TA-Cloning vector allows for two different methods of screening, firstly, the use of ampicillin and secondly, the blue and white colony screen. The incorporation of the vector into the *E. cloni* cells gave the bacteria ampicillin resistance as the vector contains a β-lactamase gene thus allowing the growth of these cells on LB + ampicillin plates. The second level of screening is the blue-white colony screen, which is informative as to whether the vector within the *E.cloni* cell contains the amplified PCR product. The site where the product is inserted disrupts the LacZ gene in the vector causing it to not be expressed. In a vector that contains no genetic insert the LacZ gene is able to be expressed, leading to the production of the β-galactosidase. β-galactosidase is able to hydrolyse Xgal a sugar included in the agar and this hydrolysis in turn results in the colony becoming a distinct blue colour. Therefore, interruption of the lacZ gene via a ligated PCR product inhibits β-galactosidase expression, preventing hydrolysis of Xgal, thus causing the colonies to stay a characteristic white colour. IPTG is a molecular reagent also incorporated into the LB agar plates which aids in triggering the expression of the LacZ gene. Therefore, from this screen it can be concluded that white cells contain the PCR product whereas blue colonies do not. To further validate this the blue-white colony screen, white colonies were selected from the plates and a PCR was run targeting the region of the ligated PCR product. On the bottom (underside) of each plate, ten white colonies were chosen and marked with a label from 1 to 10. 2µL of DEPC water was added to ten 0.2mL PCR tubes (Neptune Scientific). Each tube was then labelled and

inoculated with one of the selected colonies on the plate which was done by touching the colony with a sterile 10µL pipette tip (Denville Scientific Inc) and transferring a small portion of the colony into the DEPC water by pipetting up and down. Caution was taken not to contaminate the tubes with agar as inclusion of this in the mixture could inhibit the following PCR reaction. The primers used in the PCR mix were M13 forward (5'-GTAAAACGACGGCCAGT-3') and M13 reverse (5'-AGTGTGTCCTTTGTCGATACTG-3') which specifically targeted the ligated product inside the plasmid. To each tube containing the bacterial colony and 2µL of DEPC water (template DNA), the following was added: 1µL of M13 forward primer, 1µL M13 reverse primer giving a total volume of 4µL. A master mix was then made up in the same way as nested PCR (see section 2.4.3) with 12.5µL 10x reaction buffer (Zymo Research), 1µL dNTPs (Solis BioDyne), 0.2µL ZymoTaq DNA Polymerase, and 7.3µL of DEPC water per sample. Tubes were pulse spun then placed in the thermocycler (Bio-Rad T100) and run using selected cycles parameters (Table 5). The PCR products were subsequently run on a 1.5% agarose gel (see section 2.5) to visualise and confirm the presence of the ligated PCR product.

2.7.5 Culturing Transformed Bacteria

Colonies found to contain the ligated PCR product, as confirmed by the PCR screen were grown in LB + Ampicillin medium overnight. LB medium was made up in a 500mL glass Schott bottle (Duran) by dissolving 12.5g of LB powdered medium (USB Corporation) in 500mL of deionised water. The bottle was then autoclaved (Astell) on the pre-set media cycle to ensure sterility. Once cooled, in a sterile 50mL falcon tube (Greiner) 5mL of sterile LB medium and 5µL of 50mg/mL Ampicillin (Calbiochem) was added. A disposable sterile transfer loop was used to transfer the colony containing the correct PCR insert from the agar plate to the culture tube. The tubes were then incubated at 37˚C overnight in a shaking incubator (Alphatech).

2.7.6 Plasmid Extraction

Following successful bacterial growth (LB medium was cloudy), the plasmid was extracted. The DNA-spin™ Plasmid DNA Purification Kit (iNtRON

Biotechnology) was used to effectively isolate and remove the plasmids from the bacterial cells. 3mL of bacterial suspension was centrifuged at 13,000rpm for 1min which caused the bacteria to form a pellet in the bottom of the tube and the supernatant was discarded. Cells were resuspended in 250µL of resuspension buffer and vortexed (Chiltern) thoroughly. 250µL of lysis buffer was added to the tube and inverted gently ten times. This step allows the plasmid DNA to be released from the bacterial cell. Gentle mixing is needed as any vigorous action may cause shearing of the plasmids. Tubes were then incubated at room temperature for 3min, after which 350µL of neutralisation buffer was added to the tube, and the tube inverted ten times, followed by a 5min incubation on ice. After the incubation time tubes were centrifuged (Eppendorf) at 13,000rpm for 10min at 4˚C. Supernatant was collected and transferred to a DNA-spin column and collection tube, with care taken not to disturb the precipitate as it contained unwanted cell debris, proteins, and genomic DNA. The column was centrifuged for 1min at 13,000rpm. The flow through was discarded and 500µL of Washing Buffer A (removes trace nuclease activity) was added to the DNA-spin column and then centrifuged for 1min at 13,0000rpm. The flow through was discarded and 700µL of Washing Buffer B (removes any unwanted salts) was added to the column and again centrifuged for 1min at 13,000rpm. The flow through was discarded and columns were centrifuged once again for 1min at 13,000rpm to completely remove any residual ethanol and to completely dry the membrane. The DNA-spin column was then transferred into a new clean collection tube to prevent the addition of washing buffer contaminants and 50µL of DEPC water was added directly onto the matrix of the column. The tube was incubated for 1min at room temperature and then centrifuged a final time for 1min at 13,000rpm, which allowed the eluted DNA to be collected in the bottom of the tube. The column was then discarded and using the 2000 nanodrop (Thermo Scientific) the quality and quantity of the collected plasmid DNA was determined in the same way as described for RNA (see section 2.3).

2.7.7 Restriction Enzyme Digest

Prior to sending the isolated plasmids for sequencing, a final screening method was used to ensure the presence of the correct PCR product in the

plasmid. This was carried out through a restriction digest of the plasmid. Within the PLUG-Prime TA Cloning Vector there are restriction sites located on either side of where the PCR product is inserted (Figure 18). Therefore, two restriction enzymes can be used to exploit these sites cutting the PCR product from the plasmid. For this reaction, EcoR1 and Pst1 restriction enzymes (Roche Diagnostics) were used. In a 1.5mL RNase/DNase free snap seal microfuge tube (Scientific Specialties Inc) the plasmid was added to the restriction digest reagents (Table 7).

Table 7: Reagents and volumes used for the restriction digest of the plasmids

Reagent	Volume
Plasmid	2µL
10X SuRE Cut Buffer	2µL
EcoR1 10U/µL	2µL
Pst1 10U/µL	2µL
DEPC Water	12µL
Total	20µL

The contents of the tubes was briefly mixed by inverting the tube several times, followed by a pulse spin for 5sec. Tubes were then incubated overnight at 37˚C (Sanyo). Once incubation was complete samples were run on a 1.5% agarose gel (see section 2.5). A successful digest contained two bands, the first band with a size of around 2700bp representing the plasmid, and the second band of approximately the same size as the ligated PCR product size. The presence of this second band indicated a successful ligation.

2.8 Sequencing

Upon conformation of the correct ligated PCR product after restriction digests the extracted plasmids (section 2.7.6) were prepared for sequencing. 15µL of plasmid at a DNA concentration of 100ng/µL was required for the sequencing reaction. Using the determined plasmid concentrations through the nanodrop results the correct amount of plasmid needed at the correct concentration was obtained by following this calculation:

$$\text{Plasmid Volume } (\mu L) = \frac{(100 ng/mL \times 15 \mu L)}{\text{Plasmid Concentration } (ng/mL)}$$

The calculated plasmid volume was then added to a labelled 0.2mL DNase/RNase free PCR tube (Neptune Scientific), followed by DEPC water to make the final volume up to 15µL. The tubes were then sent to the DNA Sequencing Facility located at the University of Waikato. The plasmid was sequenced using Applied Biosystems BigDye® Terminator v3.1 Cycle Sequencing Kit, where the sequence was then run on a 3130xl Genetic Analyser System (Applied Biosystems). Plasmid sequencing was carried out using the M13 forward primer and the M13 reverse primer (sequences previously outlined in section 2.7.4). At least three plasmids for each amplified product were sequenced.

2.8.1 Sequence Analysis

Once the nucleotide sequence was obtained for either the 3' or the 5' RACE, analysis of the results could be carried out. Firstly, any vector sequence was identified and removed from the sequenced products. Secondly, the primer sequences that had been used to amplify the initial PCR product were located. Multiple alignments of sequenced products obtained from the same initial PCR were carried out using ClustalX 2.1 (Jeanmougin, *et al.*, 1998) to allow the generation of a consensus sequence. This is required to provide quality control to the sequences obtained as errors can occur during the sequencing process at a rate of approximately every 10^6 nucleotides. Each consensus sequence obtained for the 3' and the 5' RACE was then compared to the initial sequence found in the transcriptomic database to determine it was the correct sequence. Once the identity of the sequenced products had been confirmed, the sequenced 3' and 5' RACE products for the same gene were joined together, to give the full length cDNA sequence which were then translated using ExPASy translate tool (http://web.expasy.org/translate/) to determine the amino acid sequence of the AMP of interest. BLAST analysis was used to confirm the identity of the protein and ClustalX 2.1 was used to align the protein with already characterised fish AMP's. SignalP ver4.0 (Petersen *et al.*, 2011) was used to predict the presence of any signal peptides within the predicted proteins and using the alignments homology between the proteins was analysed and conclusions were drawn.

2.9 Real-Time PCR

Real-time PCR (RT-PCR) is used to monitor the amplification of a targeted gene during a PCR reaction. This method can be used to quantitatively or semi quantitatively show amplification of genes relative to each other.

2.9.1 Primer Design

Using Primer3 an online program (http://primer3.sourceforge.net/) forward and reverse primers were designed for the genes of interest and the selected housekeeping genes. As with RACE primer design the following characteristics were followed as much as possible. Primers had a GC content of 40-60% in order to ensure stability of the product. The melting temperature of the primers was between 54 and 60°C with the forward and reverse primers used in the same reaction within 1°C of each other. Primers also ended with a GC clamp as T and A residues more easily bind to DNA non-specifically. The number of possible interactions within a primer and between primers were kept to a minimum to prevent hairpin loops or primer dimers, respectively. Lastly, the primers and amplicons were BLAST searched against the transcriptome and public database to confirm that the primers targeted the correct gene of interest.

2.9.2 RNA Extraction and cDNA Synthesis

Total RNA was prepared from gill, liver and spleen tissues from three different *S. lalandi* individuals, for use in determination of primer efficiency and specificity. Preparation of high purity RNA, with no gDNA contamination was crucial for achieving the best results from RT-PCR. To achieve this, the Quick-RNA™ MiniPrep kit (Zymo) was used, which reliably and rapidly isolates DNA-free RNA from tissue. The RNA isolation and extraction protocol consisted of three steps; sample lysis, sample clearing (gDNA removal) and RNA purification. Selected tissues were added to a 2.0mL RNase/DNase free conical base screw cap tubes (Neptune) containing 600 µL of RNA Lysis Buffer. 0.2mL of 0.1mm zirconia/silica beads (BioSpec Productions) and 0.3mL of 0.5mm glass beads (BioSpec Productions) were added and the tubes and placed into a bead beater (Alphatech Systems Limited). The tissues were homogenised at 4800 oscillations

per minute for 10sec, which was repeated if the tissue did not break down completely. Homogenisation caused the lysis buffer to foam, which was cleared by centrifuge at 13,000rpm for 1min. The supernatant was then transferred into a yellow Spin-Away™ Filter, which was placed into a collection tube. The sample was centrifuged at 13,000rpm for 1min allowing DNA to bind to the columns matrix and therefore removing the contaminating gDNA from the mix. The flow-through in the collection tube was collected and used for RNA purification. To the flow-through, 1 volume of ethanol (95-100%) was added at a ratio of 1:1 and mixed well. The mixture was then transferred to a green RNA Zymo-Spin™ IIICG column in a collection tube and centrifuged 13,000rpm for 30sec. Centrifugation allowed the total RNA to bind to the columns matrix, the flow-through was discarded. To ensure complete removal of any trace gDNA, the isolated total RNA underwent DNase I treatment. 400 µl of RNA Wash Buffer was added to the column, which was then centrifuged at 13,000rpm for 30sec. The flow through discarded. For each sample to be treated, a DNase I Reaction Mix was prepared in a 2.0mL RNase/DNase free tube, which contained 5µL of DNase I DNA and 75µL of Digestion Buffer. The 80µL DNase I Reaction Mix was added directly to the column matrix and incubated at room temperature for 15min. The column was then centrifuged at 13,000rpm for 30sec and flow through discarded. 400µL of RNA Prep Buffer was added to the column and again centrifuged at 13,000rpm for 30sec. The flow-through was discarded ensuring complete removal of gDNA and the DNase I Reaction Mix. 700µl of RNA Wash Buffer was added to the column in order to wash the RNA contained in the matrix. The column was centrifuged at 13,000rpm for 30sec and the flow-through discarded. The RNA wash step was repeated where another 400µl RNA Wash Buffer was added to the column. The tube was then centrifuged 13,000rpm for 2min to ensure complete removal of the wash buffer. The RNA-spin column was then transferred into a new clean collection tube to prevent any addition of washing buffer contaminants. 30µL of DEPC water was added directly onto the matrix of the column, and incubated for 1min at room temperature to elute the RNA. The tube was then centrifuged a final time for 30sec at 13,000rpm, which allowed eluted RNA to be collected in the bottom of the tube. The column was then discarded, and a nanodrop reading carried out to establish the quality and quantity of the RNA (see section 2.3).

The RNA was immediately used for cDNA synthesis to minimise RNA degradation. To synthesise cDNA the HiSenScript™ RH(-) cDNA Synthesis Kit (iNtRON) was used. This kit is designed for the sensitive, reproducible detection and analysis of full-length cDNA copies from total RNA. For each sample the following mixture was made up in a 0.2mL DNase/RNase free PCR tube (Neptune); 1µg of total RNA, 10µL of 2X RT Reaction Solution, 1µL of Enzyme Mix Solution and DNase/RNase Free Water up to a total volume of 20µL. Tubes were then vortexed to ensure thorough mixing, pulse spun and finally incubated in a Biorad T100 thermal cycler at 42°C for 1 hour. Incubation allowed for reverse transcription of the RNA into cDNA. Following reverse transcription the reaction was inactivated by incubation at 85°C for 5min. Tubes were then stored at 4°C until being used in RT-PCR for testing primer efficiencies.

2.9.3 Real-Time PCR

RT-PCR was performed using the Rotor-Gene 6000 (Corbett). The RealMOD™ GH Green q PCR Master Mix Kit (iNtRON) was used following the manufacturer's instructions. Briefly, into a 0.2mL RNase/DNase free, thin walled, clear PCR tube (Axygen), 10µL of 2x RealMODTM GH Green qPCR Master Mix, 1µL of forward primer (10 µM), 1µL of reverse primer (10µM) and 8µL of previously synthesised cDNA was added. Amplification was carried out using the following program; Initial denature at 94°C for 5min, followed by 40-50 cycles of denaturation at 94°C for 20sec and annealing/extension at 60°C for 30sec. Fluorescent outputs were then measured and recorded at 80°C. A melt curve for each sample was performed between 72°C and 94°C to ensure 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.

2.9.4 Primer Testing and Efficiencies

Prior to determination of gene expression, the primer efficiencies were tested to establish amplification efficiencies. The previously prepared cDNA from selected tissues underwent either a 2-fold or a 4-fold serial dilution. The produced

cDNA was used at multiple concentrations, undiluted, 1:4, 1:16 and 1:64 for the housekeeping genes and undiluted, 1:2, 1:4, and 1:16 for the genes of interest. For each cDNA dilution, primer pairs were run in duplicate as well as a negative control. The determination of primer efficiencies were run in duplicate to ensure accurate results. The negative control contained DNase/RNase free water in lieu of cDNA template in order to check for contamination. 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-log10 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 determined how close the points were to the line of best fit. Efficiencies (E) of the primers were calculated using the equation $E=10^{(-1/s)}-1$, where "s" is the slope of the line. In addition, real-time products were run on a 1.5% agarose gel, using the same protocol that was previously described (section 2.5) to confirm amplification of a single product.

2.9.5 Measuring Gene Expression

The housekeeping genes were used to normalise the expression of selected genes, with expression analysis being achieved using the geometric means of these genes (Vandesompele *et al.*, 2002). Using the comparative ΔCq method (Silver *et al.*, 2006), relative expression levels were determined. This method was a variation of the Livak method (Livak & Schmittgen, 2001) which is used to evaluate gene expression, where $\Delta Cq = 2^{Cq(ref)-Cq(target)}$.

Chapter Three: Results

3.1 Bioinformatics

Known AMP genes and their corresponding amino acid sequence were compiled from several fish species (Figure 19). Cathelicidins, piscidins, hepcidins, defensins, and other groups of AMPs were identified from searches of public databases or from published literature. Several of these sequences were then used to search the available yellowtail kingfish (*Seriola lalandi*) and pouched lamprey (*Geotria australis*) transcriptomic library to identify genetic fragments similar to the input sequence. The transcriptomic library used for *S. lalandi* in this research was derived from spleen tissue of an adult individual. Whereas, the transcriptomic libraries generated for *G. australis* were derived from liver, kidney, skin and gill tissue of adult individuals. Candidate gene sequences were then generated using de novo assembly in Geneious from identified fragments. Within these libraries sequences were then aligned with known AMP sequences from fish to confirm that the correct genetic product had been identified.

From the *S. lalandi* transcriptomic library ten different AMPs were identified (see Appendix IV), hepcidin, moronecidin, two high density lipoproteins (apoA-I and apoA-II), piscidin, epinecidin, two histone derived AMPs (H2A and H2B), haemoglobin derived, and NK-lysin. Each identified sequence was aligned with a known AMP gene of the same type to determine sequence homology (see Appendix V). Whereas, from the *G. australis* transcriptomic library only five AMPs were identified (see Appendix VI), defensin-like, haemoglobin derived, NK-lysin, and two histone derived AMPs, H2A and H2B. Each identified sequence was aligned with a known AMP gene of the same type to determine sequence homology (see Appendix VII). From this two potential *S. lalandi* AMP genes (moronecidin and hepcidin) and one *G. australis* AMP gene (defensin-like) were chosen for further analysis. All other significant hits from the transcriptomic library were saved as potential candidate genes for future study.

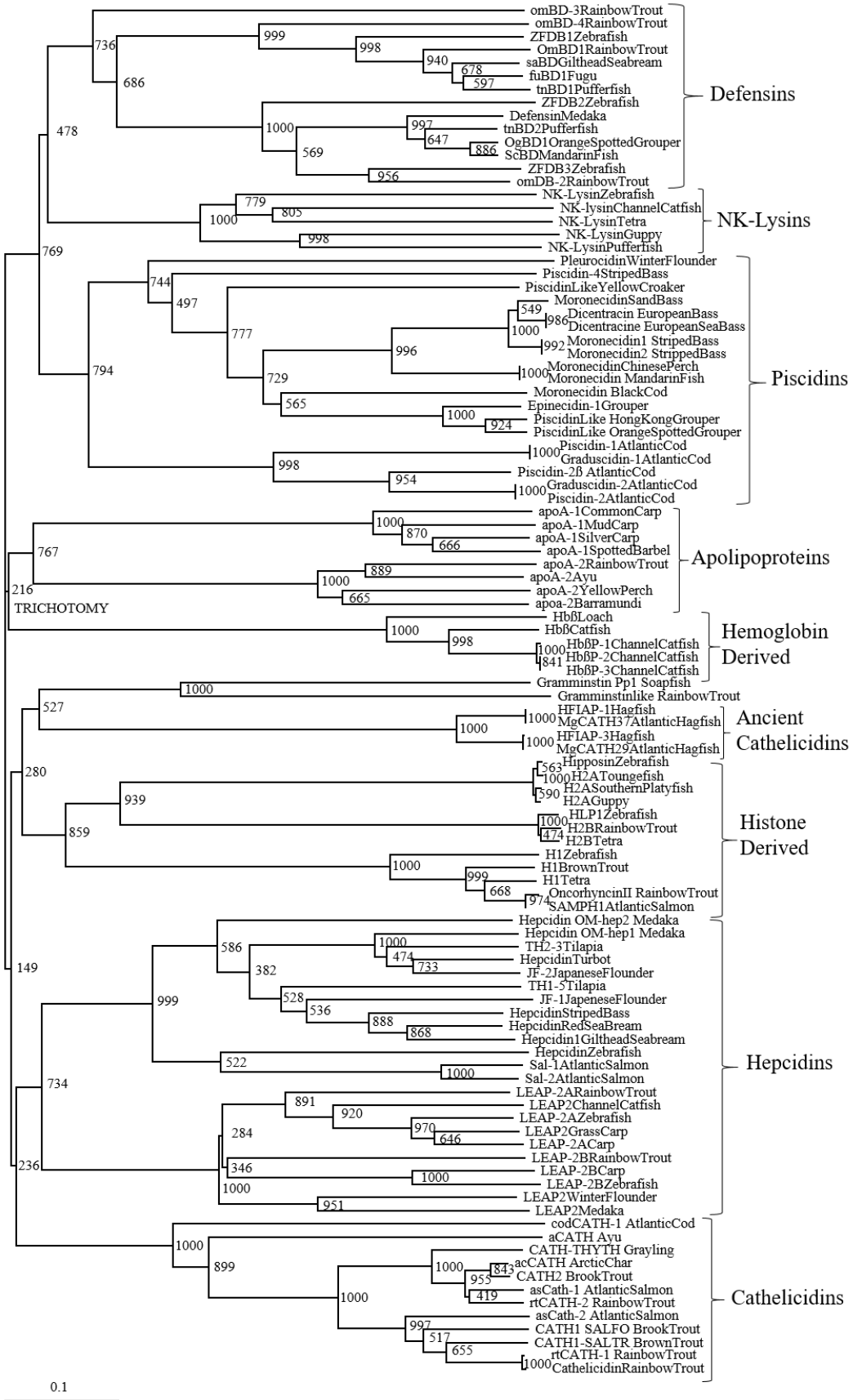

Figure 19: Range of AMP sequences identified in fish and available in public databases. Identification for each sequence is listed in appendix III.

3.1.1 Yellowtail Kingfish (*S. lalandi*)

Hepcidin and moronecidin were two AMP genes identified from the *S. lalandi* transcriptomic library and showed good homology when aligned with other known fish AMP sequences. A potential moronecidin gene was identified and aligned against duskytail grouper (*Epinephelus bleekeri*) nucleotide sequence (Figure 20). The potential *S. lalandi* moronecidin gene was then translated and the protein sequence aligned against the duskytail grouper moronecidin protein (Figure 21).

```
Potential S. lalandi moronecidin   GGCCAAGGGCCAAGGCGAGACACGGRCCTCWSAGCGGCCAAGGCGCAGTC
Duskytail grouper moronecidin      --------------------------------------------------

Potential S. lalandi moronecidin   ATCAGGAAGGATGAAGTTCATCGCCCTGTTTCTTGTGTTGTCACTGGTCG
Duskytail grouper moronecidin      ----------ATGAGGTGCATCGCCCTCTTTTTTGTGTTGTCGCTGGTGG
                                            **** ** ********* *** ********** ***** *

Potential S. lalandi moronecidin   TCCTCATGGCTGAACCTGGGGAAGGTTTTTTTCACCACATTCTCTCGGGA
Duskytail grouper moronecidin      TCCTCATGGCTGAACCCGGGGAGGGGTTCTTTTTCCACATCATCAAAGGA
                                   ********************* ***** ** ** ***  ******  **   ***

Potential S. lalandi moronecidin   ATTTTTCACGTTGGCAAAATGATCCACGGCGCGATCCAAAGGAGGAGACA
Duskytail grouper moronecidin      CTCTTTCACGCTGGCAAGATGATCCATGGACTTATCCACAGGAGACGACA
                                    * ******* ****** ******** **    ***** *****  ****

Potential S. lalandi moronecidin   T-GGCATGACAGAGCTAGAGCGATGCGGGTGGAGCGGTCGT--CTCTGGA
Duskytail grouper moronecidin      TCGACATGGGATGGA-AGAGCTGCAAGACCTGGACCAACGTGCCTTTGAA
                                   * * ****  *  *  *****     *    *  *   ***  ** ** *

Potential S. lalandi moronecidin   TCAGG-----------------------------------------------
Duskytail grouper moronecidin      CGAGAGAAAGCTTTTGCCTGAGTCTACAATAGCCCAGTGAAGGAGCTACT
                                     **

Potential S. lalandi moronecidin   --------------------------------------------------
Duskytail grouper moronecidin      CATTGTTAACACAAAAACGAAAAGATTTTTGTTTTTGAGTATAGGAAGTA

Potential S. lalandi moronecidin   --------------------------------------------------
Duskytail grouper moronecidin      TTGGCTCAATTGGGTAACCAAAATATTTTACATTGATCTAATCGATTTTG

Potential S. lalandi moronecidin   -------------------------------------------------
Duskytail grouper moronecidin      AAAAAAACGTTATTCGAAATAAATCTGGAATCTGTGTTACGCAAAAGC
```

Figure 20: Moronecidin nucleotide sequence obtained from the *S. lalandi* transcriptomic library aligned against a duskytail grouper moronecidin gene (Accession number, HQ437912.1).

```
Potential S. lalandi moronecidin    GQGPRRDTXLXAAKAQSSGRMKFIALFLVLSLVVLMAEPGEGFFHHILSG
Duskytail grouper moronecidin       --------------------MRCIALFFVLSLVVLMAEPGEGFFFHIIKG
                                                        *: ****:****************.**:.*

Potential S. lalandi moronecidin    IFHVGKMIHGAIQRR--RHGMTELERCGWSGRLWIR-----
Duskytail grouper moronecidin       LFHAGKMIHGLIHRRRHRHGMEELQ--DLDQRAFEREKAFA
                                    :**.****** *:**  **** **:  . . * : *
```

Figure 21: *S. lalandi* potential moronecidin protein sequence aligned against a duskytail grouper moronecidin protein (Accession number, ADY86110.1).

The potential hepcidin gene identified from the *S. lalandi* transcriptomic database was aligned against the large yellow spotted croaker (*Larimichthys crocea*) nucleotide sequence (Figure 22). The potential *S. lalandi* hepcidin gene was then translated and the protein sequence aligned against the large yellow croaker hepcidin protein (Figure 23).

```
Potential S. lalandi hepcidin     --------------------------------------------------
Large yellow croaker hepcidin     ATGAAGGCATTCAGCATTGCAGTTGCAGTGACACTCGTGCTCGCCTTTAT

Potential S. lalandi hepcidin     -GGCCAAGGCGAGAGCTCTGCTGTCCCATTCCACGGGGTGCGAGAGCTGG
Large yellow croaker hepcidin     TTGCATTCTGGAGAGCTCTGCTGTCCCATTCACCGGGGTGCAAGAGCTGG
                                    **      **********************  ******** ********

Potential S. lalandi hepcidin     AAGAGGCAGGAAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCA
Large yellow croaker hepcidin     AGGAGGCAGGGAGCAATGACACTCCAGTTGCGGCACATCAAGAAATGTCA
                                  * ******** ******************** ***** ****** ******

Potential S. lalandi hepcidin     ATGGCATCGTGGATGATGCCGAATCCCGTCAGGCAGAAGCGCCAGAGCCA
Large yellow croaker hepcidin     ATGGAATCGTGGATGATGCCCAATCACATCAGGCAGAAGCGTCAGAGCCA
                                  **** *************** **** * ************* ********

Potential S. lalandi hepcidin     CCTCTCCATGTGCCGCTGGTGCTGCAACTGCTGCACGGCCAACAAGGGCT
Large yellow croaker hepcidin     CCTCTCCTTGTGCCGCTGGTGCTGCAACTGCTGCAAGAGCAACAAGGGCT
                                  ******* *************************** *  ***********

Potential S. lalandi hepcidin     GCGGTTTCTGCTGCAGGTTCTGAGGATTCCCGCCACAGCCTCGAAATATT
Large yellow croaker hepcidin     GCGGTTTCTGCTGCAGGTTC------------------------------
                                  ********************

Potential S. lalandi hepcidin     AATTTATTTTACTTCTTTTTGCTTTACCCCAGGAAATGACCCTCTTTC
Large yellow croaker hepcidin     ---------------------------------------------
```

Figure 22: Hepcidin nucleotide sequence obtained from the *S. lalandi* transcriptomic library aligned against a large yellow croaker hepcidin gene (Accession number, DQ307050.1).

```
Potential S. lalandi hepcidin      ------------------GQGESSAVPFHGVRELEEAGSNDTPVVARQEMS
Large yellow croaker hepcidin      MKAFSIAVAVTLVLAFICILESSAVPFTGVQELEEAGSNDTPVAAHQEMS
                                                    ******* **:************.*:****

Potential S. lalandi hepcidin      MASWMMPNPVRQKRQSHLSMCRWCCNCCTANKGCGFCCRF-GFPPQPRNI
Large yellow croaker hepcidin      MESWMMPNHIRQKRQSHLSLCRWCCNCCKSNKGCGFCCRF----------
                                   * ****** :*********:********.:**********

Potential S. lalandi hepcidin      NLFYFFLLYPRK-PSF
Large yellow croaker hepcidin      ----------------
```

Figure 23: *S. lalandi* potential hepcidin protein sequence aligned against a large yellow croaker hepcidin protein (Accession number, ABC18307.1).

3.1.2 Lamprey

A defensin-like peptide was identified from the *G. australis* transcriptomic library using an already characterised protein sequence from sea lamprey (*Petromyzon marinus*; Accession number, Q10996.1). There is no nucleotide sequence available for this protein, so an alignment comparing four different sequences identified from the lamprey transcriptomic library was generated (Figure 24). However, the protein sequence translated from the identified nucleotide sequences shows good homology when aligned with the sea lamprey defensin-like protein (Figure 25). Other AMP sequences were also identified in the lamprey transcriptomic library and aligned (Appendix VII).

```
G. australis 1  ------------------AATGTTAAAGGTTTGCTGTAATGCACAGTGGTTATCTTAAAT
G. australis 2  ------------------AATGTTAAAGGTTTGCTGTAATGCACAGTGGTTATCTTAAAT
G. australis 3  AGATGAAGATGGGCGGCCGGGAGGAGAAGGTCGCYGKRKY-YGTCGKSAYGWYCMCTCAT
G. australis 4  ----------------------------------AGAATT-CACCAGRCKGATC----AT
                                                   *               *    **

G. australis 1  G--TTTGAACAATAAACCAGTTATAGTATGAATAGTG--TGKACWYTMSTCAGCTTGTCC
G. australis 2  G--TTTGAACAATAAACCAGTTATAGTATGAATAGTG--TGKACWYTMSTCAGCTTGTCC
G. australis 3  GGCTCTTCTTCGTGCGCTGTTGCTGCTGGTGGTGGTGACTGGGCTCTCGTCAGCTTGTCC
G. australis 4  GGCTCTTCTTCGTGCGCTGTTGCTGCTGGTGGTGGTGACTGGGCTCTCGTCAGCTTGTCC
                *  * *      *   *   *  *  *     * ***  **  *  *  ***********

G. australis 1  CTGCGGCAAAAGAAGATGCTGCGTTCGAGGCTTGACGGTTTACTGCTGCTT-GCAGACAG
G. australis 2  CTGCGGCAAAAGAAGATGCTGCGTTCGAGGCTTGACGGTTTACTGCTGCTT-GCAGACAG
G. australis 3  CTGCGGCAAAAGAAGATGCTGCGTTCGAGGCTTGACGGTTTACTGCTGCTTTGCAGACAG
G. australis 4  CTGCGGCAAAAGAAGATGCTGCGTTCGAGGCTTGACGGTTTACTGCTGCTTTGCAGACAG
                ************************************************ ********

G. australis 1  GGAGGAAA-CGGAGTGATGCAAGGAAGCAGAATTCACCAAGCGTTGGATTGTTCACCCAC
G. australis 2  GGAGGAAA-CGGAGTGATGCAAGGAAGCAGAATTCACCAAGCGTTGGATTGTTCACCCAC
G. australis 3  GGAGGAAAACGGAGTGATGCAAGTGACCATGGTGCCGGCACAGGCTGTTT-CTC-CCTAC
G. australis 4  GGAAGAAAACGGAGTGATGCAAGTGACCATGGTGCCGGCACAGGCTGNTTTCTC-CCTAC
                *** **** **************  * **   * *    *  *   * **  ** ** **

G. australis 1  TAATAGGGAACGTGAGCTGGGTTAGACCGTCGTGAGACAGGTTAGTTTACCC--------
G. australis 2  TAATAGGGAACGTGAGCTGGGTTAGACCGTCGTGAGACAGGTTAGTTTACCC--------
G. australis 3  AACGCCCACGCCCGCTCCTGCTCAGGC-GCTGTCTGCTCGGCCAATGGAGAATCTCGCTG
G. australis 4  AACGCCCACGCCCGCTCCTGCTCAGGC-GCTGTCTGCTCGGCC-----------------
                 *        *  *  *  * * ** * *  **  *   **

G. australis 1  ---------------
G. australis 2  ---------------
G. australis 3  CCTGCCCCATGTCTG
G. australis 4  ---------------
```

Figure 24: Defensin-like nucleotide alignment comparing four different sequences identified in the lamprey transcriptomic library.

```
Potential G. australis defensin-like    R-RWAAGRRRSXXXSXXXLMALLRALLLLVVVTGLSSACPCGKRRCCVRG
Petromyzon marinus defensin-like        ------------------------------------CPCGRRRCCVRG
                                                                            ****:*******

Potential G. australis defensin-like    LTVYCCFADREENGVMQVTMVPAQAVSPYNAHARSCSGAVCSANGESRCL
Petromyzon marinus defensin-like        LNVYCCF-----------------------------------------
                                        *.*****

Potential G. australis defensin-like    PHV
Petromyzon marinus defensin-like        ---
```

Figure 25: *G. australis* defensin-like protein sequence translated from the identified nucleotide sequence aligned against the sea lamprey defensin-like protein (Accession number, Q10996.1).

To compare the homology of the identified *G. australis* defensin-like protein to a known mammalian defensin, it was aligned to human alpha defensin-1 (Figure 26). Overall there is low sequence homology, although conserved cysteine and arginine residues between the proteins can be seen.

```
G. australis transcriptome ----------------------------------------------------
Human alpha defensin-1     MRTLAILAAILLVALQAQAEPLQARADEVAAAPEQIAADIPEVVVSLAWD

G. australis transcriptome ---------------CPC-------GKRR---CCVRG-LTVYCCL
Human alpha defensin-1     ESLAPKHPGSRKNMACYCRIPACIAGERRYGTCIYQGRLWAFCC-
                                           * *       *:**   *  :* * .:**
```

Figure 26: Sequence alignment of the *G. australis* defensin-like protein identified from the transcriptomic library aligned with human alpha defensin-1 (Accession number, NP_004075.1). Highlighted are the cysteine and arginine residues.

3.2 Rapid Amplification of cDNA Ends (RACE)-PCR

3.2.1 RNA Extraction

3’ and 5’ RACE was carried out on RNA from adult *S. lalandi* and *G. australis* tissues. Four *S. lalandi* tissue types (spleen, gills, liver, and kidney) were used, whereas, only kidney tissue was used in *G. auatralis*. Total RNA was extracted from these tissues and quantified using the Nanodrop 2000 (Table 8). The nanodrop showed the RNA purity of each tissue to be 1.7 or above. In addition, the concentration for each sample was used to calculate the volume of RNA needed for 5’ and 3’ RACE.

Table 8: Nanodrop results from RNA extraction of various *S. lalandi* tissues and one *G. australis* tissue.

Tissue Type	RNA Concentration (ng/µL)	260/280	260/230
***S. lalandi* Kidney**	1164.6	2.01	2.10
***S. lalandi* Liver**	179731	1.80	1.90
***S. lalandi* Gill**	1721.5	2.07	2.18
***S. lalandi* Spleen**	6393.9	1.72	1.82
***G. australis* Kidney**	597	2.06	2.25

3.2.2 Yellowtail Kingfish 3' RACE

3.2.2.1 Primer Design

The partial hepcidin and moronecidin sequences identified from the *S. lalandi* transcriptomic library were used to design 3' RACE primers. Two forward primers were designed close to the start of the sequences. The first forward primer (F1) and second forward primer (F2) are outlined for moronecidin (Figure 27) and hepcidin (Figure 28)

GGCCAAGGGCCAAGGCGAGACACGGRCCTCWSAGCGGCCAAGGCGCAGTCATCAGGAAGGAT
GAAGTTCATCGCCCTGTTTCTTGTGTTGTCACTGGTCGTCCTCATGGCTGAACCTGGGGAAG
GTTTTTTTCACCACATTCTCTCGGGAATTTTTCACGTTGGCAAAATGATCCACGGCGCGATC
CAAAGGAGGAGACATGGCATGACAGAGCTAGAGCGATGCGGGTGGAGCGGTCGTCTCTGGAT
CAGG

Figure 27: Candidate *S. lalandi* moronecidin sequence with F1 primer outlined by a box and F2 primer highlighted in blue.

GGCCAAGGCGAGAGCTCTGCTGTCCCATTCCACGGGGTGCGAGAGCTGGAAGAGGCAGGAAG
CAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGCATCGTGGATGATGCCGAATC
CCGTCAGGCAGAAGCGCCAGAGCCACCTCTCCATGTGCCGCTGGTGCTGCAACTGCTGCACG
GCCAACAAGGGCTGCGGTTTCTGCTGCAGGTTCTGAGGATTCCCGCCACAGCCTCGAAATAT
TAATTTATTTTACTTCTTTTTGCTTTACCCCAGGAAATGACCCTCTTTC

Figure 28: Candidate *S. lalandi* hepcidin sequence with F1 primer outlined by a box and F2 primer highlighted in blue.

3.2.2.2 Nested PCR

The two primers designed for each *S. lalandi* AMP sequence were used in nested 3' RACE-PCR in an effort to isolate the 3' ends of the moronecidin and hepcidin genes. The first round of nested PCR was carried out on the 3' RACE cDNA, using either the forward primers F1 or F2 for each gene along with the outer 3' RACE reverse primer. PCR products were run on a 1.5% agarose gel (Figure 29). Gel imaging showed the presence of multiple bands when using the F1 and F2 moronecidin primers (lane 1 and 2 consecutively). The presence of multiple bands was also noted for the F1 hepcidin primer (lane 3). In contrast, nothing appeared to be amplified when using the F2 hepcidin primer (lane 4).

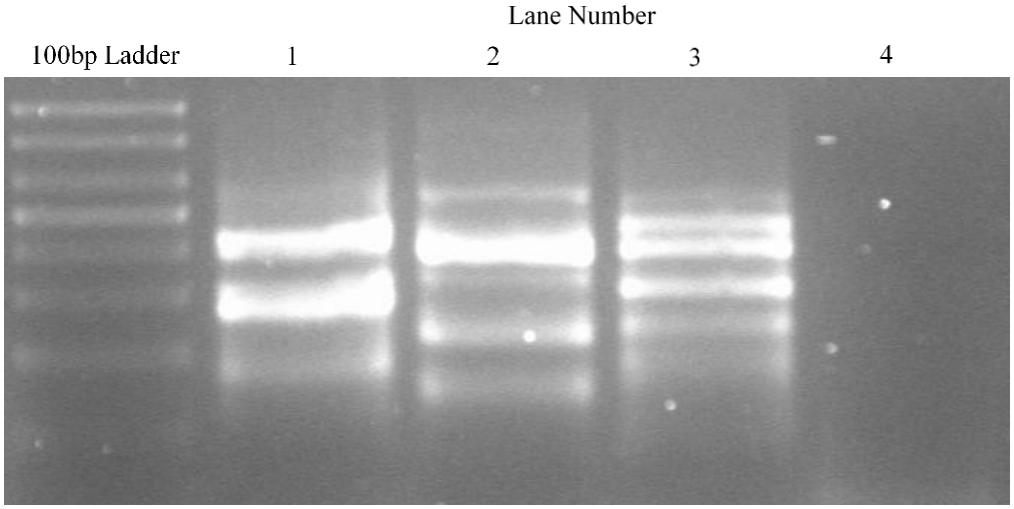

Figure 29: Gel image showing the first round of nested PCR. Products obtained were using the F1 and F2 primers for moronecidin and hepcidin alongside the 3' RACE outer reverse primer. The template DNA used was a combination of all *S. lalandi* tissues from which RNA was previously isolated. Lane numbering is as follows; lane 1 moronecidin F1, lane 2 moronecidin F2, lane 3 hepcidin F1, and lane 4 hepcidin F2. Refer to appendix VIII for ladder band sizing.

PCR products obtained from round one nested PCR using the F1 and 3' RACE outer primer for both moronecidin and hepcidin were subsequently used as the template for the second round. The forward primer F1 or F2 for each gene were used alongside the 3' RACE inner primer. PCR products were run on an agarose gel (Figure 30). Multiple faint bands could be seen using the F1 primer for moronecidin (lane 1) and hepcidin (lane 3), whereas, a single strong band was amplified when using F2 for each gene (lane 2 and lane 4). The single isolated bands for moronecidin and hepcidin show product sizes of approximately 400bp for moronecidin and 500bp for hepcidin.

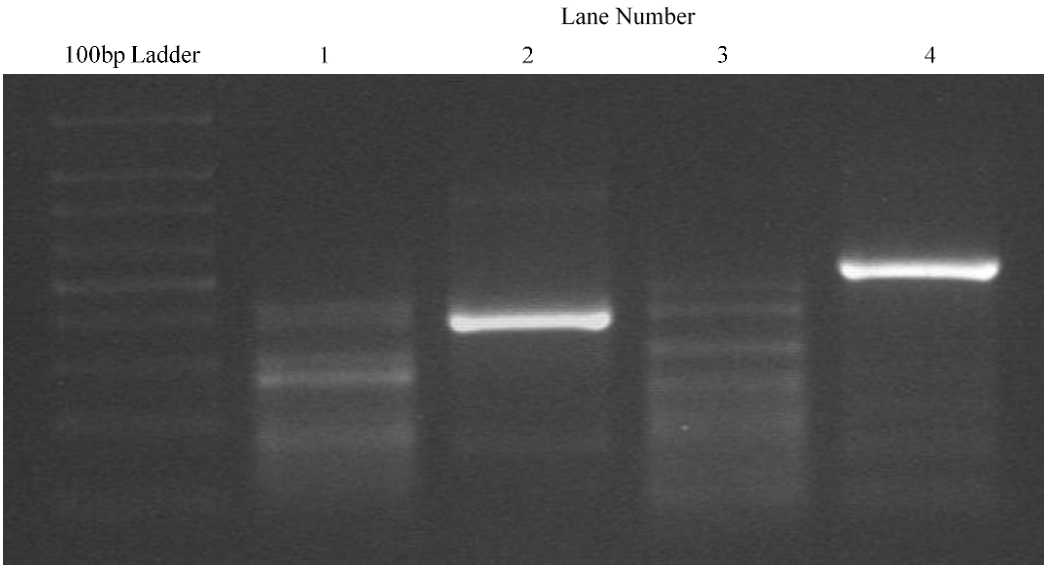

Figure 30: Gel image showing the second round of nested PCR. Products obtained were using the reverse 3' RACE inner primer alongside the F1 or F2 *S. lalandi* moronecidin or hepcidin primers. Lane numbering is as follows; lane 1 moronecidin F1, lane 2 moronecidin F2, lane 3 hepcidin F1, and lane 4 hepcidin F2. Refer to appendices VIII for ladder band sizing.

In each case the first round of nested PCR using the F1 forward primer and reverse 3' RACE outer primer first isolated a number of fragments, however, the second round of nested PCR using the F2 forward primer and reverse 3' RACE inner primer increased specificity allowing the isolation of a single major product band. Following the successful amplification of these products using nested PCR, fresh products were taken on to be cloned for sequencing.

3.2.2.3 Ligation and Bacterial Transformation

Each PCR product obtained after the second round of nested PCR were ligated into a cloning vector followed by transformation into chemically competent *E. cloni* cells (Appendix XVII). Transformed bacterial cells were grown up on LB + AMP + Xgal + IPTG plates, and underwent a blue and white colony screen. Transformed cells for both moronecidin (Plate 1, Figure 31) and hepcidin (Plate 2, Figure 31) were grown up on these selective plates.

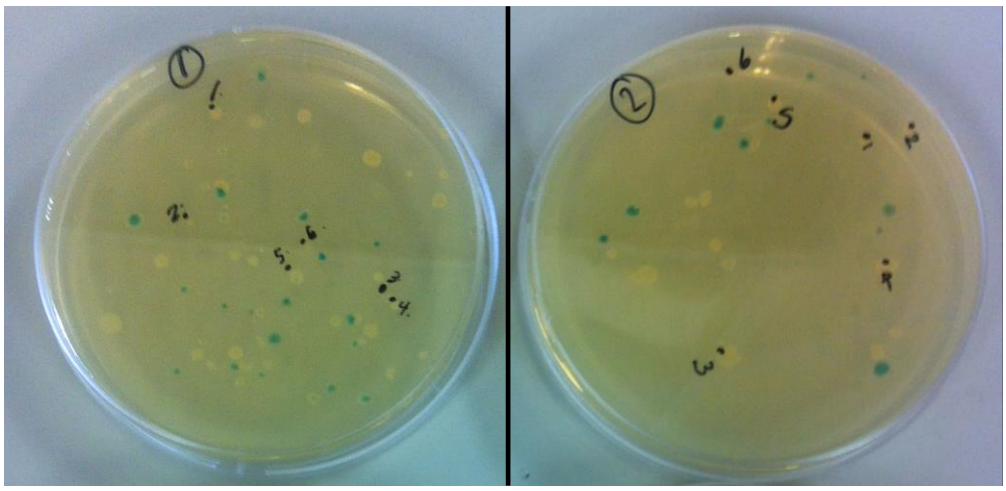

Figure 31: Blue and white colony screen for moronecidin (plate 1) and hepcidin (plate 2).

White colonies indicated cells that contained the cloning vector with a successfully ligated PCR product. However, blue colonies contained a cloning vector but no ligated PCR product. Six white colonies from each plate were selected and screened using conventional PCR to determine if they contained PCR products of the correct size. M13 forward and M13 reverse primers were used to select for the ligated gene, the expected product sizes for each gene included part of the cloning vector where the M13 primers bound. PCR products were run on an agarose gel for moronecidin (Figure 32) and hepcidin (Figure 33), where products of ~550bp were expected for moronecidin and ~650bp were expected for hepcidin.

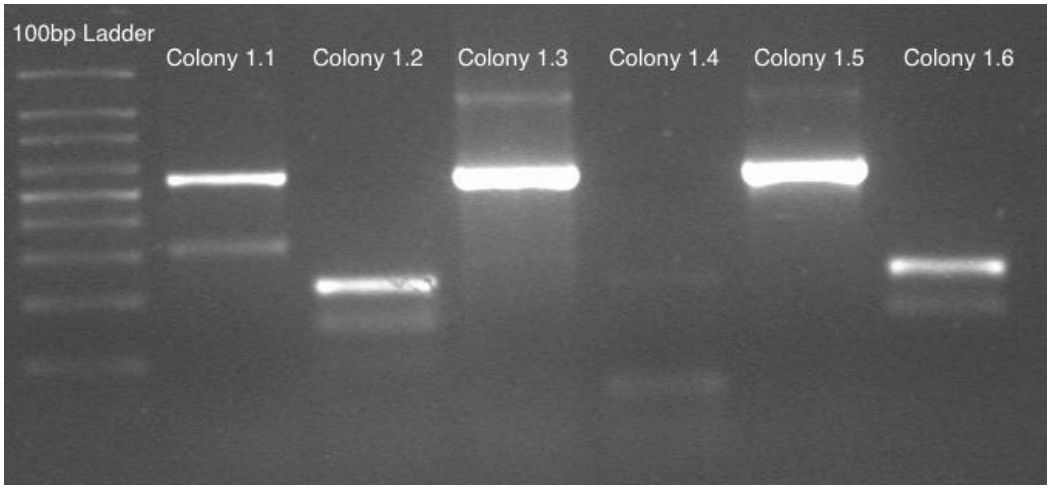

Figure 32: Gel image showing PCR products obtained from six colonies screened from the moronecidin plate. Refer to appendix VIII for ladder band sizing.

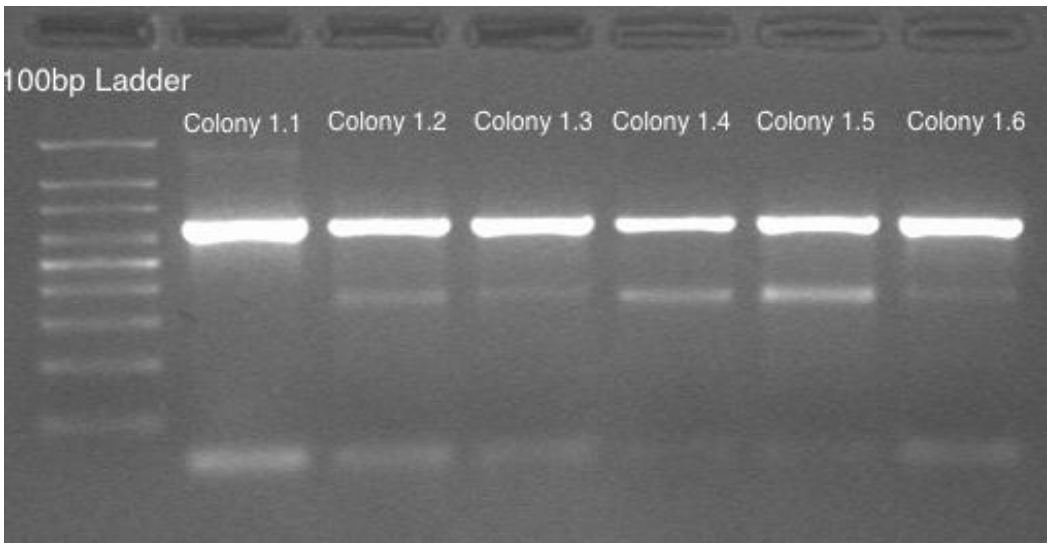

Figure 33: Gel image showing PCR products obtained from six colonies screened from the hepcidin plate. Refer to appendix VIII for ladder band sizing.

From the moronecidin colonies analysed using PCR, three colonies were successful in showing gene products of the correct size, colonies 1.1, 1.3 and 1.5. Whereas, the PCR screening of hepcidin indicated all colonies contained the correct PCR product. All screened colonies of the correct size were selected to be grown in LB + ampicillin medium for 24 hours (Figure 34) in order to increase colony concentration.

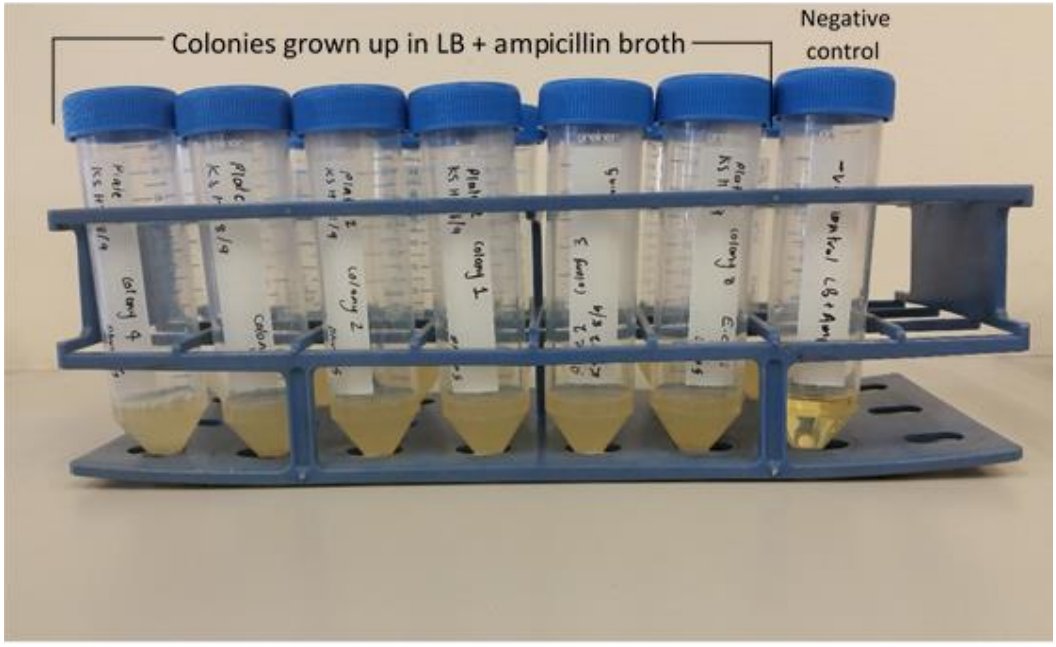

Figure 34: Example of transformed *E. cloni* cells grown up in LB + Ampicillin medium for 24 hours.

3.2.2.4 Plasmid Extraction and Restriction Digest

Following colony growth in LB + ampicillin medium, plasmids were extracted from the bacteria. A restriction digest was carried out on the extracted plasmids to confirm the ligated moronecidin (Figure 35) and hepcidin (Figure 36) PCR product was still present. Each restriction digest was run on an agarose gel.

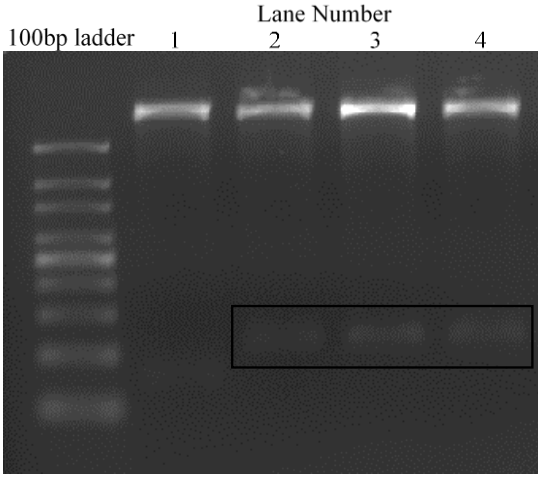

Figure 35: Moronecidin restriction digest. Outlined by the black box shows an insert that matches the size of the moronecidin PCR product. Refer to appendix VIII for ladder band sizing.

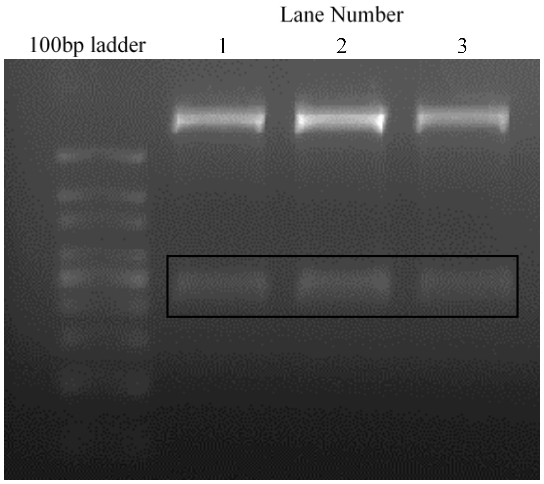

Figure 36: Hepcidin restriction digest. Outlined by the black box shows an insert that matches the size of the hepcidin PCR product. Refer to appendix VIII for ladder band sizing.

The restriction digest carried out on the moronecidin products (Figure 35) showed large bands at the top of the gel (>1500bp) which are the plasmids, whereas the smaller fainter bands are the ligated products. Lanes 2 to 4 showed moronecidin products of the correct size ~400bp. The restriction digest carried out on the hepcidin products (Figure 36) showed large plasmid bands, whereas, the

small faint bands are the ligated product. Lanes 1 to 3 show a genetic insert of the correct size ~500bp for hepcidin, outlined by the black box. Each plasmid that showed an insert of the expected size for moronecidin and hepcidin were sent for sequencing and the results analysed.

3.2.2.5 Sequencing Results

Three samples for each cloned product were sent for sequencing to the DNA Sequencing Facility located at the University of Waikato. Once sequencing was completed the inserted product had to be identified from the vector sequence. First the polyA tail was identified as this is characteristic of all mRNA sequences in eukaryotes. Following the polyA tail was the 3' RACE inner primer, with anything after the primer sequence being part of the vector and therefore could be removed. Next the F2 forward primer sequence could be identified so the vector sequence appearing before this could also be removed, leaving the actual amplified moronecidin (Figure 37) and hepcidin (Figure 38) 3' RACE sequenced products.

Monorecidin - Colony 1.1
ATCAGGAGGAGAGTTCATCGCCCTGTTTCTTGTGTTGTCACTGGTCGTCCTCATGGTTGAACCTGGGG
AAGGTTTTTTTCACCACATTCTCTCGGGAATTTTTCACGTTGGCAAAATGATCCACGGCGCGATCCAA
AGGAGGAGACATGGCATGACAGAGCTAGAGCAGGGGTAGTTTGACCGAGATCGGGCTGATTTTGCCTA
GACCTCCTATGACCTATAATGTTTCACCTGAAGGAGTCACTGTGAAGCAATTCACACACAATTGTCTA
ATGGTGTTATTCTTGGGTTCTTTGGAAAAATATGATTCATCAATTCAAATAAAATTGCATTTTAAAGA
GTTAAAAAAAAAAACCTATAGTGAGTCGTATTAATTCGGATCCGCG

Monorecidin - Colony 1.3
ATCAGGAAGGAGAGTTCATCGGCCTGTTTCTTGTGTTGTCACTGGTCGTCCTCATGGCTGAACCTGGG
GAAGGTTTTTTTCACCACATTCTCTCGGGAATTTTTCACGTTGGCAAAATGATCCACGGCGCGATCCA
AAGGAGGAGACATGGCATGACAGAGCTAGAGCAGGAGCAGTTTGACCGAGATCGGGCTGATTTTGCCT
AGACCTCCTATGACCTATAATGTTTCACCTGAAGGAGTCACTGTGAAGCAATTCACACACAATTGTCT
AATGGTGTTATTCTTGGGTTCTTTGGAAAAATATGATTCATCAATTCAAATAAAATTGCATTTTAAAG
AGTAAAAAAAAAAACCTATAGTGAGTCGTATTAATTCGGATCCGCG

Monorecidin - Colony 1.5
ATCAGGAGGAGAAGTTCATCGCCCTGTTTCTTGTGTTGTCACTGGTCGTCCTCATGGCTGAACCTGGG
GAAGGTTTTTTTCACCACATTCTCTCGGGAATTTTTCACGTTGGCAAAATGATCCACGGCGCGATCCA
AAGGAGGAGACATGGCATGACAGAGCTAGAGCAGGGGCAGTTTGACCGAGATCGGGCTGATTTTGCCT
AGACCTCCTATGACCTATAATGTTTCACCTGAAGGAGTCACTGTGAAGCAATTCACACACAATTGTCT
AATGGTGTTATTCTTGGGTTCTTTGGAAAAATATGATTCATCAATTCAAATAAAATTGCATTTTAAAG
AGTTAAAAAAAAAAACCTATAGTGAGTCGTATTAATTCGGATCCGCG

Figure 37: *S. lalandi* moronecidin gene sequences amplified using 3' RACE. The F2 primer is highlighted in red, the polyadenylation sequence is highlighted in green, the polyA tail is highlighted in yellow and the reverse 3' RACE inner primer highlighted in blue.

Hepcidin - Colony 1.2

AGCTGGAGAGGCAGGAAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGTATCGTGGA
TGATGCCGAATCCCGTCAGGCAGAAGCGCCAGAGTCACCTCTCCGTGTGCCGCTGGTGCTGCAACTGC
TGCACGGCCAACAAGGGCTGCGGTTTCTGCTGCAGGTTTTGAGGATTCCCGCCACAGCCTCGAAATAT
TAATTTATTTTACTTCTTTTTGCTTTACCCCAGGAAATGACCCTCTTTCTTGACTTCTTTCTCCAATG
CATTGCCTGTGATATTCACTTTTTTTTTGTATCATTTTTGATGGTATGATTCATAGAAGAGCACTGGAA
ACAAATGAGAAGCTATTCTGTGTATTTATTTTGTATGTTTGTAATACTTTCACCCTGGAACTCTTTTT
GTAAACTCAATGGCATGTCAAGACTGTTTCTTTTTATCTAAATTAAATCTGTAATAAATAATGAACAT
TTGAAGCAAAAAAAAAAAACCTATAGTGAGTCGTATTAATTCGGATCCGCG

Hepcidin - Colony 1.4

AGCTGGAGAGGCAGGAAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGTTCGTGGAT
GATGCCGAATCCCGTCAGGCAGAAGCGCCAGAGCCACCTCTCCATGTGCCGCTGGTGCTGCAACTGCT
GCACGGCCAACAAGGGCTGCGGTTTCTGCTGCAGGTTTTGAGGATTCCCGCCACAGCCTCGAAATATT
AATTTATTTTACTTCTTTTTGCTTTACCCCAGGAAATGACCCTCTTTCTTGACTTCTTTCTCCAATGC
ATTGCCTGTGATATTCACTTTTTTTTTGTATCATTTTTGATGGTATGATTCATAGAAGAGCACTGGAAA
CAAATGAGAAGCTATTCTGTGTATTTATTTTGTATGTTTGTAATACTTTCACCCTGGAACTCTTTTTG
TAAACTCAATGGCATGTCAAGACTGTTTCTTTTTATCTAAATTAAATCTGTAATAAATAATGAACATT
TGAAGCAAAAAAAAAAACCTATAGTGAGTCGTATTAATTCGGATCCGCG

Hepcidin - Colony 2.3

AGCTGGAGAGGCAGGAAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGCATCGTGGA
TGATGCCGAATCCCGTCAGGCAGAAGCGCCAGAGCCACCTCTCCATGTGCCGCTGGTGCTGCAACTGC
TGCACGGCCAACAAGGGCTGCGGTTTCTGCTGCAGGTTCTGAGGATTCCCGCCACAGCCTCGAAATAT
TAATTTATTTTACTTCTTTTTGCTTTACCCCAGGAAATGACCCTCTTTCTTGACTTCTTTCTCCAATG
CATTGCCTGTGATATTCACTTTTTTTTTGTATCCTTTTTGATGGTATGATTCATAGAAGAGCACTGGAA
ACAAATGAGAAGCTATTCTGTGTATTTATTTTGTATGTTTGTAATACTTTCACCCTGGAACTCTTTTT
GTAAACTCAATGGCATGTCAAGACTGTTTCTTTTTATCTAAATTAGATCTGTAATAAATAATGAACAT
TTGAAGCAAAAAAAAAAGAAACCTATAGTGAGTCGTATTAATTCGGATCCGCG

Figure 38: *S. lalandi* hepcidin gene sequences amplified using 3' RACE. The F2 primer is highlighted in red, the polyadenylation sequence is highlighted in green, the polyA tail is highlighted in yellow and the reverse 3' RACE inner primer highlighted in blue.

All moronecidin sequences (Figure 39) and hepcidin sequences (Figure 41) were aligned using ClustalX 2.1 to produce a consensus sequence for each gene. The consensus sequence for moronecidin (Figure 40) and hepcidin (Figure 42) does not contain the 3' RACE reverse primer sequence or the gene specific primer sequences for moronecidin and hepcidin.

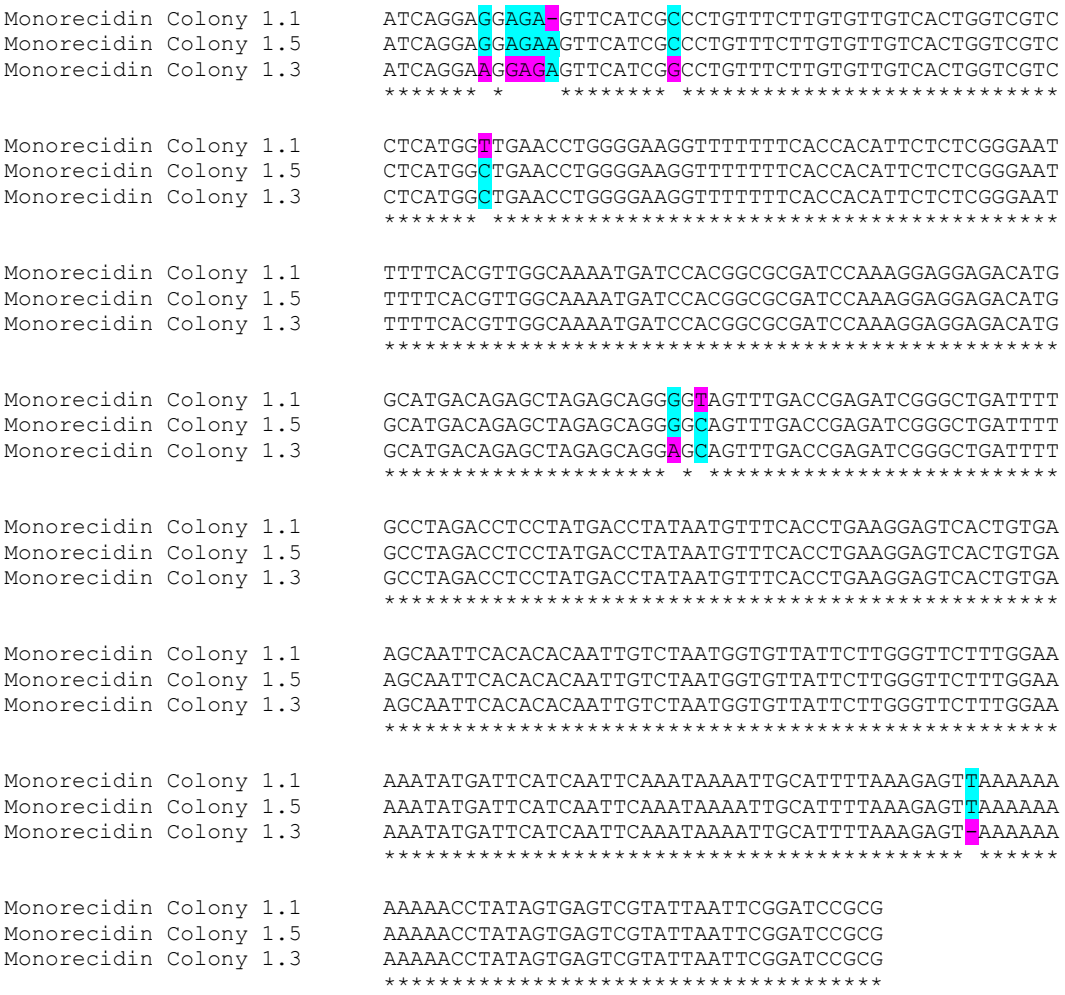

Figure 39: Alignment of the three products sequenced for the *S. lalandi* moronecidin 3' RACE product. Regions where sequences do not match are highlighted.

ATCGCCCTGTTTCTTGTGTTGTCACTGGTCGTCCTCATGGCTGAACCTGGGGAAGGTTTTTT
TCACCACATTCTCTCGGGAATTTTTCACGTTGGCAAAATGATCCACGGCGCGATCCAAAGGA
GGAGACATGGCATGACAGAGCTAGAGCAGGGGCAGTTTGACCGAGATCGGGCTGATTTTGCC
TAGACCTCCTATGACCTATAATGTTTCACCTGAAGGAGTCACTGTGAAGCAATTCACACACA
ATTGTCTAATGGTGTTATTCTTGGGTTCTTTGGAAAAATATGATTCATCAATTCAAATAAAA
TTGCATTTTAAAGAGTTAAAAAAAAAAA

Figure 40: Moronecidin 3' RACE consensus sequence generated from the product alignment.

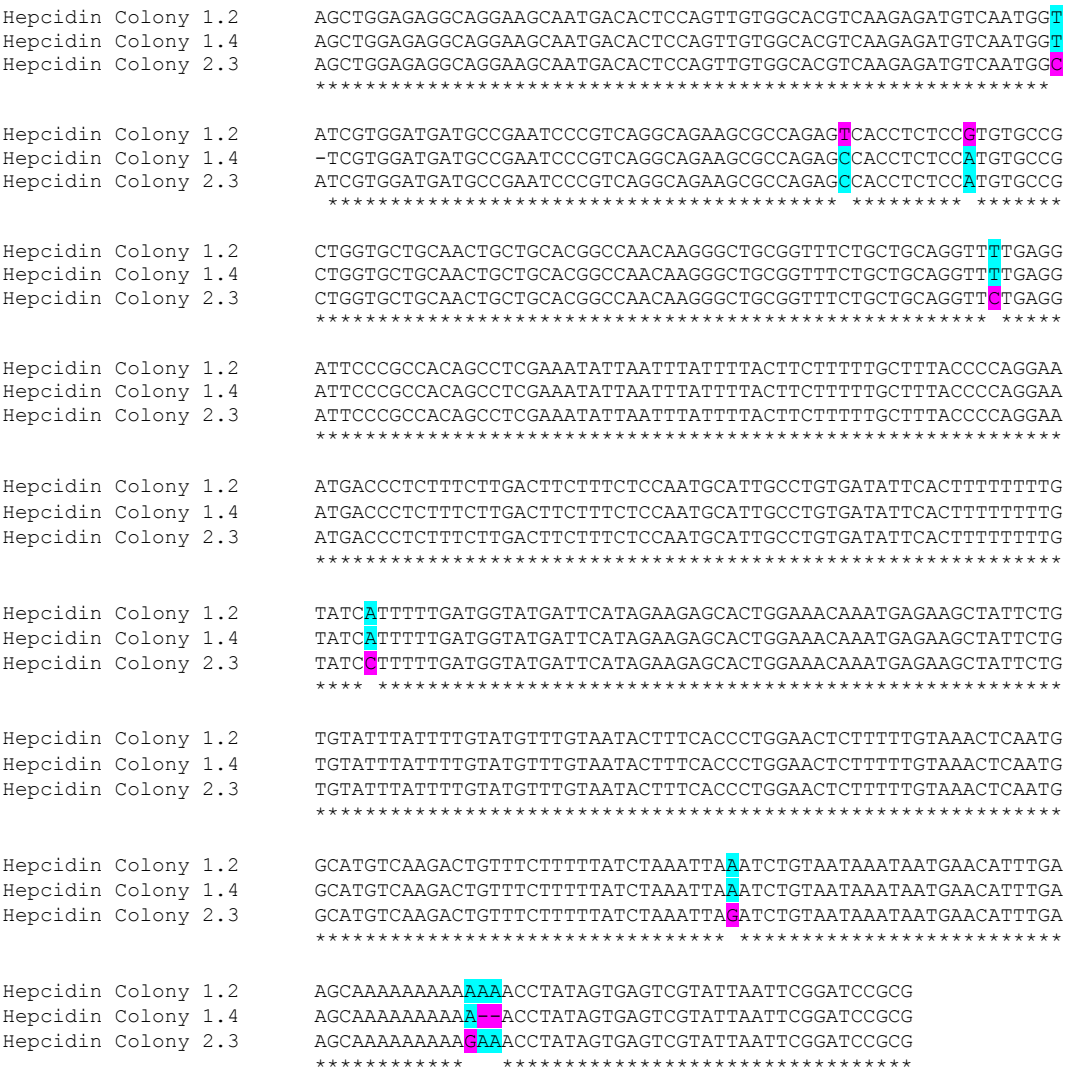

Figure 41: Alignment of the three products sequenced for the *S. lalandi* hepcidin 3' RACE product. Regions where sequences do not match are highlighted.

CAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGTATCGTGGATGATGCCGAATC
CCGTCAGGCAGAAGCGCCAGAGCCACCTCTCCATGTGCCGCTGGTGCTGCAACTGCTGCACG
GCCAACAAGGGCTGCGGTTTCTGCTGCAGGTTTTGAGGATTCCCGCCACAGCCTCGAAATAT
TAATTTATTTTACTTCTTTTTGCTTTACCCCAGGAAATGACCCTCTTTCTTGACTTCTTTCT
CCAATGCATTGCCTGTGATATTCACTTTTTTTTTGTATCATTTTTGATGGTATGATTCATAGA
AGAGCACTGGAAACAAATGAGAAGCTATTCTGTGTATTTATTTTGTATGTTTGTAATACTTT
CACCCTGGAACTCTTTTTGTAAACTCAATGGCATGTCAAGACTGTTTCTTTTTATCTAAATT
AAATCTGTAATAAATAATGAACATTTGAAGCAAAAAAAAAAAAA

Figure 42: Hepcidin 3' RACE consensus sequence generated from the product alignment.

3.2.3 Lamprey 3’ RACE

3.2.3.1 Primer Design

The partial defensin-like sequence identified from the *G. australis* alignment (Figure 24) where a consensus was found between all the sequences was used to design 3’ RACE primers. As there was limited sequence data, two sets of forward primers were designed, the first set F1A and F2A (Figure 43) and the second set F1B and F2B (Figure 44).

TCAGCTTGTCCCTGCGGCAAAAGAAGATGCTGCGTTCGAGGCTTGACGGTTTACT
GCTGCTT

Figure 43: Candidate defensin-like sequence with the first set of primers shown, F1A outlined in a box and F2A highlighted in blue.

TCAGCTTGTCCCTGCGGCAAAAGAAGATGCTGCGTTCGAGGCTTGACGGTTTACT
GCTGCTT

Figure 44: Candidate defensin-like sequence with the second set of primers shown, F1B outlined in a box and F2B highlighted in blue.

3.2.3.2 Nested PCR

The two primer sets designed for the defensin-like gene were used in nested 3’ RACE-PCR in an effort to isolate the 3’ ends of the gene. The first round of nested PCR was carried out using either the forward primer F1A or F1B with the reverse 3’ RACE outer primer. PCR products were run on an agarose gel where multiple bands can be seen using both the F1A and F1B defensin-like primers (Figure 45).

PCR products obtained from round one nested PCR using the F1A and F1B primers were used as the template for the second round. The second round primers used were F2A with the PCR mixture produced by F1A in round 1 and F2B with the PCR mixture produced by F1B in the first round. In each case the reverse 3’ RACE inner primer was used (Figure 46). Much like the first round of nested PCR multiple bands could be seen using F2A and F2B primers. Further

optimisation will be required to improve gene product specificity before being taken on to be ligated, cloned and sequenced.

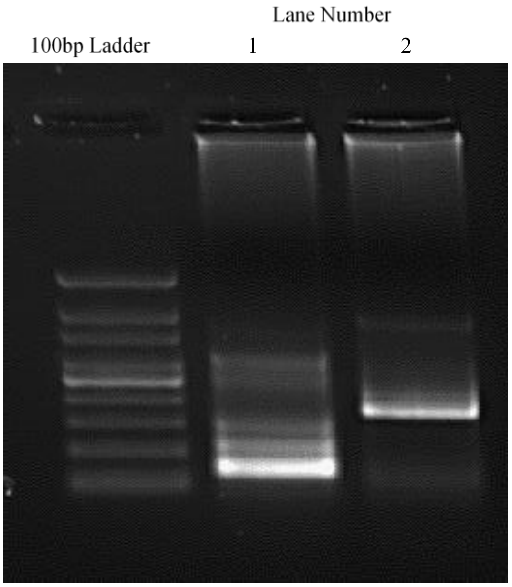

Figure 45: Gel image showing the first round of nested PCR. Products obtained when using the F1A (Lane 1) and F1B (Lane 2) primers for the defensin-like peptide with the reverse 3' RACE outer primer. The template DNA used was kidney tissue from *G. australis*. Refer to appendix VIII for ladder band sizing.

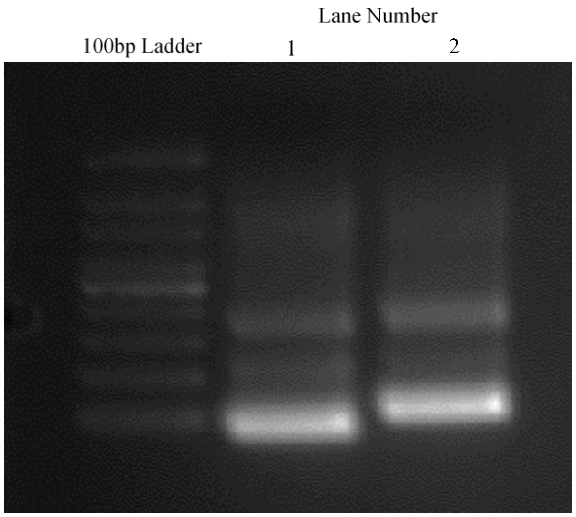

Figure 46: Gel image showing the second round of nested PCR. Products obtained were using the reverse 3' RACE inner primer alongside the F2A (Lane 1) or F2B (Lane 2) *G. australis* defensin-like primers. Refer to appendices VIII for ladder band sizing.

3.2.4 Yellowtail Kingfish 5’ RACE

3.2.4.1 Primer Design

The partial hepcidin and moronecidin sequences confirmed using 3’ RACE were used to design 5’ RACE primers. Two reverse primers were designed close to the end of the sequences. The first reverse primer (R1) and second reverse primer (R2) can be seen outlined for moronecidin (Figure 47) and hepcidin (Figure 48).

ATCGCCCTGTTTCTTGTGTTGTCACTGGTCGTCCTCATGGCTGAACCTGGGGAAGGTTTTTT
TCACCACATTCTCTCGGGAATTTTTCACGTTGGCAAAATGATCCACGGCGCGATCCAAAGGA
GGAGACATGGCATGACAGAGCTAGAGCAGGGGCAGTTTGACCGAGATCGGGCTGATTTTGCC
TAGACCTCCTATGACCTATAATGTTTCACCTGAAGGAGTCACTGTGAAGCAATTCACACACA
ATTGTCTAATGGTGTTATTCTTGGGTTCTTTGGAAAAATATGATTCATCAATTCAAATAAAA
TTGCATTTTAAAGAGTTAAAAAAAAAAA

Figure 47: Moronecidin 5' RACE reverse primers designed from 3' RACE consensus sequence. R2 primer is outlined by a box and R1 primer is highlighted in blue.

CAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGTATCGTGGATGATGCCGAATC
CCGTCAGGCAGAAGCGCCAGAGCCACCTCTCCATGTGCCGCTGGTGCTGCAACTGCTGCACG
GCCAACAAGGGCTGCGGTTTCTGCTGCAGGTTTTGAGGATTCCCGCCACAGCCTCGAAATAT
TAATTTATTTTACTTCTTTTTGCTTTACCCCAGGAAATGACCCTCTTTCTTGACTTCTTTCT
CCAATGCATTGCCTGTGATATTCACTTTTTTTTGTATCATTTTTGATGGTATGATTCATAGA
AGAGCACTGGAAACAAATGAGAAGCTATTCTGTGTATTTATTTTGTATGTTTGTAATACTTT
CACCCTGGAACTCTTTTTGTAAACTCAATGGCATGTCAAGACTGTTTCTTTTTATCTAAATT
AAATCTGTAATAAATAATGAACATTTGAAGCAAAAAAAAAAAA

Figure 48: Hepcidin 5' RACE reverse primers designed from 3' RACE consensus sequence. R2 primer is outlined by a box and R1 primer is highlighted in blue.

3.2.4.2 Nested PCR

The two primers designed for each *S. lalandi* AMP gene were used in nested 5’ RACE-PCR in an effort to isolate the 5’ ends of the moronecidin and hepcidin genes. There were two mixtures of tissues used for construction of the 5’ RACE cDNA, a mixture of kidney and spleen (KS) tissue and a mixture of liver and gill (LG) tissue. The first round of nested PCR was carried out using the reverse primers R1 or R2 for each gene along with the outer 5’ RACE reverse primer. PCR products were run on an agarose gel (Figure 49).

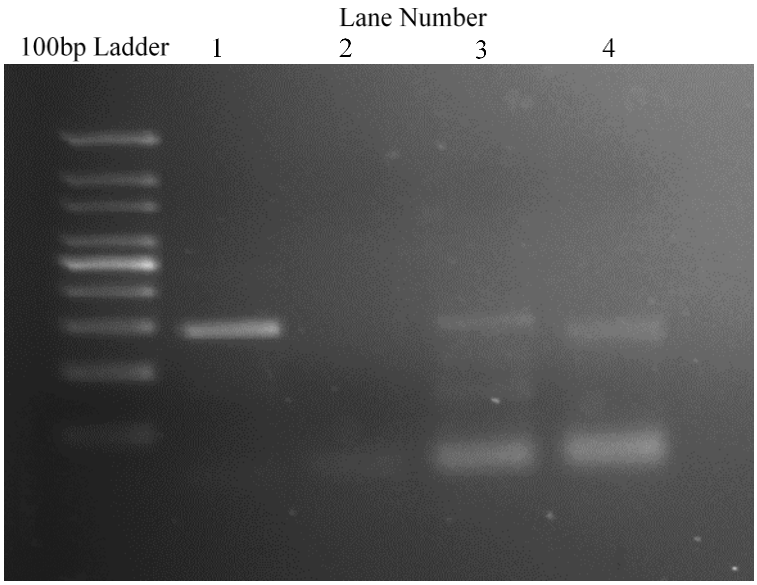

Figure 49: Gel image showing the first round of nested PCR. Products obtained when using the R1 primers for moronecidin and hepcidin alongside the forward 5' RACE outer primer. In lane 1 the R1 hepcidin primer was used on KS tissues. In lane 2 the R1 moronecidin primer was used on KS tissues. In lane 3 the R1 hepcidin primer was used on LG tissues. In lane 4 moronecidin R1 primer was used on LG tissues. Refer to appendix VIII for ladder band sizing.

Multiple bands can be seen using the R1 moronecidin and hepcidin primers in the LG tissues (lanes 3 and 4). However, there was only one band seen for the R1 hepcidin primer in KS tissue (lane 1), and no bands were amplified when using the R1 moronecidin primer in KS tissue (lane 2). All PCR products obtained from round one nested PCR using the R1 and 5' RACE outer primer for both moronecidin and hepcidin were used as the template for the second round. The moronecidin or the hepcidin reverse R2 primers alongside the forward 5' RACE inner primer were used and run on an agarose gel (Figure 50)

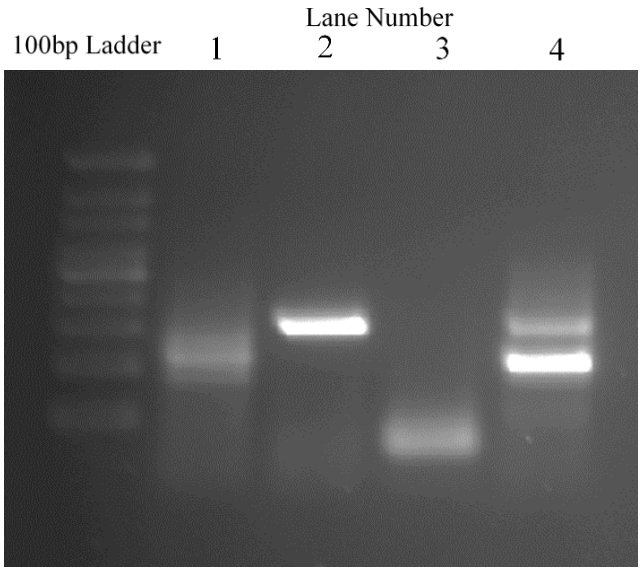

Figure 50: Gel image showing the second round of nested PCR. Products obtained when using the R2 primers for moronecidin and hepcidin alongside the forward 5' RACE inner primer. In lane 1 and 3 the R2 hepcidin primer was used on the corresponding lane template from round 1. In lane 2 and 4 the R2 moronecidin primer was used on the corresponding lane template from round 1. Refer to appendix VIII for ladder band sizing.

Moronecidin showed good amplification (lanes 2 and 4) with the R2 primer giving a product from the KS tissue of approximately 400bp, whereas, two moronecidin products were amplified from LG tissue of approximately 250bp and 400bp. For moronecidin the larger amplified product was used for sequencing. For hepcidin in lane 1 an amplified product could be seen but it is not very specific, therefore, round 2 nested PCR was repeated for hepcidin in both tissue types using different PCR conditions. The PCR products were run on an agarose gel (Figure 51). Lane 1 clearly shows the R2 hepcidin primer in KS tissue amplified a single prominent product at ~500bp. However, no bands appeared in lane 2 with the LG tissue. Following the successful amplification of products for moronecidin and hepcidin using nested PCR, fresh products were taken and ligated into the cloning vector for sequencing.

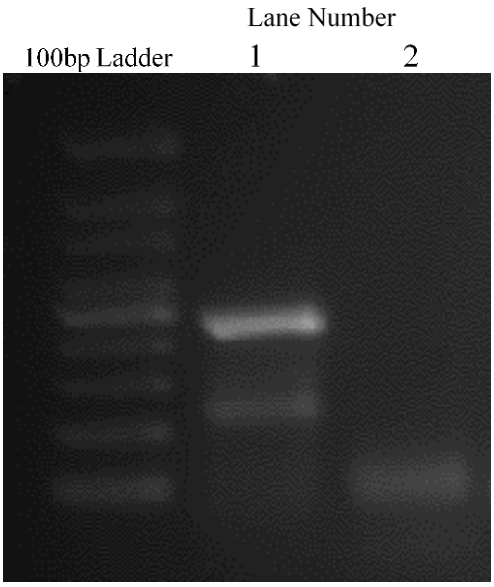

Figure 51: Gel image showing the repeated second round of nested PCR. Products obtained when using the R2 primers for hepcidin alongside the forward 5' RACE inner primer. In lane 1 the R2 hepcidin primer was used with template based on KS tissue from round 1. In lane 2 the R2 hepcidin primer was used with template based on LG tissue from round 1. Refer to appendix for ladder band sizing.

3.2.4.3 Ligation and Bacterial Transformation

The PCR products obtained after the second round of nested PCR were ligated into a cloning vector followed by transformation into chemically competent *E. cloni* cells. Cloned and transformed cells were grown up on LB + AMP + Xgal + IPTG plates with one plate for each PCR product. Colonies for both moronecidin (Plate 1, Figure 52) and hepcidin (Plate 2, Figure 52) grew on the selective plates.

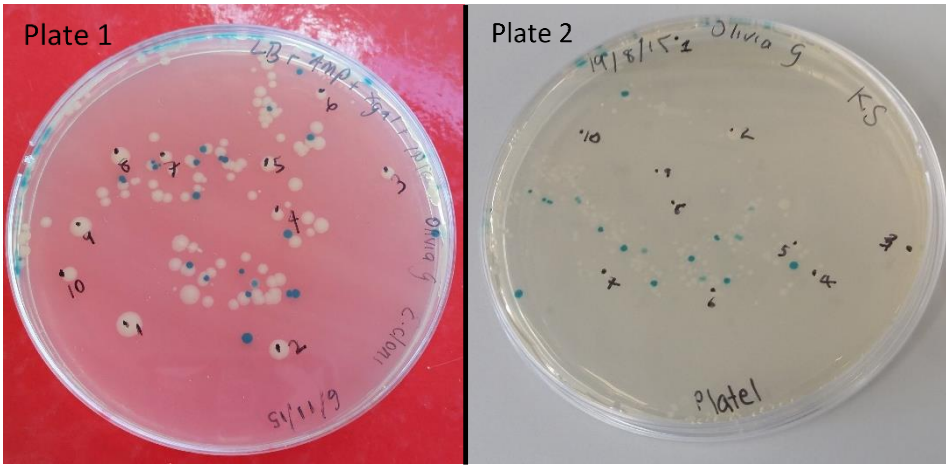

Figure 52: Blue and white colony screen for moronecidin (plate 1) and hepcidin (plate 2).

Ten white colonies indicating that cells contained the cloning vector with a successfully ligated PCR product, were selected from each plate and screened using PCR to determine if they contained ligated products of the correct size. M13 forward and M13 reverse primers were used to screen the colonies. When products were run on an agarose gel expected product sizes for moronecidin (Figure 53) were approximately ~550bp and hepcidin (Figure 54) were approximately ~650bp.

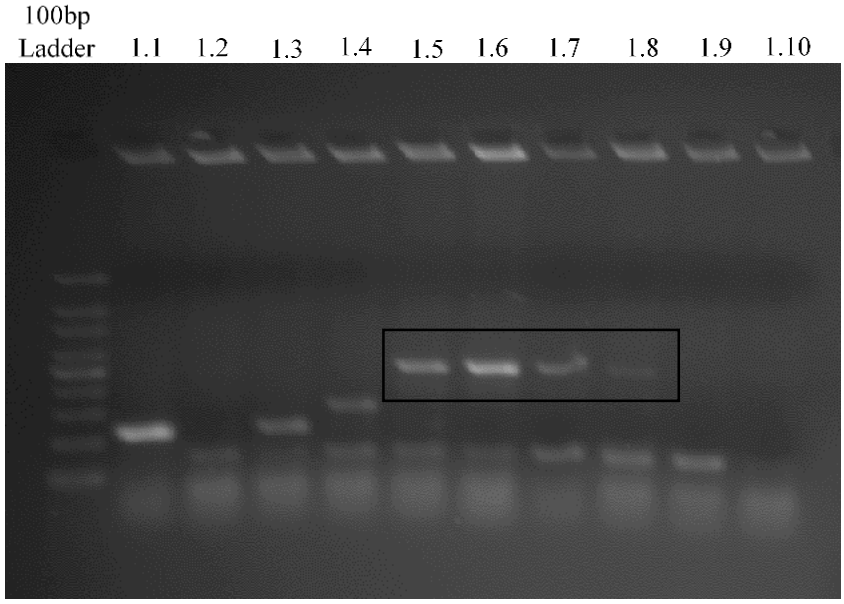

Figure 53: Gel image showing PCR products obtained from ten colonies on the moronecidin plate. The black box highlights ligated products of the correct size for moronecidin (~550bp). Refer to appendix VIII for ladder band sizing.

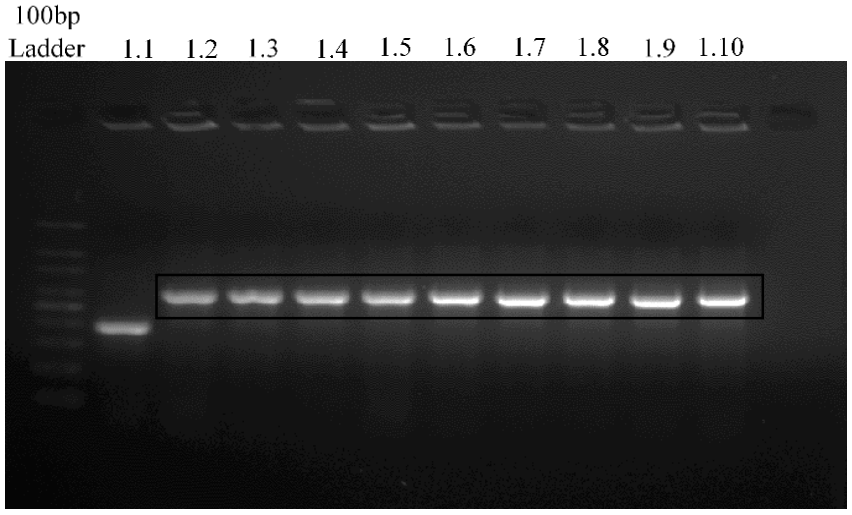

Figure 54: Gel image showing PCR products obtained from ten colonies on the hepcidin plate. The black box highlights ligated products of the correct size for hepcidin (~650bp). Refer to appendix VIII for ladder band sizing.

From the moronecidin colonies screened, four colonies showed products of the correct size, colonies 1.5, 1.6, 1.7 and 1.8. However, the screening of the hepcidin colonies indicated nine colonies contained the correct PCR product, with colony 1.1 which contained a smaller genetic insert. All successfully screened colonies were grown in LB + AMP medium for 24 hours (Figure 34) in order to increase colony concentration.

3.2.4.4 Plasmid Extraction and Restriction Digest

Following colony growth in LB + Ampicillin medium, plasmids were extracted from the bacteria and a restriction digest was carried out on the plasmids to confirm the ligated PCR product was still present. Restriction digests for moronecidin (Figure 55) and hepcidin (Figure 56) were run on a 1.5% agarose gel.

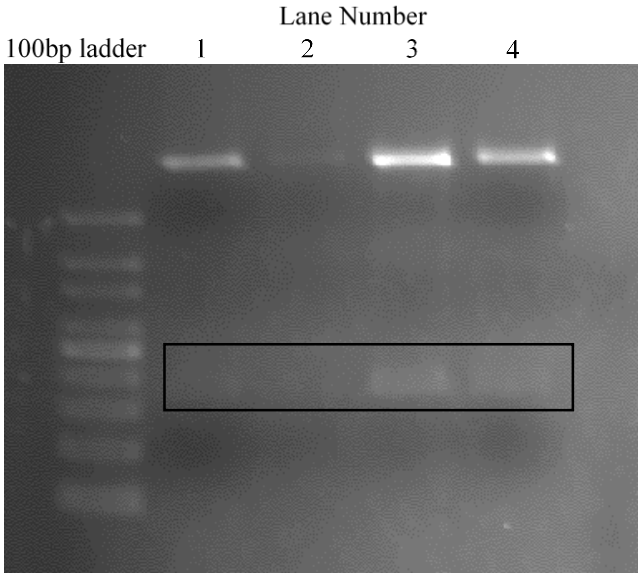

Figure 55: Moronecidin restriction digest. Outlined by the black box shows a band that matches the size of the moronecidin product. Refer to appendix VIII for ladder band sizing.

The restriction digest carried out on the moronecidin plasmids shows a large band at the top of the gel (>1500bp) which indicates the presence of the plasmid. Whereas, the smaller fainter bands show the product inserts. All lanes showed a product insert of the expected size of ~400bp indicating it is likely the moronecidin product initially amplified.

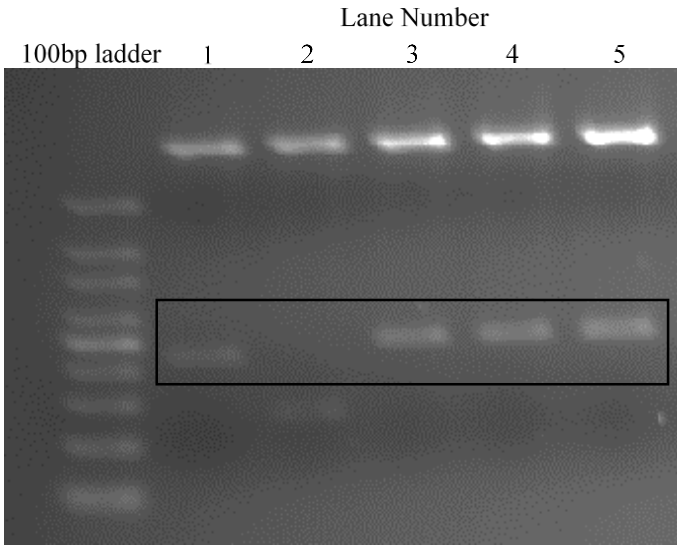

Figure 56: Hepcidin restriction digest. Outlined by the black box shows a band that matches the size of the hepcidin product. Refer to appendix VIII for ladder band sizing.

The restriction digest carried out on the hepcidin plasmid also showed a larger plasmid band with lanes 1, 3, 4 and 5 showing a product insert of the expected size ~500bp for hepcidin, outlined by the black box. Plasmids that contained the correct size product for moronecidin and hepcidin were sent for sequencing.

3.2.4.5 Sequencing Results

Samples for each gene were sent for sequencing to the DNA Sequencing Facility located at the University of Waikato. Once sequencing was completed the ligated product had to be identified from the plasmid. First the 5' RACE inner primer was identified, followed by the R2 gene specific primers used to amplify the moronecidin (Figure 57) and hepcidin (Figure 58) products.

All moronecidin sequences (Figure 59) and hepcidin sequences (Figure 61) were aligned using ClustalX 2.1 to produce a consensus sequence for each gene. The consensus sequence for moronecidin (Figure 60) and hepcidin (Figure 62) does not contain the 5' RACE forward primer sequence or the gene specific primer sequences for moronecidin and hepcidin.

Moronecidin – Colony 1.5
CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAAAGGCATCTATCCACTCAGAGATTTGTCTTG
TCTTTGACATCAGTTTTTTGACTCTTGACTCAGTCATCAGGAAGGATGAAGTTCATCGGCCTGTTTCT
TGTGTTGTCACTGGTCGTCCTCATGGCTGAACCTGGGGAAGGTTTTTTTCACCACATTCTCTCGGGAA
TTTTTCACGTTGGCAAAATGATCCACGGCGCGATCCAAAGGAGGAGACATGGCATGACAGAGCTAGAG
CAGGAGCAGTTTGACCGAGATCGGGCTGATTTTGC

Moronecidin – Colony 1.6
CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAAAGGCATCTATCCACTCAGAGATTTGTCTTG
TCTTTGACATCAGTTTTTTGACTCTTGACTCAGTCATCAGGAAGGATGAAGTTCATCGGCCTGTTTCT
TGTGTTGTCACTGGTCGTCCTCATGGCTGAACCTGGGGAAGGTTTTTTTCACCACATTCTCTCGGGAA
TTTTTCACGTTGGCAAAATGATCCACGGCGCGATCCAAAGGAGGAGACATGGCATGACAGAGCTAGAG
CAGGAGCAGTTTGACCGAGATCGGGCTGATTTTGC

Moronecidin – Colony 1.8
CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAATAGGCATCTATCCACTCAGAGATTTGTCTTG
TCTTTGACATCAGTTTTTTGACTCTTGACTCAGTCATCAGGAAGGATGAAGTTCATCGGCCTGTTTCT
TGTGTTGTCAGTGGTCGTCCTCATGGCTGAACCTGGGGAAGGTTTTTTTCACCACATTCTCTCGGGAA
TTTTTCACGTTGGCAAAATGATCCACGGCGCGATCCAAAGGAGGAGACATGGCATGACAGAGCTAGAG
CAGGAGCAGTTTGACCGAGATCGGGCTGATTTTGC

Figure 57: *S. lalandi* 5' RACE moronecidin product sequences. The forward 5' RACE inner primer is highlighted in blue and the F2 primer is highlighted in red.

Hepcidin – Colony 1.2
CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAAGACGGCGGAAAGAGTCGAGAAGGAGCCGAC
AGGGGTCACAGAAAAGATCTGACGAAATCCACTTGCCCAGACAATCACCATCCATCACTGGAGCTGAA
GAACAAAGACACAGTCGTGCTCTTCGGTGGCCTGACACCCATGAGAAAGAGGAACCCTCAACTCTAAT
CTTCTCAGGATTTAATCACTAAATCATTTAAAACATCCCATAAAATGAAGGCATTCAGCATTGCAGTT
GCAGTGACACTCGTGCTCGCCTTTATTTGCATTCTGGAGAGCTCTGCTGTCCCATTCCACGGGGTGCG
AGAGCTGGAAGAGGCAGGAAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGCATCGT
GGATGATGCCGAATCCCGTCAGGCAGAA

Hepcidin – Colony 1.3
CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAAGACGGCGGAAAGAGTCGAGAAGGAGCCGAC
AGGGGTCACAGAAAAGATCTGACGAAATCCACTTGCCCAGACAATCACCATCCATCACTGGAGCTGAA
GAACAAAGACACAGTCGTCCTCTTCGGTGGCCTGACACCCATGAGAAAGAGGAACCCTCAACTCTAAT
CTTCTCAGGATTTAATCACTAAATCATTTAAAACATCCCATAAAATGAAGGCATTCAGCATTGCAGTT
GCAGTGACACTCGTGCTCGCCTTTATTTGCATTCTGGAGAGCTCTGCTGTCCCATTCCACGGGGTGCG
AGAGCTGGAAGAGGCAGGAAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGCATCGT
GGATGATGCCGAATCCCGTCAGGCAGAA

Hepcidin – Colony 1.4
CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAAGACGGCGGAAAGAGTCGAGAAGGAGCCGAC
AGGGGTCACAGAAAAGATCTGACGAAATCCACTTGCCCAGACAATCACCATCCATCACTGGAGCTGAA
GAACAAAGACACAGCCGTGCTCTTCGGTGGCCTGACACCCATGAGAAAGAGGAACCCTCAACTCTAAT
CTTCTCAGGATTTAATCACTAAATCATTTAAAACATCCCATAAAATGAAGGCATTCAGCATTGCAGTT
TTTTTTGGCAGAGGGTGCTCGCCTTTATTTGCATTCTGGAGAGCTCTGCTGTCCCATTCCACGGGGTG
CGAGAGCTGGAAGAGCCAAAAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGCCAATGCATCGT
GGATGATGCCGAATCCCGTCAGGCAGAA

Hepcidin – Colony 1.5
CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAAGACGGCGGAAAGAGTCGAGAAGGAGCCGAC
AGGGGTCACAGAAAAGATCTGACGAAATCCACTTGCCCAGACAATCACCATCCATCACTGGAGCTGAA
GAACAAAGACACAGTCGTCCCCTTCGGTGGCCTGACACCCATGAGAAAGAGGAACCCTCAACTCTAAT
CTTCTCAGGATTTAATCACTAAATCATTTAAAACATCCCATAAAATGAAGGCATTCAGCATTGCAGTT
GCAGTGACACTCGTGCTCGCCTTTATTTGCATTCTGGAGAGCTCTGCTGTCCCATTCCACGGGGTGCG
AGAGCTGGAAGAGGCAGGAAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGCATCGT
GGATGATGCCGAATCCCGTCAGGCAGAA

Figure 58: *S. lalandi* 5' RACE hepcidin product sequences. The forward 5' RACE inner primer is highlighted in blue and the F2 primer is highlighted in red.

```
Moronecidin_1.5      CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAAAGGCATCTATCCACTCAGAGAT
Moronecidin_1.6      CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAAAGGCATCTATCCACTCAGAGAT
Moronecidin_1.8      CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAIAGGCATCTATCCACTCAGAGAT
                     ************************************ ********************

Moronecidin_1.5      TTGTCTTGTCTTTGACATCAGTTTTTTGACTCTTGACTCAGTCATCAGGAAGGATGAAGT
Moronecidin_1.6      TTGTCTTGTCTTTGACATCAGTTTTTTGACTCTTGACTCAGTCATCAGGAAGGATGAAGT
Moronecidin_1.8      TTGTCTTGTCTTTGACATCAGTTTTTTGACTCTTGACTCAGTCATCAGGAAGGATGAAGT
                     ************************************************************

Moronecidin_1.5      TCATCGGCCTGTTTCTTGTGTTGTCACTGGTCGTCCTCATGGCTGAACCTGGGGAAGGTT
Moronecidin_1.6      TCATCGGCCTGTTTCTTGTGTTGTCACTGGTCGTCCTCATGGCTGAACCTGGGGAAGGTT
Moronecidin_1.8      TCATCGGCCTGTTTCTTGTGTTGTCAGTGGTCGTCCTCATGGCTGAACCTGGGGAAGGTT
                     ************************* ********************************

Moronecidin_1.5      TTTTTCACCACATTCTCTCGGGAATTTTTCACGTTGGCAAAATGATCCACGGCGCGATCC
Moronecidin_1.6      TTTTTCACCACATTCTCTCGGGAATTTTTCACGTTGGCAAAATGATCCACGGCGCGATCC
Moronecidin_1.8      TTTTTCACCACATTCTCTCGGGAATTTTTCACGTTGGCAAAATGATCCACGGCGCGATCC
                     ************************************************************

Moronecidin_1.5      AAAGGAGGAGACATGGCATGACAGAGCTAGAGCAGGAGCAGTTTGACCGAGATCGGGCTG
Moronecidin_1.6      AAAGGAGGAGACATGGCATGACAGAGCTAGAGCAGGAGCAGTTTGACCGAGATCGGGCTG
Moronecidin_1.8      AAAGGAGGAGACATGGCATGACAGAGCTAGAGCAGGAGCAGTTTGACCGAGATCGGGCTG
                     ************************************************************

Moronecidin_1.5      ATTTTGC
Moronecidin_1.6      ATTTTGC
Moronecidin_1.8      ATTTTGC
                     *******
```

Figure 59: Alignment of the three sequenced samples for the *S. lalandi* moronecidin 5' RACE product. Regions where the sequence do not match are highlighted.

```
AAAAGGCATCTATCCACTCAGAGATTTGTCTTGTCTTTGACATCAGTTTTTTGACTCTTGAC
TCAGTCATCAGGAAGGATGAAGTTCATCGGCCTGTTTCTTGTGTTGTCACTGGTCGTCCTCA
TGGCTGAACCTGGGGAAGGTTTTTTTCACCACATTCTCTCGGGAATTTTTCACGTTGGCAAA
ATGATCCACGGCGCGATCCAAAGGAGGAGACATGGCATGACAGAGCTAGAGCAGGAGCAGTT
T
```

Figure 60: Moronecidin 5' RACE product consensus sequence generated from the product alignment.

```
Hepcidin_1.3      CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAAGACGGCGGAAAGAGTCGAGAAG
Hepcidin_1.5      CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAAGACGGCGGAAAGAGTCGAGAAG
Hepcidin_1.2      CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAAGACGGCGGAAAGAGTCGAGAAG
Hepcidin_1.4      CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAAGACGGCGGAAAGAGTCGAGAAG
                  ************************************************************

Hepcidin_1.3      GAGCCGACAGGGGTCACAGAAAAGATCTGACGAAATCCACTTGCCCAGACAATCACCATC
Hepcidin_1.5      GAGCCGACAGGGGTCACAGAAAAGATCTGACGAAATCCACTTGCCCAGACAATCACCATC
Hepcidin_1.2      GAGCCGACAGGGGTCACAGAAAAGATCTGACGAAATCCACTTGCCCAGACAATCACCATC
Hepcidin_1.4      GAGCCGACAGGGGTCACAGAAAAGATCTGACGAAATCCACTTGCCCAGACAATCACCATC
                  ************************************************************

Hepcidin_1.3      CATCACTGGAGCTGAAGAACAAAGACACAGTCGTCCTCTTCGGTGGCCTGACACCCATGA
Hepcidin_1.5      CATCACTGGAGCTGAAGAACAAAGACACAGTCGTCCCTTCGGTGGCCTGACACCCATGA
Hepcidin_1.2      CATCACTGGAGCTGAAGAACAAAGACACAGTCGTGCTCTTCGGTGGCCTGACACCCATGA
Hepcidin_1.4      CATCACTGGAGCTGAAGAACAAAGACACAGCCGTGCTCTTCGGTGGCCTGACACCCATGA
                  ***************************** *** * ***********************

Hepcidin_1.3      GAAAGAGGAACCCTCAACTCTAATCTTCTCAGGATTTAATCACTAAATCATTTAAAACAT
Hepcidin_1.5      GAAAGAGGAACCCTCAACTCTAATCTTCTCAGGATTTAATCACTAAATCATTTAAAACAT
Hepcidin_1.2      GAAAGAGGAACCCTCAACTCTAATCTTCTCAGGATTTAATCACTAAATCATTTAAAACAT
Hepcidin_1.4      GAAAGAGGAACCCTCAACTCTAATCTTCTCAGGATTTAATCACTAAATCATTTAAAACAT
                  ************************************************************

Hepcidin_1.3      CCCATAAAATGAAGGCATTCAGCATTGCAGTTGCAGT-GACACTCG-TGCTCGCCTTTAT
Hepcidin_1.5      CCCATAAAATGAAGGCATTCAGCATTGCAGTTGCAGT-GACACTCG-TGCTCGCCTTTAT
Hepcidin_1.2      CCCATAAAATGAAGGCATTCAGCATTGCAGTTGCAGT-GACACTCG-TGCTCGCCTTTAT
Hepcidin_1.4      CCCATAAAATGAAGGCATTCAGCATTGCAGTTTTTTTTGGCAGAGGGTGCTCGCCTTTAT
                  ********************************    * * **   * *************

Hepcidin_1.3      TTGCATTCTGGAGAGCTCTGCTGTCCCATTCCACGGGGTGCGAGAGCTGGAAGAGGCAGG
Hepcidin_1.5      TTGCATTCTGGAGAGCTCTGCTGTCCCATTCCACGGGGTGCGAGAGCTGGAAGAGGCAGG
Hepcidin_1.2      TTGCATTCTGGAGAGCTCTGCTGTCCCATTCCACGGGGTGCGAGAGCTGGAAGAGGCAGG
Hepcidin_1.4      TTGCATTCTGGAGAGCTCTGCTGTCCCATTCCACGGGGTGCGAGAGCTGGAAGAGCCAA-
                  ************************************************** **

Hepcidin_1.3      AAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGCATCGTGGATGATGCC
Hepcidin_1.5      AAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGCATCGTGGATGATGCC
Hepcidin_1.2      AAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGCATCGTGGATGATGCC
Hepcidin_1.4      AAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGCCAATG-CATCGTGGATGATGCC
                  ************************************ ***** ****************

Hepcidin_1.3      GAATCCCGTCAGGCAGAA
Hepcidin_1.5      GAATCCCGTCAGGCAGAA
Hepcidin_1.2      GAATCCCGTCAGGCAGAA
Hepcidin_1.4      GAATCCCGTCAGGCAGAA
                  ******************
```

Figure 61: Alignment of the three sequenced samples for the *S. lalandi* hepcidin 5' RACE product. Regions where the sequences do not match are highlighted.

```
AAAGACGGCGGAAAGAGTCGAGAAGGAGCCGACAGGGGTCACAGAAAAGATCTGACGAAATC
CACTTGCCCAGACAATCACCATCCATCACTGGAGCTGAAGAACAAAGACACAGTCGTCCTCT
TCGGTGGCCTGACACCCATGAGAAAGAGGAACCCTCAACTCTAATCTTCTCAGGATTTAATC
ACTAAATCATTTAAAACATCCCATAAAATGAAGGCATTCAGCATTGCAGTTGCAGTGACACT
CGTGCTCGCCTTTATTTGCATTCTGGAGAGCTCTGCTGTCCCATTCCACGGGGTGCGAGAGC
TGGAAGAGGCAGGAAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGCATCG
TGGATGATGCC
```

Figure 62: Hepcidin 5' RACE product consensus sequence generated from the product alignment.

3.2.5 Lamprey 5’ RACE

3.2.5.1 Primer Design

The partial defensin-like sequence identified from the *G. australis* alignment (Figure 24) where a consensus was found between all sequences and used to design 5’ RACE primers. As there was limited sequence data, two sets of reverse primers were designed, the first set R1A and R2A (Figure 63) and the second set R1B and R2B (Figure 64).

TCAGCTTGTCCCTGCGGCAAAAGAAGATGCTGCGTTCGAGGCTTGACGGTTTACT
GCTGCTT

Figure 63: Candidate defensin-like sequence with the first set of primers shown, R1A outlined in a box and R2A highlighted in blue.

TCAGCTTGTCCCTGCGGCAAAAGAAGATGCTGCGTTCGAGGCTTGACGGTTTACT
GCTGCTT

Figure 64: Candidate defensin-like sequence with the second set of primers shown, R1B outlined in a box and R2B highlighted in blue.

3.2.5.2 Nested PCR

The two reverse primer sets designed for the defensin-like gene were used in nested 5’ RACE-PCR in an effort to isolate the 5’ ends of the gene. The first round of nested PCR was carried out using either the reverse primer R1A or R1B with the forward 5’ RACE outer primer. PCR products were run on an agarose gel (Figure 65).

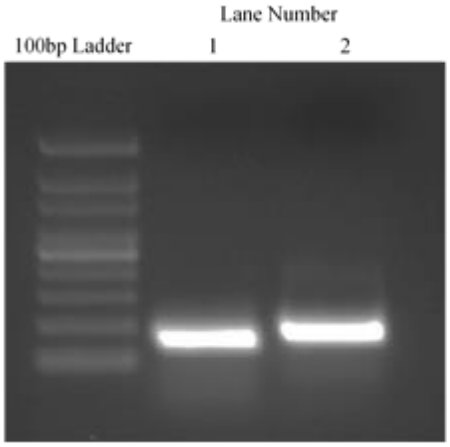

Figure 65: Gel image showing the first round of nested PCR. Products obtained when using the R1A (Lane 1) and R1B (Lane 2) primers for the defensin-like peptide with the forward 5' RACE outer primer. The template DNA used was from kidney of *G. australis*. Refer to appendix VIII for ladder band sizing.

The isolation of a single major product band was seen when using either the R1A or R1B defensin-like primers. PCR products obtained from round one nested PCR using the R1A and R1B primers were used as a template for the second round. The second round primers used were R2A with the PCR mixture produced by R1A in round 1 and R2B with the PCR mixture produced by R1B in the first round. In each case the forward 5' RACE inner primer was used. PCR products were run on an agarose gel (Figure 66).

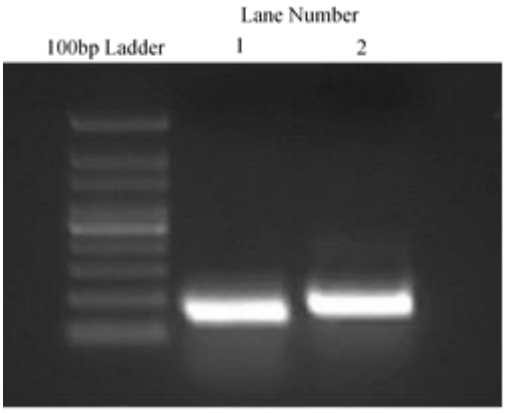

Figure 66: Gel image showing the second round of nested PCR. Products obtained when using the R2A (Lane 1) or R2B (Lane 2) primers for the defensin-like peptide with the forward 5' RACE inner primer. Refer to appendix VIII for ladder band sizing.

Much like the first round of nested PCR only single bands could be seen using R2A and R2B primers (150bp and 200bp) but these appear too small for the actual 5' end. Further optimisation is requires to improve gene product specificity and size before being taken on to be ligated, cloned and sequenced.

3.3 Sequencing Results

The 3’ RACE and 5’ RACE consensus sequences for moronecidin and hepcidin were aligned to determine the site of overlap. Once the overlap site was identified they were joined together resulting in the full sequenced moronecidin (Figure 67) and hepcidin (Figure 68) genes.

```
AAAAGGCATCTATCCACTCAGAGATTTGTCTTGTCTTTGACATCAGTTTTTTGACTCTTGAC
TCAGTCATCAGGAAGGATGAAGTTCATCGGCCTGTTTCTTGTGTTGTCACTGGTCGTCCTCA
TGGCTGAACCTGGGGAAGGTTTTTTTCACCACATTCTCTCGGGAATTTTTCACGTTGGCAAA
ATGATCCACGGCGCGATCCAAAGGAGGAGACATGGCATGACAGAGCTAGAGCAGGAGCAGTT
TGACCGAGATCGGGCTGATTTTGCCTAGACCTCCTATGACCTATAATGTTTCACCTGAAGGA
GTCACTGTGAAGCAATTCACACACAATTGTCTAATGGTGTTATTCTTGGGTTCTTTGGAAAA
ATATGATTCATCAATTCAAATAAAATTGCATTTTAAAGAGTTAAAAAAAAAAA
```

Figure 67: Full moronecidin cDNA sequence obtained from 3’ and 5’ RACE.

```
AAAGACGGCGGAAAGAGTCGAGAAGGAGCCGACAGGGGTCACAGAAAAGATCTGACGAAATC
CACTTGCCCAGACAATCACCATCCATCACTGGAGCTGAAGAACAAAGACACAGTCGTCCTCT
TCGGTGGCCTGACACCCATGAGAAAGAGGAACCCTCAACTCTAATCTTCTCAGGATTTAATC
ACTAAATCATTTAAAACATCCCATAAAATGAAGGCATTCAGCATTGCAGTTGCAGTGACACT
CGTGCTCGCCTTTATTTGCATTCTGGAGAGCTCTGCTGTCCCATTCCACGGGGTGCGAGAGC
TGGAAGAGGCAGGAAGCAATGACACTCCAGTTGTGGCACGTCAAGAGATGTCAATGGCATCG
TGGATGATGCCGAATCCCGTCAGGCAGAAGCGCCAGAGCCACCTCTCCATGTGCCGCTGGTG
CTGCAACTGCTGCACGGCCAACAAGGGCTGCGGTTTCTGCTGCAGGTTTTGAGGATTCCCGC
CACAGCCTCGAAATATTAATTTATTTTACTTCTTTTTGCTTTACCCCAGGAAATGACCCTCT
TTCTTGACTTCTTTCTCCAATGCATTGCCTGTGATATTCACTTTTTTTTGTATCATTTTTGA
TGGTATGATTCATAGAAGAGCACTGGAAACAAATGAGAAGCTATTCTGTGTATTTATTTTGT
ATGTTTGTAATACTTTCACCCTGGAACTCTTTTTGTAAACTCAATGGCATGTCAAGACTGTT
TCTTTTTATCTAAATTAAATCTGTAATAAATAATGAACATTTGAAGCAAAAAAAAAAAAA
```

Figure 68: Full hepcidin cDNA sequence obtained from 3’ and 5’ RACE.

To determine the correct genes had been identified, sequences were translated using ExPasy for moronecidin (Figure 69) and hepcidin (Figure 70). Both showed good homology when aligned with other known fish AMP protein sequences. For moronecidin the *S. lalandi* protein sequence was aligned against a striped sea bass (*Morone saxatilis*) moronecidin protein sequence (Figure 71). Whereas, for *S. lalandi* hepcidin the obtained protein sequence was aligned against a turbot (*Scophthalmus maximus*) hepcidin protein sequence (Figure 72). Based on these alignments the positioning of the signal peptide, mature peptide and pro-domain could be determined.

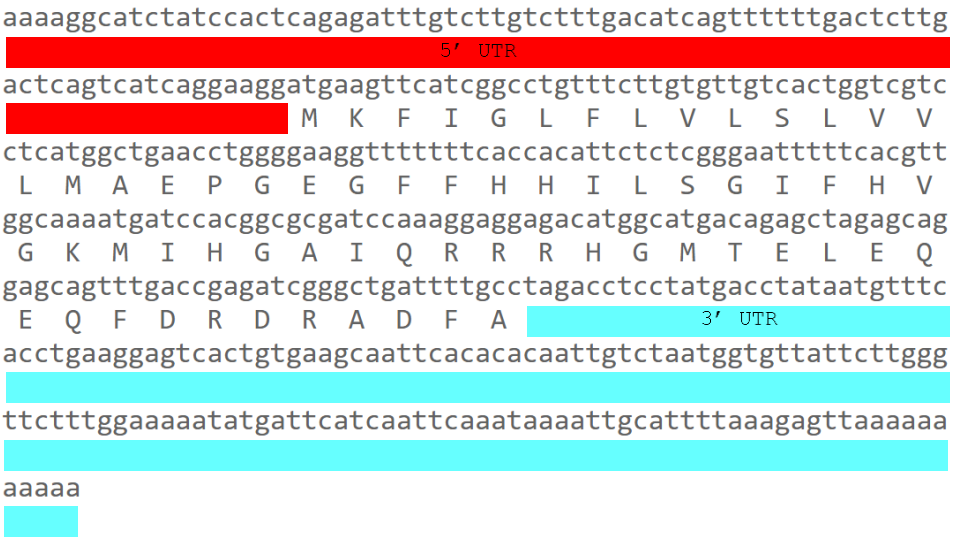

Figure 69: *S. lalandi* moronecidin protein sequence, translated from the full hepcidin gene using ExPASy Translate tool. The 5' UTR is highlighted in red and the 3' UTR is highlighted in blue

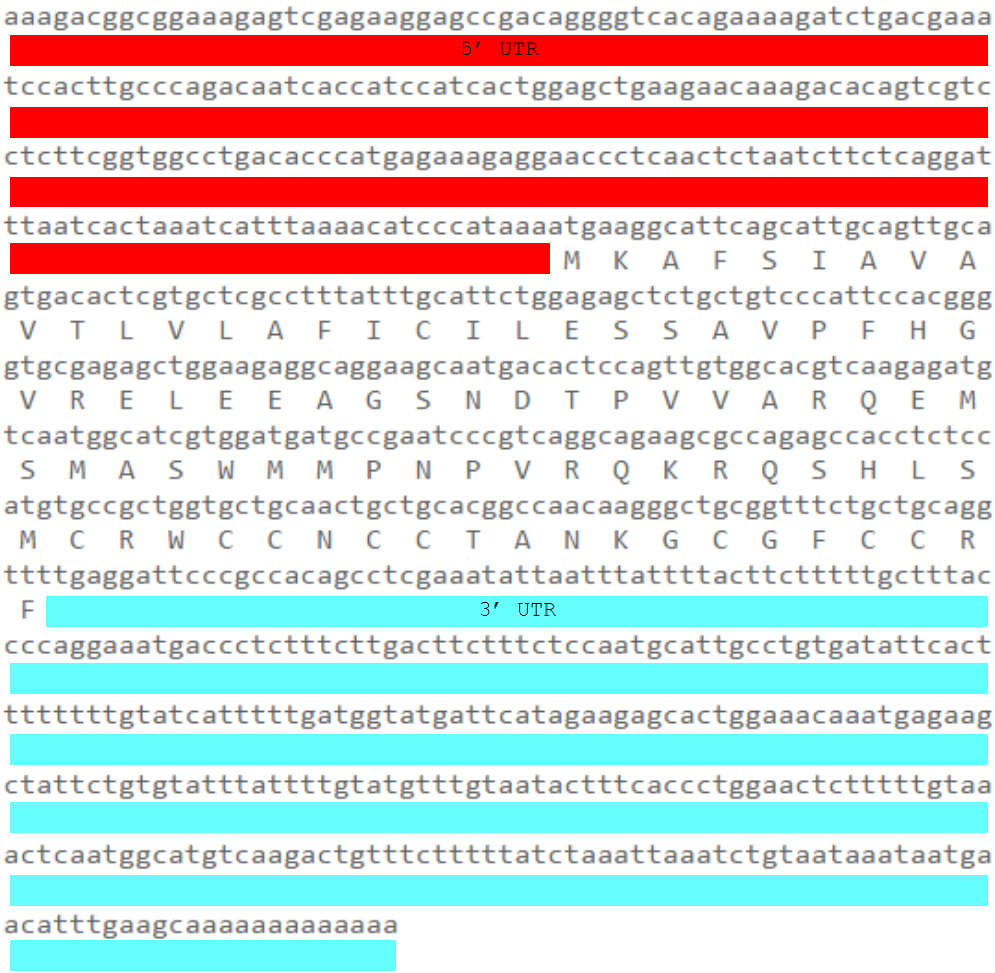

Figure 70: *S. lalandi* hepcidin protein sequence, translated from the full hepcidin gene using ExPASy Translate tool. The 5' UTR is highlighted in red and the 3' UTR is highlighted in blue

```
S. lalandi          MKFIGLFLVLSLVVLMAEPGEGFFHHILSGIFHVGKMIH----G--AIQRRRHGMTELEQ
M. saxatilis        MKCATLFLVLSMVVLMAEPGDAFFHHIFRGIVHVGKTIHRLVTGGKAEQDQQDQQYQQEQ
                    **   ******:********:.*****: **.**** **    *  * * ::.   : **

S. lalandi          -EQ-------FDRDRADFA
M. saxatilis        QEQQAQQYQRFNRERAAFD
                     **       *:*:** *
```

Figure 71: *S. lalandi* moronecidin protein sequence aligned against a striped sea bass moronecidin protein (Accession number, Q8UUG0.1). The signal peptide is outlined in blue, the mature peptide in red and the pro-domain in yellow.

```
S. lalandi          MKAFSIAVAVTLVLAFICILESSAVPFHGVRELEEAGSNDTPVVARQEMSMASWMMPNPV
S. maximus          MKAFSIAVAVTLVLAFVCILESSAVPFPGVQELEEAGSNDTPAAAHQETSMEPWTVPSHI
                    ****************:********** **:***********..*:** ** .* :*. :

S. lalandi          RQKRQSHLSMCRWCCNCCTANKGCGFCCRF
S. maximus          RQKRQSHISLCRWCCNCCKANKGCGFCCKF
                    *******:*:********.*********:*
```

Figure 72: *S. lalandi* hepcidin protein sequence aligned against a turbot moronecidin protein (Accession number, AAX92670.1). The signal peptide is outlined in blue, the pro-domain in yellow and the mature peptide in red.

Comparison of the identified *S. lalandi* moronecidin protein sequence against several other known fish protein sequences showed high sequence homology (Figure 73). The *S. lalandi* moronecidin protein sequence was aligned against Chinese perch (*Siniperca chuatsi*; Accession number, AAV65044.1), sand bass (*Morone chrysops*; Accession number, AAL57318.1), striped beakfish (*Oplegnathus fasciatus*; Accession number, BAM99885.1), and duskytail grouper (*Epinephelus bleekeri*; Accession number, ADY86110.1) moronecidin proteins. Comparison of known fish hepcidin protein sequences including that identified in *S. lalandi* shows some sequence homology when aligned against a known human (*Homo sapien*) hepcidin (Figure 74). *S. lalandi* hepcidin protein sequence was aligned against human (Accession number, NP066998), large yellow croaker (*Larimichthys crocea*; Accession number, ABC18307.1) and gilthead seabream (*Sparus aurata*; Accession number, ABV01929.1) moronecidin proteins. Particular conservation is noted in the functionally important cysteine residues of the mature peptide.

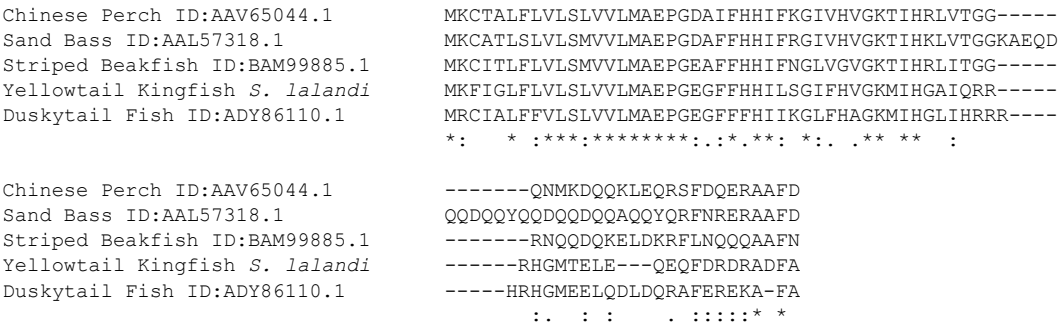

Figure 73: *S. lalandi* moronecidin protein sequence alignment with other known fish moronecidin proteins.

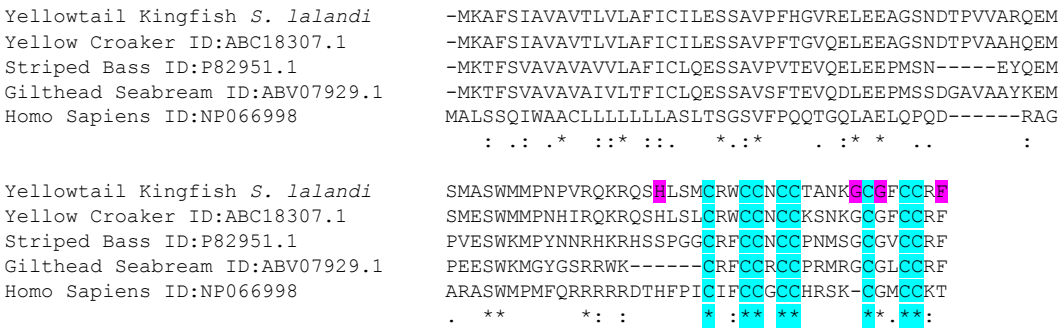

Figure 74: *S. lalandi* hepcidin protein sequence alignment with other known fish hepcidin proteins. Note the conservation of cysteines between all sequences in the mature peptide. Also highlighted in the *S. lalandi* hepcidin are the conserved residues in the mature peptide, histidine at position 3, glycine at position 20, glycine at position 22 and a phenylalanine at position 27.

3.4 Real-Time PCR

The efficiency of each pair of primers designed (Table 9) for RT-PCR first had to be determined. At 100% efficiency primers would be doubling the amount of template DNA after each PCR cycle; however, primer pairs often deviate from this ideal scenario. Therefore, calculating exact efficiencies was used to determine if the designed primers could successfully be used. Determination of primer efficiency for the housekeeping genes (GAPDH and β-actin) and the genes of interest (moronecidin and hepcidin) was achieved by producing a calibration curve where serially diluted template of known concentrations was used. An example of how this was done is shown using GAPDH. Using cDNA at different dilutions (Figure 75) Ct values were calculated by placing a threshold line on the Y-axis of the amplification plot. The dilution series for the housekeeping genes were: undiluted, 1:4, 1:16 and 1:64, whereas, the dilutions for the genes of interest

(moronecidin and hepcidin) were, undiluted, 1:2, 1:4, and 1:8. 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 76) and the products were run on an agarose gel (Figure 77) showing the presence of only one product.

Table 9: Primer sequences designed for RT-PCR

Primer	Sequence
GAPDH-F1	GCTCATCTCTTGGTATGACAATG
GAPDH-R1	TGCATGTACATCAGCAGG
B-actin-F1	TACAACGAGCTGAGAGTTGC
B-actin-R1	GTTGAAGGTCTCGAACATGAT
Moronecidin-F1	CGTCCTCATGGCTGAACCTG
Moronecidin-R1	ATCTCGGTCAAACTGCTCCTG
Hepcidin-F1	GTGCTCGCCTTTATTTGCATTCTG
Hepcidin-R1	CGGGATTCGGCATCATCCAC

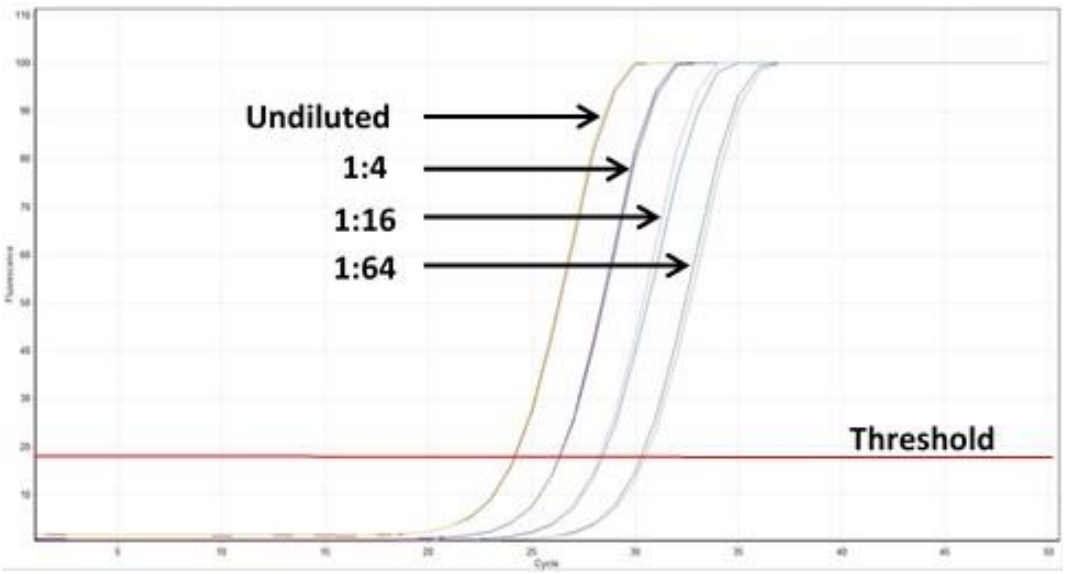

Figure 75: Calibration curve for RT-PCR obtained from GAPDH primers. Different dilutions of template cDNA were used and the threshold was set to obtain the corresponding Ct values.

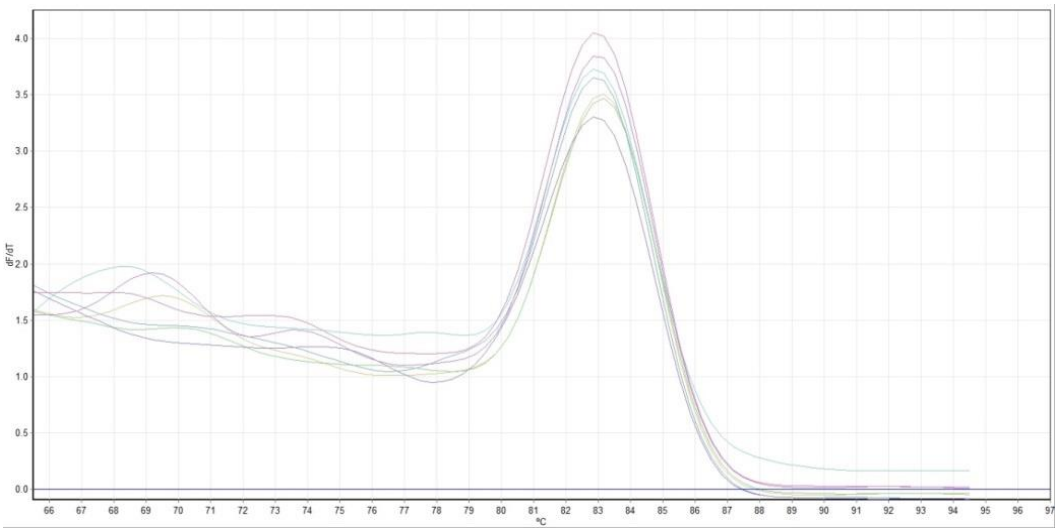

Figure 76: Melt curve obtained using GAPDH primers. The presence of one peak showed that only one product was formed during the run.

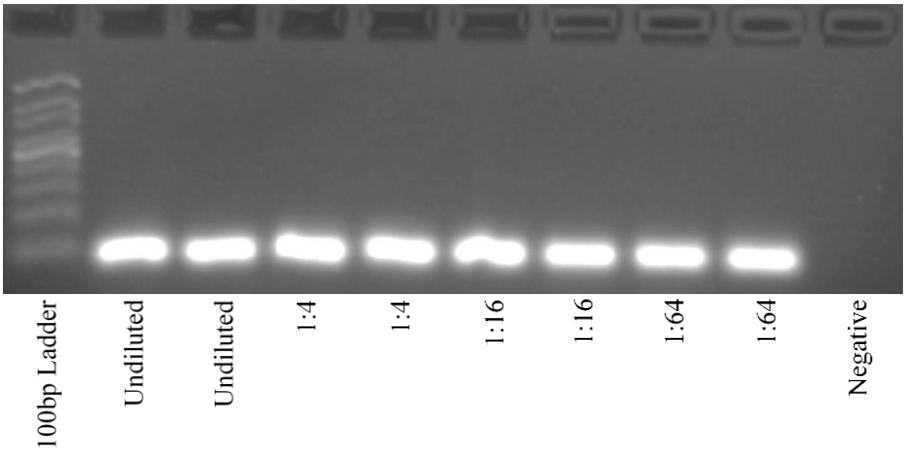

Figure 77: Gel image showing the GAPDH RT-PCR products. Dilutions of undiluted, 1:4, 1:16 and 1:64 were performed in duplicate and run on a 1.5% agarose gel. Refer to appendix VIII for ladder band sizing

The GAPDH Ct values were plotted on a graph against the log of the initial amount of template cDNA (Figure 78). A line of best fit was drawn through these points which produced a line equation and R^2 value. The line equation for GAPDH was y = -3.1749x + 28.046 and the R^2 value was 0.99. The closer the line gradient is to -3.33, the closer the amplification efficiency is to the 100%. The gradient for the GAPDH primers was -3.17, using the equation $E= 10^{(-1/s)}-1$, to determine primer efficiency, GAPDH primers were $10^{(-1/-3.1749)}-1 = 1.06$ or 106% efficient.

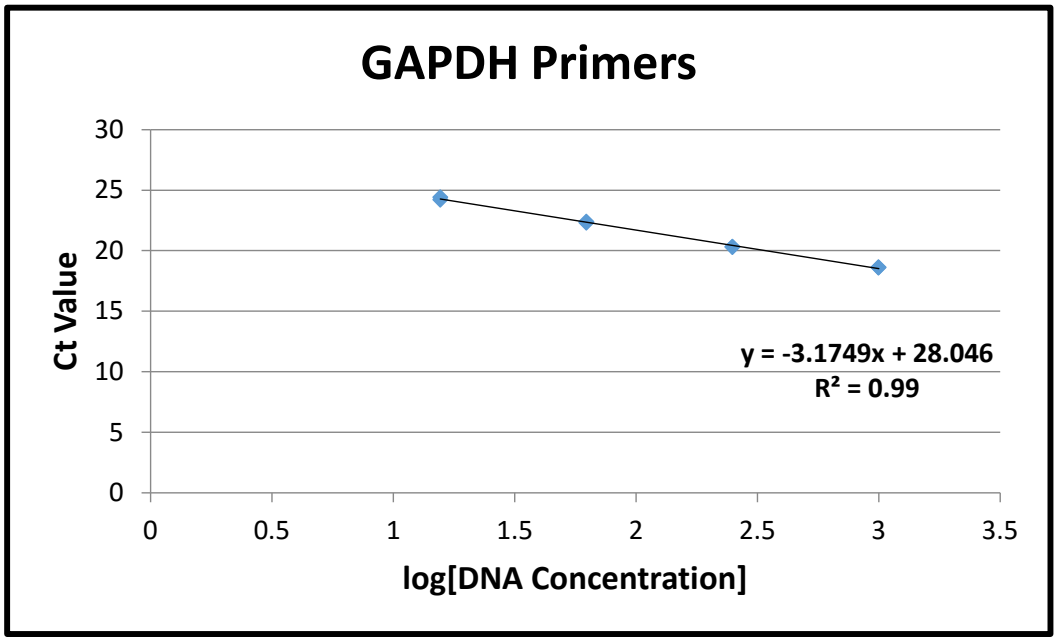

Figure 78: Standard curve for GAPDH with Ct values plotted against the log of the template concentration. The equation of the line and the R^2 value is shown.

Subsequently, 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 79), moronecidin (Figure 81) and hepcidin (Figure 83) were determined, followed by their corresponding melt curves, for β-actin (Figure 80), moronecidin (Figure 82) and hepcidin (Figure 84). The efficiency plots for β-actin, moronecidin and hepcidin primers gave efficiencies of 98%, 82% and 84% respectively and each melt curve showed that only one product was being isolated.

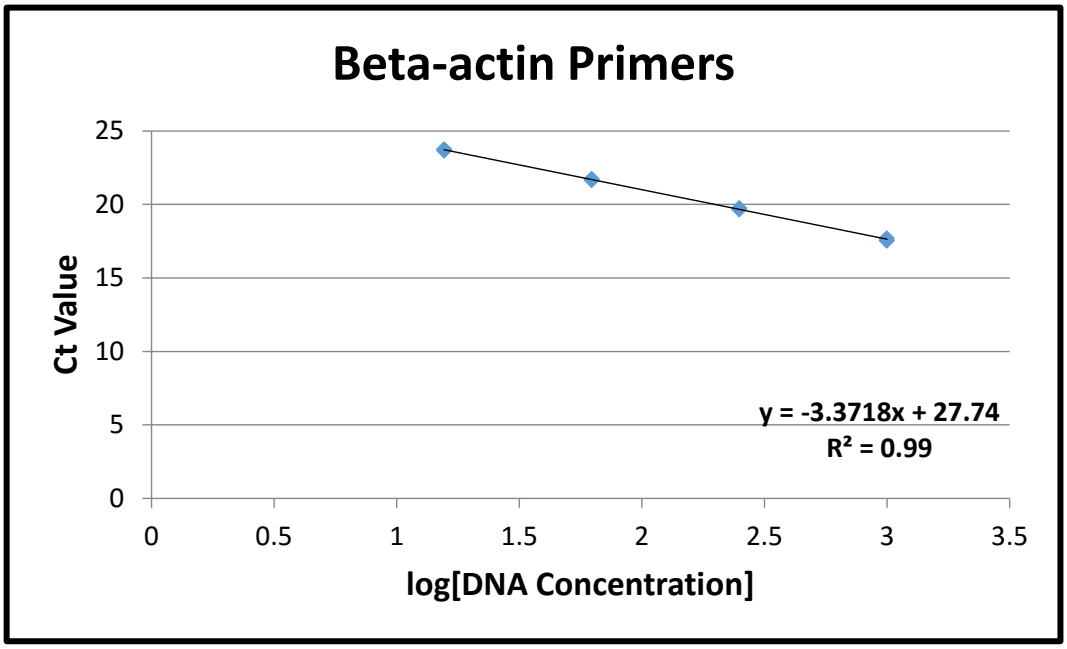

Figure 79: Standard curve for β-actin with Ct values plotted against the log of the template concentration. The equation of the line and the R^2 value are shown.

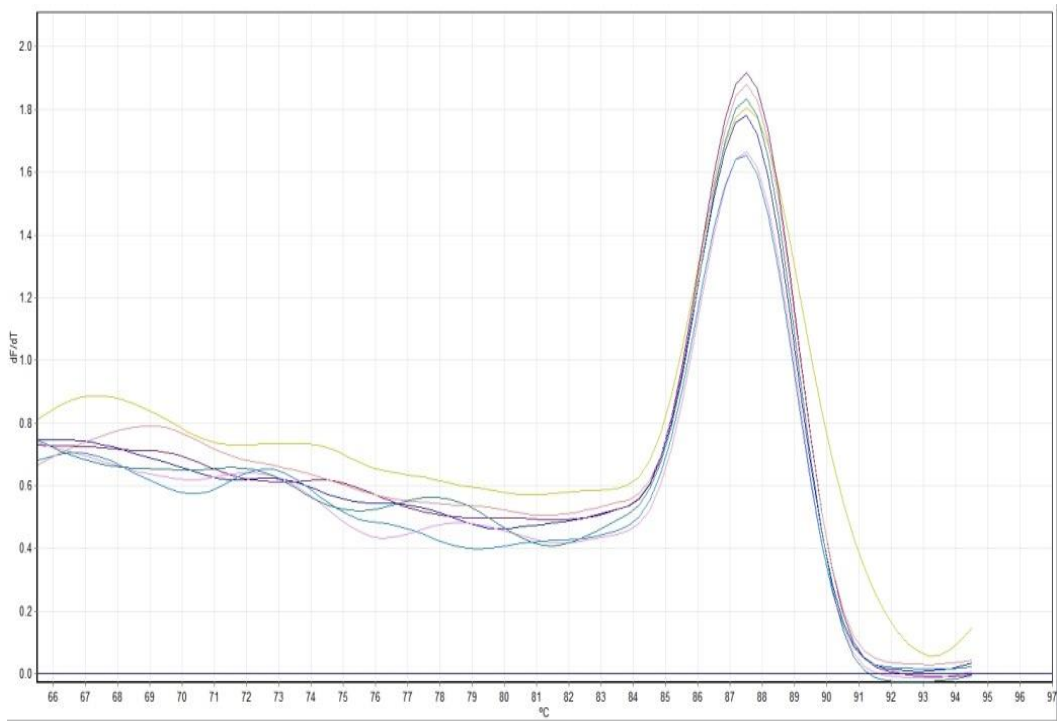

Figure 80: The melt curve obtained using β-actin primers. The presence of one peak showed that only one product was formed during the run.

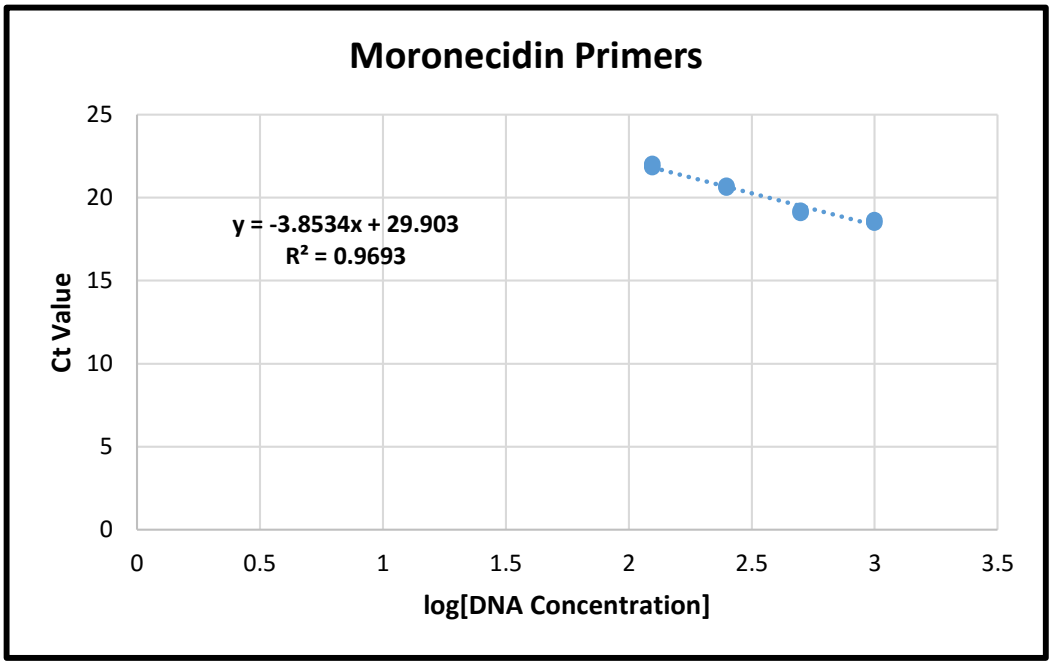

Figure 81: Standard curve for moronecidin with Ct values plotted against the log of the template concentration. The equation of the line and the R2 value are shown.

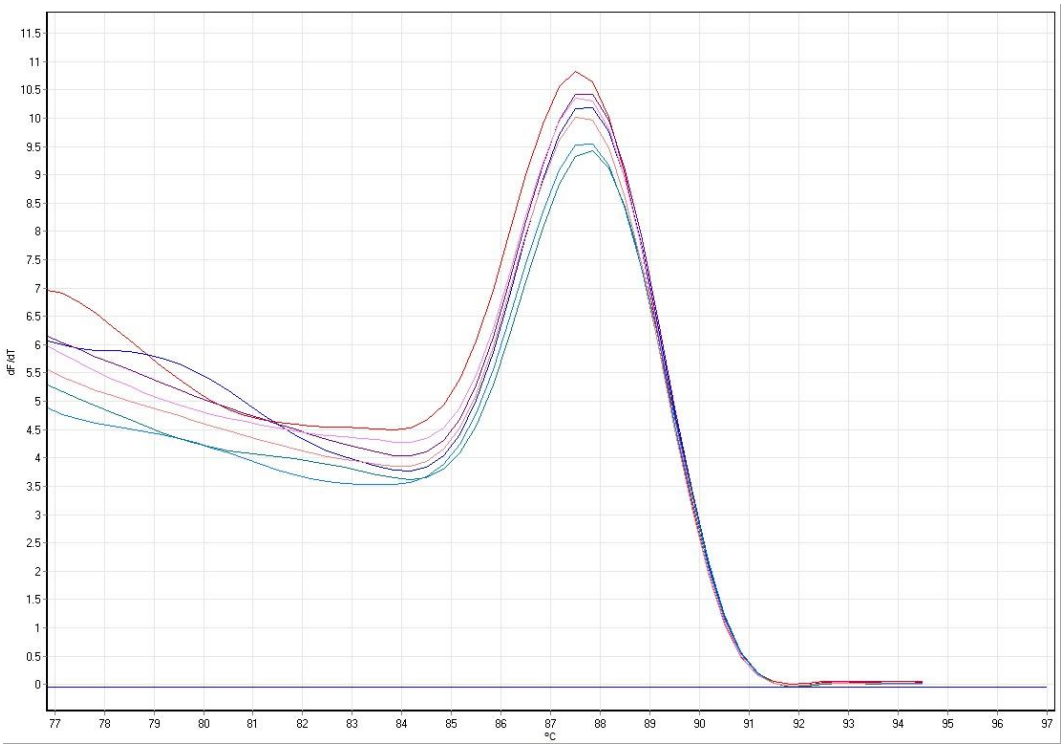

Figure 82: The melt curve obtained using moronecidin primers. The presence of one peak showed that only one product was formed during the run.

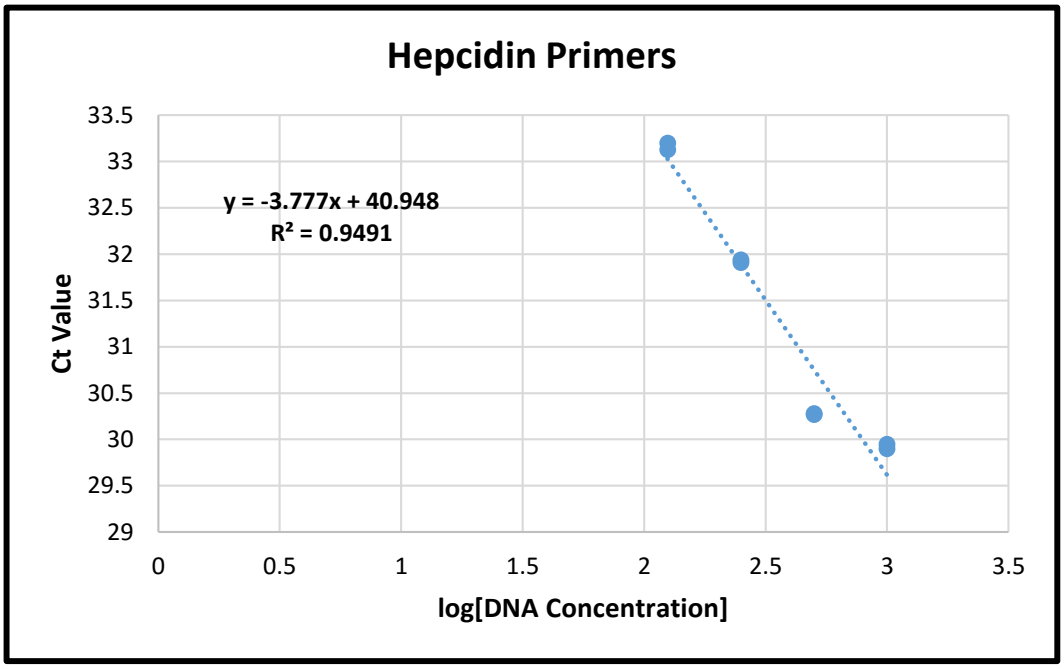

Figure 83: Standard curve for hepcidin with Ct values plotted against the log of the template concentration. The equation of the line and the R2 value are shown.

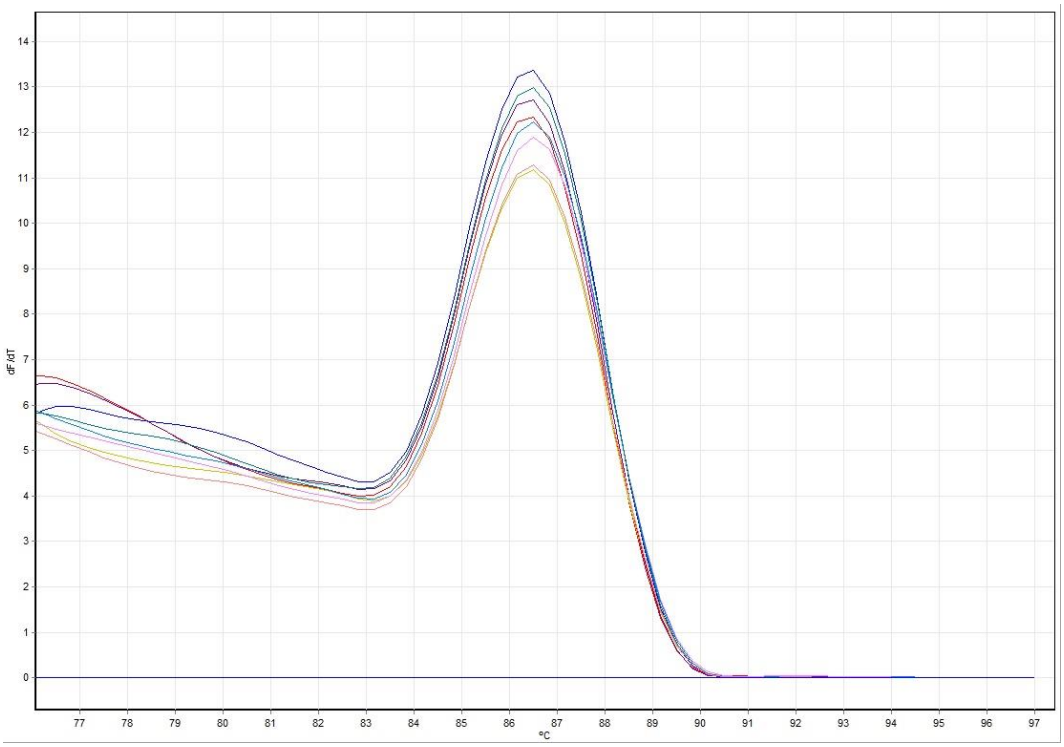

Figure 84:The melt curve obtained using hepcidin primers. The presence of one peak showed that only one product was formed during the run.

3.5 Expression of the Genes of Interest

The relative expression of the genes of interest was determined. The data was obtained by taking the geometric mean of three replicate RT-PCR runs (see Appendix IX and X). Moronecidin expression is highest in the spleen, followed closely by the gill and lowest expression was seen in the liver (Figure 85). Whereas hepcidin showed highest expression in the liver with significantly lower expression levels in the gill and spleen (Figure 86). In comparison to each other moronecidin is overall more highly expressed in all tissue types.

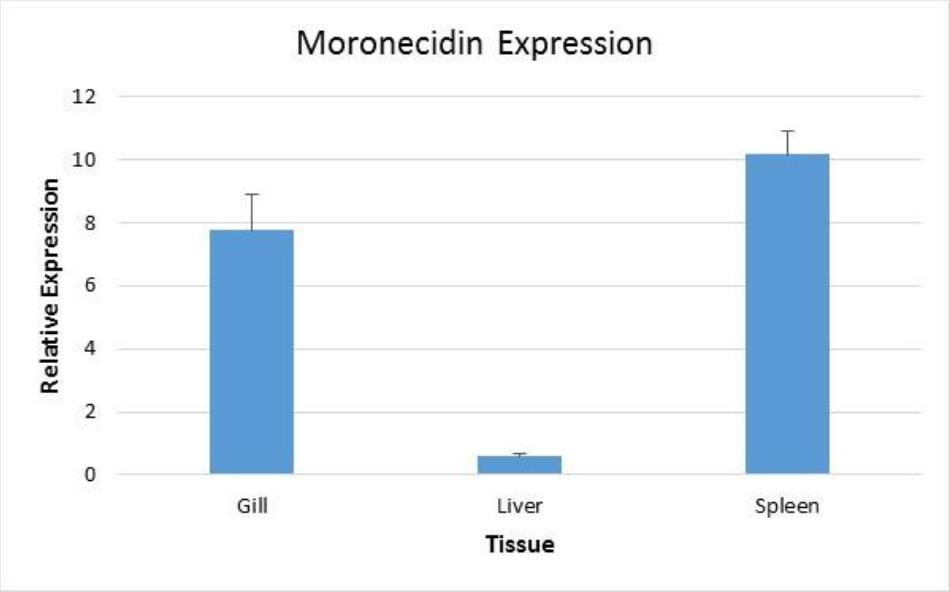

Figure 85: Relative expression of *S. lalandi* moronecidin (n=3).

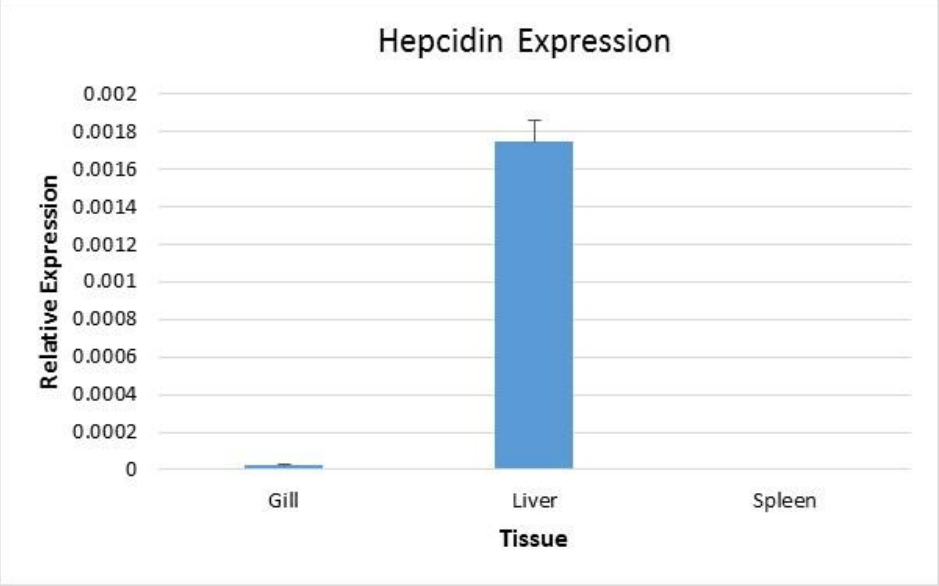

Figure 86: Relative expression of *S. lalandi* hepcidin (n=3).

Chapter Four: Discussion

Currently, very little is known about the immune systems of yellowtail kingfish (*Seriola lalandi*) and pouched lamprey (*Geotria australis*). The aim of this investigation was to identify and characterise AMPs, an important component of the innate immune system, from a jawed (*S. lalandi*) and jawless (*G. australis*) vertebrate. Initially, bioinformatic approaches were used to identify various AMPs in transcriptomic libraries created from *S. lalandi* spleen tissue, and *G. australis*, liver, gill, skin and kidney tissue. A range of molecular techniques were used to confirm the predicted sequences, followed by RT-PCR in order to examine expression levels in selected *S. lalandi* tissues.

4.1 Transcriptomic Database Searching

The *S. lalandi* and *G. australis* genomes have not yet been sequenced, but transcriptomic libraries have been produced in order to search for immune genes of interest. Several fish species do have sequenced genomes including the zebrafish (*D. rerio*) (Howe *et al.*, 2013), Atlantic salmon (*S. salar*) (Davidson *et al.*, 2010) and rainbow trout (*O. mykiss*) (Berthelot *et al.*, 2014). Due to the availability a lot has been learnt about the components of fish immune systems and gene identification in species without an available genome has become significantly easier as specific sequence characteristics of immune genes can be used to search available transcriptomic libraries. Whilst having a complete genome is useful for a species of interest, transcriptomic libraries provide a much quicker and cheaper alternative to genome sequencing (Tachibana, 2015). A transcriptomic library is essentially a database of expressed genes and they can be produced at specific points in an organisms development, at different stages of disease progression and within different tissues, depending on the genes of interest being studied or the research focus (Storvall, 2016). RNA-seq is a high throughput sequencing technology which is free of many limitations found with other transcriptomic approaches such as, microarray and tag-based sequencing methods (Wang, *et al.*, 2009). RNA-seq has been used for various practical applications, such as: **1) Novel transcript discovery** (Qian *et al.*, 2014). For

example, RNA-seq has led to the finding of more than 550 distinct non-coding RNAs in a zebrafish embryo (Ulitsky *et al.*, 2011). Non-coding RNAs play an essential role in many cellular processes where these newly discovered transcripts will promote genome annotation and lead the way for future functional studies. **2) Identification of gene associated single nucleotide polymorphisms (SNPs)** (Qian, *et al.*, 2014). For example, 342,104 intra-specific SNPs have been identified in channel catfish using RNA-seq explaining 90% of differences between individuals (Liu *et al.*, 2011). **3) Quantification of transcript levels** (Qian, *et al.*, 2014). This can be essential in understanding gene up/down regulation in response to changing environment and/or intrinsic programs.

Transcriptomics has also begun to play a major role in the understanding of the immune system of fish through the identification of immune related genes (Storvall, 2016). Comparison of fish transcriptomic expression before and after an immune challenge can give insight into, host immune responses and specific pathways involved in immunity, therefore, aiding in the development of immune based therapies for fish diseases. In turbot (*Scophathalamus maximus*) immune gene expression has been analysed in individuals infected by *Enteromyxum leei*, which causes emaciation of the fish as it inhabits the gut (Robledo *et al.*, 2014). Using transcriptomics, upregulation of chemokines and cytokines was noted in infected individuals. Another example in channel catfish (*I. punctatus*) looked at transcriptome expression after exposure to *Flavobacterium columnare.* This is a gram negative bacterium which plays a role in infectious outbreaks in fish, as a response to this microbe *I. punctatus* interferon gene expression was significantly upregulated (Sun *et al.*, 2012). Understanding how *F. columnare* affects the immune system of fish lays the foundation for therapeutic insights.

The transcriptomic library used for *S. lalandi* in this research was derived from spleen tissue of a healthy adult individual (greater than 75cm in length). The spleen was selected due to its immunological properties as it contains immune related cells such as erythrocytes, neutrophils, and granulocytes (Moore & Hawke, 2004). The spleen also contains genetically expressed immune transcripts, involved in antigen processing, haematopoiesis and antibody production (Moore & Hawke, 2004). The transcriptomic libraries generated for *G. australis* were

derived from liver, kidney, skin, and gill tissue of healthy and diseased adult individuals. The diseased individuals were affected by lamprey reddening syndrome (LRS), a fatal infection, currently with unknown origins. LRS is characterised by red patchy skin and gills resulting in extensive haemorrhaging and ultimately death (Mitland *et al.*, 2014). Therefore, due to the effects of LRS on the skin and the gills and a need to understand this disease in lamprey, these tissues were chosen to create a transcriptomic library. The liver and kidney tissues were also chosen due to the role they play in immunological responses. The liver facilitates local immune responses in fish while also producing immunoregulatory peptides and proteins (Uribe, *et al.*, 2011; Secombes & Wang, 2012). Whereas, the kidney is considered to be analogous to mammalian bone marrow producing vital immune cells such as granulocytes (Tort, *et al.*, 2003). For the sake of this research the transcriptomic libraries from healthy and diseased *G. australis* individuals were combined to search for AMP sequences.

Amino acid sequences of AMPs already identified and characterised in other fish species were used to perform a search within the *S. lalandi* and *G. australis* generated transcriptomic libraries. They were searched using tblastn, where a selected protein sequence is aligned to a nucleotide database which has been translated into all six reading frames (Gertz, *et al.*, 2006). Genetic fragments obtained from tblastn searches then underwent de novo assembly generating a larger consensus sequence. The consensus sequence was then translated using the ExPASy translate tool (Gasteiger *et al.*, 2003) and the resultant protein sequences used in an online BLAST search to confirm the preliminary genetic sequence.

Transcriptomic library searches for candidate AMP genes produced many results for *S. lalandi*, including piscidins, high density lipoproteins and NK-lysin (see Appendix V). A large range of AMPs were able to be identified using tblastn searching due to the number of AMP sequences available in public databases for closely related fish species. As AMPs have highly conserved domains, a significant number of hits were made in order to generate consensus sequences. For this investigation the genes moronecidin and hepcidin were chosen for further study as they were easily identified from the transcriptomic library and showed good homology when aligned with multiple genes from various species. In

contrast, when searching the *G. australis* transcriptomic library only a small selection of likely AMP sequences could be identified (see Appendix VI). The consensus sequences generated from the tblastn searches found, an NK-lysin, a defensin-like sequence, a haemoglobin derived AMP and two histone derived AMPs (see Appendix VII). The limited number of AMP consensus sequences identified within the *G. australis* transcriptomic library is likely due to the primitive nature of the organism. The closest living relative to lamprey are hagfish, which are significantly different therefore limiting the amount of AMP sequences that could be identified using a homology approach. To further identify AMPs in lamprey, the transcriptome would need to be searched manually using specific characteristics of AMPs, such as patterns of conserved cysteine residues and highly cationic sequences. Out of the limited number of AMPs identified the defensin-like *G. australis* sequence was chosen for further study in this investigation due to its primitive nature, as it is also found in plants, and could provide insights into evolution.

Sequences resulting from the transcriptome were used as the backbone to design primers for the amplification and confirmation of the targeted sequences (moronecidin, hepcidin, and defensin-like AMPs). Primers were designed for the three selected gene sequences in an attempt to amplify the full cDNA sequence using 3' and 5' RACE-PCR. Regions within the consensus sequences with the highest quality were used to design primers, with quality determined by overlapping regions with an already confirmed AMP nucleotide sequence. For hepcidin and moronecidin overlapping areas were defined by comparison to other known AMPs of the same kind. The identified hepcidin was aligned with the known large yellow croaker (*L. crocea*) hepcidin (Accession number, DQ307050.1) which showed high sequence homology particularly at the beginning of the sequence. Therefore, as close to this part of the sequence is where the primers were designed. The identified moronecidin was aligned with a known duskytail grouper (*E. bleekeri*) moronecidin (Accession number, HQ437912.1) which showed high sequence homology towards the middle of the sequence, therefore primers were designed here.

In comparison, the defensin-like protein identified in the *G. australis* transcriptome had no nucleotide sequences for comparison, as the *Petromyzon marinus* sequence (Accession number, Q10996.1) within the public database was submitted as a protein sequence (Conlon & Sower, 1996). Therefore, the multiple consensus sequences that had been identified in the transcriptome search were aligned and primers designed in the area of highest homology. Designing primers in these areas increased the chance for specific amplification and reduced the likelihood of mismatching. Standard primer design recommendations were used (Dieffenbach *et al.*, 1993) which included: 1) Having a GC content between 40-60%, which is important as too many runs of GC bases may stabilise non-specific primer annealing. 2) Having a GC clamp at the end of the primer, which assists in the primer binding to the template. Overall, meeting primer design features such as these, aids in increasing amplification efficiency. However, due to the low amount of sequence available and low sequence quality which occurred during transcriptome generation, primers could not always be designed to meet all specifications. Therefore, primers as close to these specifications as possible were designed, which is not uncommon practice.

4.2 Conformation of AMP cDNA Sequence

Following bioinformatics and primer design, RNA extraction was carried out on *S. lalandi* kidney, spleen, liver and gill tissue, and a mixture of *G. australis* kidney tissues from healthy and diseased individuals. Similar to the tissues chosen for transcriptomic library generation, the tissues selected for gene sequence conformation are known to be important in fish immunity and were the tissues that the potential AMP sequences had originally been identified in the RNA-seq transcriptomes.

Rapid amplification of cDNA ends (RACE) is a PCR technique which aids in cloning of full length cDNA when only a part of the cDNA sequence is available. Traditionally cDNA sequences were obtained from clones isolated from a plasmid or phage library (Okayama & Berg, 1982). Isolating a full length clone of cDNA provided certainty that the entire mRNA protein coding sequence had been identified, however, partial clones were frequently obtained that lacked the

5’ end of the mRNA transcript. To combat this issue multiple and repeated screens of the cDNA library were carried out in the hope of obtaining a full length clone. In the 1980s a better solution was developed where a general PCR was used to amplify the 5’ end of cDNA (Frohman *et al.*, 1988). This more modern RACE methods varied in design to traditional methods. Here total RNA is first reverse transcribed into cDNA (Yeku & Frohman, 2011) and at this point a 5’ RACE adapter is added to the cDNA creating a 5’ RACE library. Amplification between the adapter and gene specific primer can then be carried out using nested PCR. However, the major limitation here is there is no specific selection for mRNA, all RNA is an acceptable template. In this study, RNA Ligase Mediated (RLM) RACE was used, which is a major improvement on the more modern approach. The FirstChoice RLM-RACE kit was used which specifically selects for the 5’ end of mRNA through the targeting of the 5’ cap structure, favouring the selection of full length mRNA products. Here the 5’ cap structure is removed and a specific adapter sequence is attached creating the 5’ RACE library. From this the target sequence can be selected for by using complementary adapter primers and gene sequence specific primers during nested PCR. 3’ RACE has always been relatively straight forward as it uses the naturally occurring polyA tail that exists at the 3’ end of vertebrate mRNA transcripts where an adapter is added during the generation of cDNA. Therefore, 3’ RACE is typically easier generating results faster.

Nested PCR is used after generation of the 5’ RACE and 3’ RACE libraries in order to reduce non-specific amplification, as only one primer specific to the gene of interest is used in each PCR. In nested PCR two sets of primers are used in the two successive runs of PCR with the second set targeting a sequence within the first amplified product (Yeku & Frohman, 2011). This type of PCR is used as it is highly unlikely non-specific products would contain binding sites for both sets of primers, providing more confidence in the amplified PCR product.

4.2.1 *S. lalandi* Moronecidin

Four moronecidin primers were designed, two forward primers for 3’ RACE nested PCR and two reverse primers for 5’ RACE nested PCR. The first

round of 3’ RACE nested PCR resulted in multiple sized bands being amplified when using both the F1 and F2 forward primers. However, in the second round of nested PCR that used the template generated using the F1 primer and using the F2 primer, only a single band was isolated. After cloning and sequencing this resulted in a product of 338bp, which clearly contained the 3’ end of the *S. lalandi* moronecidin. A clear polyA and polyadenylation site could be seen which indicated this. Similar to 3’ RACE the first round of nested PCR for 5’ RACE resulted in multiple bands. After the second round of nested PCR two bands were present within the liver and gill tissue, with no defined bands in the kidney and spleen tissue. The two bands that were produced from liver and spleen tissue were of significantly different sizes, one at 250bp and the other 400bp. Both were sent for sequencing, the smaller band resulted in a product of 198bp, which upon analysis had no similarity to other known moronecidin sequences. However the larger moronecidin band resulted in a product of 249bp where similarity to other known moronecidin sequences could be seen and significant overlap was found with the 3’ RACE product. Upon generation of the consensus sequences and the combining of the 3’ RACE and 5’ RACE products, the final moronecidin cDNA sequence was 425bp. This was translated and the sequence analysed for features common to moronecidin proteins.

Moronecidin is an alpha helical, positively charged AMP which acts via membrane disruption forming transmembrane channels in bacteria (Sun, *et al.*, 2007). This creates severe permeability problems killing or inhibiting growth of the bacterial cells. Analysis of the moronecidin 425bp product generated from *S. lalandi* RACE-PCR resulted in a protein sequence of 69aa, which contained a signal peptide of 22aa, a mature peptide of 18aa and a pro-domain of 25aa. When compared to other fish, similar protein configurations can be found, such as in the hybrid striped bass (*M. saxatalis*) which has a predicted pre-pro-peptide of 79aa consisting of a 22aa signal peptide, a 22aa mature peptide, and a 35aa pro domain (Lauth, *et al.*, 2002). Since the discovery of moronecidin in hybrid striped bass it has consequently been discovered in Atlantic cod (*Gadus morhua*), European bass (*Dicentarachus labrax*), rainbow trout (*O. mykiss*), winter flounder (Sun, *et al.*, 2007) and now yellowtail kingfish (*S. lalandi*). However, there is a lot of difficulty in defining which species moronecidin has been identified in due to the

confusion associated with piscidin family members and the nomenclature that has been used in different fish species.

Comparison of the protein sequence with other moronecidins showed high sequence homology in the signal peptide region, especially between *S. lalandi* and *M. saxatalis* with 73% similarity. There is slightly less conservation in the mature peptide with 64% similarity, with a poorly conserved C terminus, which was seen in the alignment between *S. lalandi* and *M. saxatilis*. However, the N-terminus of moronecidin mature peptides have been shown to be highly conserved and are specifically rich in histidine and phenylalanine (Bae *et al.*, 2014) and these mature peptide characteristics can also be seen in the moronecidin found in *S. lalandi*. Compared to the signal and mature peptide sequences, the pro-domain shows very little sequence conservation with only 31% similarity between *S. lalandi* and *M. saxatilis,* which is typical of the pro-domain in many AMPs that have been characterised in fish. However, the pro-domain of piscidin AMPs are shown to contain a repeating XQQ motifs (where X represents Asp, Tyr, Glu or Ala) (Sun, *et al.*, 2012). The *S. lalandi* moronecidin does not contain this XQQ motif in the pro-domain, which suggests it is much more similar to moronecidin and pleurocidin. Interestingly, the pro-domain of the *M. saxatilis* moronecidin does contain six repeated XQQ motifs, which does raise the question about the actual identity of this protein. The piscidin family contains several different members which include moronecidin, pleurocidin, piscidin, epinecidin and gaduscidin. Currently, with each of these family members there is no overall definition, which, results in significant overlap in sequence and name differentiation leading to the confusion that exists. An example of this overlap is where moronecidin and piscidin proteins were isolated from sea bass but have since been discovered to be the same gene (Silphaduang & Noga, 2001). In future, a general consensus needs to be developed to distinguish the differences between piscidin family members which could ultimately be achieved through functional studies. However, due to the overlap of all piscidin family members it may make more sense that piscidin is used to name all of these AMPs in the future.

4.2.2 *S. lalandi* Hepcidin

Four hepcidin primers were designed, two forward primers for 3' RACE nested PCR and two reverse primers for 5' RACE nested PCR. The first round of 3' RACE nested PCR resulted in multiple sized band being amplified when using the F1 primer and using just the F2 primer in round one of nested PCR resulted in no bands being present. However, using the F2 primer in the second round of nested PCR resulted in a single strong band, which after cloning and sequencing resulted in a 3' RACE cDNA product of 428bp in length which clearly contained the 3' end of the *S. lalandi* hepcidin. Clear polyA tail and polyadenylation (Proudfoot, 2011) sites could be seen which indicated this. In the first round of 5' RACE nested PCR only a single band was amplified using R1. The same band was isolated again in the second round of nested PCR using R2. Cloning and sequencing of the 5' RACE nested PCR amplicons resulted in a product of 383bp in length where similarity to other known moronecidin sequences could be seen and significant overlap with the 3' RACE product was found. Upon generation of the consensus sequences and the combining of the 3' RACE and 5' RACE products, the final hepcidin cDNA sequence was 804bp.

Analysis of the hepcidin 804bp product resulted in a protein sequence of 90aa with a signal peptide of 24aa, a pro-domain of 40aa and a mature peptide of 26aa. This configuration is identical to the hepcidin found in *S. maximus* (Chen, et al., 2007). The similarity found between the *S. lalandi* and *S. maximus* hepcidin is extremely high with the signal peptide sharing 96% similarity, the pro-domain sharing 68% similarity, and the mature peptide sharing 85% similarity. Overall, the signal peptide and mature peptide show the highest sequence conservation, whereas the pro-domain is less well maintained. This hepcidin sequence conservation between species potentially indicates conserved functionality between fish (Ke *et al.*, 2015).

Hepcidin is a β-sheet hairpin AMP with eight conserved cysteine residues that form four disulphide bridges (Masso-Silva & Diamond, 2014). Hepcidin has been identified in a wide range of organisms including amphibians, fish and mammals (Falco, *et al.*, 2012). The defining characteristic between the hepcidin found in such a wide array of organisms is the cysteine saturation in the mature

peptide (Douglas, *et al.*, 2003). For example, little sequence similarity was detected between human and fish hepcidin in this study, however, the characteristic cysteine residues are very well conserved in the same location between all these species. Due to the preservation of these cysteine residues it can be assumed that the hepcidin disulphide bridge formation must be functionally important.

Similar to pleurocidin the protein sequence of fish hepcidin is highly similar between species (Douglas, *et al.*, 2003). Up to seven hepcidin gene isoforms have been identified in Japenese black porgy (*Acanthopargus schlegelii*) (Yang *et al.*, 2007), therefore, indicating several duplication and diversification events (Masso-Silva & Diamond, 2014). In comparison, mammals contain only a single hepcidin gene which functions both as an AMP and as an iron regulator. As fish contain several hepcidin genes the suggestion is, different hepcidins may carry out different functions (Neves *et al.*, 2015). Further analysis of hepcidin variants found in fish has also determined they can be clustered into two groups, hamp-1 and hamp-2 (Masso-Silva & Diamond, 2014). Under experimental conditions it has been shown that hamp-1 expression was up-regulated in response to elevated iron levels and infection, whereas hamp-2 expression was highly upregulated in response to infection where changing iron levels had no effect (Neves, *et al.*, 2015). This study concluded hamp-1 may play more of a role in iron regulation and hamp-2 in infections. Apart from the conserved cysteine residues teleost hamp-1 contains four conserved amino acids in the mature peptide, a histidine at position 3, a glycine at position 20, another glycine at position 22, and a phenylalanine at position 27 (Lee *et al.*, 2011). Based on these characteristics it can be concluded that the hepcidin isolated from *S. lalandi* is more similar to hamp-1, as the mature peptide contains all of these conserved residues. In comparison to hamp-1, hamp-2 has high variability in protein length and sequence, with heterogenous structure and charge. Due to the high microbicidal activity that is characteristic of hamp-2, it would be ideal to go back and specifically search the *S. lalandi* transcriptome, to determine if hamp-2 can be found. Some antimicrobial activity has been detected in fish hamp-1, therefore, the isolated *S. lalandi* hepcidin would still be of interest in bioactivity studies. For example, hamp-1 in spotted scat (*Scatophagus argus*) showed antibacterial

activity against gram positive and gram negative bacteria, in some cases more effective than hamp-2. However, no microbicidal activity was detected for hamp-1 against viruses, where hamp-2 showed strong antiviral activity. Therefore, the antimicrobial activity of hamp-1 may be slightly more limited in the breadth of micro-organisms it is effective against in comparison to hamp-2.

4.2.3 Defenisn-Like

Two sets of defensin-like forward primers were designed for 3' RACE, the first set F1A and F2A and the second set F1B and F2B. For 5' RACE two sets of reverse primers were also designed, the first set R1A and R2A and the second set R1B and R2B. This was because the size of the consensus sequence obtained from the transcriptome was short and features important for good primer design had to be compromised. The first round of 3' RACE nested PCR using F1A and F1B primers resulted in multiple sized bands being amplified. A similar result was seen in round 2 of nested PCR using F2A and F2B with the presence of multiple bands. There were two bands that were specifically distinct, one at 100bp which is likely primer dimer, and a second at 350bp. Ideally PCR parameters would need to be optimised to maximise the efficiency of the primers in order to more specifically select for the 350bp product. Another method that could have been utilised is to cut the 350bp band out of the agarose gel from which the DNA could be purified. In the first round of 5' RACE nested PCR only a single band was amplified using R1A and R1B reverse primers. The same band was isolated again in the second round of nested PCR using R2A and R2B primers. Using set A primers a band of approximately 150bp was amplified, whereas, using set B primers amplified a slightly larger product of around 200bp. Further optimisation of these primers again is needed to determine if these small sized isolates are from the gene of interest. Due to time constraints this work was not taken further but does open up work for a future project.

Defensins (α-defensins, β-defensins, and θ-defensins) are a family of AMPs that are characteristically arginine rich with six conserved cysteine residues forming three intramolecular disulphide bonds (Bateman *et al.*, 1996). Corticostatin was proposed as an alternative name for α-defensins as it was found

that rabbit α-defensin bound to the adrenocorticotrophic hormone (ACTH) receptor which are located on the surface of adrenal cells (Lehrer & Lu, 2012). This activity of α-defensins as antagonists for ACTH receptors has led to their classification as corticostatin-defensins (Raj & Dentino, 2002). Research looking at protein expressed in pacific lamprey skin found a cysteine and arginine rich peptide termed lamprey corticostatin related peptide (LCRP) with some structural similarity to mammalian corticostatin-defensins (Conlon & Sower, 1996). Using the LCRP protein a similar sequence in the *G. australis* transcriptome was identified, this corticostatin-defensin like protein was then aligned against human α-defensin-1. Overall, there was low sequence similarity between human α-defensin-1 and the identified *G. australis* defensin-like sequence from the transcriptome, although conserved cysteine and arginine residues were present, indicating the identified protein as being functionally similar. The ability to verify the presence of the corticostatin-defensin like peptide and its mRNA sequence would provide significant evolutionary insights, as lamprey are the most primitive living vertebrate (Raj & Dentino, 2002).

Unfortunately, in the Pacific lamprey, there is no nucleotide sequences currently available for LCRP, therefore, the expected size for the full *G. australis* defensin-like sequence from RACE-PCR is unknown. Cryptdin a corticostatin-defensin-like precursor in mice has an mRNA sequence length of 450 to 480 nucleotides (Ouellette *et al.*, 1989), and this could give an indication of size, although further analysis is required to confirm this. Having only a small region of the protein currently makes exact identification impossible.

4.3 Real-Time PCR

Primers were designed for the characterised *S. lalandi* moronecidin and hepcidin genes, as well as the housekeeping genes GAPDH and β-actin which were used as reference genes. The expression of each of the genes were targeted in three available *S. lalandi* tissues spleen, liver and gill from three individual adult fish. To begin with each set of reference AMP and reference gene primers had their amplification efficiency established. As the amplification efficiencies of each primer set was high it was found appropriate to use the $\Delta Cq = 2^{Cq(ref)} -$

$^{Cq(target)}$. RT-PCR was then carried out on each tissue sample and the relative expression of each gene was determined using the comparative ΔCq test (Bird *et al.*, 2005).

In *S. lalandi* tissues moronecidin showed highest expression levels in the spleen followed closely by the gill and significantly lower expression in the liver. In comparison, moronecidin expression is similar in the hybrid striped bass which has been shown to have relatively high expression in the gill and spleen and relatively undetectable levels in the liver (Lauth, *et al.*, 2002). Expression of moronecidin in healthy rock sea bream showed ubiquitous detection in all tissues tested, where highest expression was noted in the gill with decreasing expression in liver and spleen. These results do show some variability to *S. lalandi* moronecidin expression levels as expression in the liver was higher than the spleen (Bae, *et al.*, 2014). However, there does appear to be a general consensus with high ubiquitous expression levels of moronecidin in all tissues. It has been proposed that the high level of moronecidin expression in the tissues of healthy fish may be due to the presence of moronecidin in circulating immune cells such as peripheral blood lymphocytes (PBLs) and red blood cells (RBCs) (Bae, *et al.*, 2014).

Further to moronecidin expression in healthy fish, expression levels have also been analysed in fish exposed to bacterial and viral challenges. Hybrid striped bass exposed to *Streptococcus iniae* showed increased expression of moronecidin in all tissues although the differences were not shown to be statistically significant (Lauth, *et al.*, 2002). Another study in rock sea bream analysing moronecidin expression in response to bacterial and viral infection was analysed in the spleen and gill (Bae, *et al.*, 2014). In the spleen inducible moronecidin expression was noted with increasing levels over a 7 day period in response to *Edwardsiella tarda, S. iniae*, and red sea bream iridovirus (RSIV). In the gill increasing expression of moronecidin over 7 days was also noted in response to *E. tarda, S, iniae* although no detectable induction was found in response to RSIV (Bae, *et al.*, 2014). Overall, there appears to be differing opinions on the inducible expression of moronecidin, therefore it needs to be explored in more depth. It is possible moronecidin is only induced in response to

particular pathogens. Further investigation of moronecidin expression should be analysed in more tissues from healthy *S. lalandi* and in tissues from immune compromised *S. lalandi*.

In comparison to moronecidin, hepcidin showed highest expression in *S. lalandi* liver tissue with significantly lower expression levels in the gill and spleen. Although, relative to each other moronecidin showed much higher constitutive expression in all tissue types in comparison to hepcidin. It may be that hepcidin is a more inducible AMP in comparison to moronecidin which shows more constitutive expression as has been shown in other studies (Douglas, *et al.*, 2003; Valero, *et al.*, 2013; Bae, *et al.*, 2014). As previously mentioned, fish have been shown to contain two hepcidins, hamp-1 and hamp-2, expression has been characterised in a wide range of fish species. In turbot hamp-1 was detected mostly in the liver, with moderate expression in the spleen and lowest detection levels in the gills, and hamp-2 is also predominantly detected in the liver. As the isolated hepcidin from *S. lalandi* is also predominantly in the liver and limited tissues were studied it is not entirely clear from which of the two hepcidins *S. lalandi* is related to. However, the tissue specific pattern of hepcidin expression in the liver is characteristic of hepcidin (Douglas, *et al.*, 2003).

Expression levels have also been explored in immune challenged fish. Upon bacterial infection expression of hepcidin increases in the liver spleen and gills or most adult fish (Douglas, *et al.*, 2003; Chen, *et al.*, 2007; Huang *et al.*, 2007). Turbot hepcidin showed high inducible expression after bacterial challenge with *Aeromonas salmonidica (Douglas, et al., 2003).* This is also in line with Atlantic salmon infected with *Aeromonas salmonidica* showing increased expression in most tissues tested, whereas healthy individuals had barely detectable levels. The time-course of hepcidin expression is highest after 3-6 hours post infection with decreasing levels thereafter, putting it in line with other immune proteins such as IL-1β (Yang, *et al.*, 2007; Li *et al.*, 2013). In addition, in mammals tumour cells lines (SAF-1 and L-1210) and fungi (*Saccharomyces cerevisiae*) have shown to induce hepcidin expression (Cuesta, *et al.*, 2011; Masso-Silva & Diamond, 2014). However, this remains to be studied in fish.

4.4 Problems and Limitations

There were a number of problems encountered throughout the course of this research, limiting all aims being completed. The initial step of this research involved bioinformatics, where the literature was searched for known fish AMPs. The difficulty surrounding this is the multitude of information available when defining AMPs. There is significant overlap in the naming of AMPs where some are characterised by structure and others by sequence homology. Therefore, a substantial amount of time was spent reading a broad range of literature in order to gain an understanding of fish AMPs and their organisation. Another restriction is that a significant amount of the work carried out on fish AMPs is limited to functional mature peptides and not the whole sequence. In order to search the transcriptomic library the entire pre-pro-peptide sequence was required, meaning that many AMPs that have an available mature peptide sequence were not able to be used as the sequence was too short, limiting the search of the transcriptome. A major short coming with the *G. australis* transcriptomic database mining was that they are such an evolutionary distinct animal where their genome is significantly different to bony fish, limiting the amount of transcriptome hits possible. A way to overcome this issue is by manually searching the transcriptome looking for characteristic features of known AMPs. This can include features such as, high cysteine residue content. Another method that could be used to search the *G. australis* transcriptome is sequences from other organisms, not limited to fish AMPs, thus, increasing the likelihood of matches. Unfortunately, these approaches are time consuming and were outside the scope of this project. However, the work presented in this thesis has provided an in depth study of AMPs available in the two species that were investigated.

Primer design was another issue as not all primers were able to amplify a product. A significant amount of amplification protocols were trialled in an attempt to optimise primers. In some instances, very blurry products were amplified and in this instance increasing the annealing temperature from 54˚C to 58˚C resulted in a more defined band. Also generation of new RACE libraries aided in the generation of more specific results. Once amplification of genes by PCR generated consistent results, experimental research was also delayed due to issues with bacterial cloning and transformation. The pLUG-Prime TA-cloning

vectors used for ligation of PCR products, were left overnight at 4˚C to ensure maximum ligation efficiency. The ligated plasmids were then transformed into chemically competent *E. cloni* cells and grown up on LB + ampicillin agar plates. Several attempts at this resulted in agar plates densely populated with white colonies, in contrast to the desired effect being a combination of blue and white colony growth. Initially it was thought the white colony growth was due to degraded IPTG and Xgal, therefore new stocks of this were acquired. When this made no difference, new plates were then made and the Xgal and IPTG were incorporated into the agar itself. This made a small difference where some blue colonies were then visible, however, a PCR screen was carried out on ten of the white colonies but none of them contained the correct product inserts (see Appendix XI). Even though some blue colonies were present the overall growth on the plate was still extremely dense. Therefore the ampicillin concentration was increased to 100µg/mL which eventually gave an agar plate with the expected combination of blue and white colonies. A PCR screen showed positive results with colonies containing PCR product inserts of the correct size.

The final procedure that also caused some delay was the restriction digest which is used to confirm the presence of a PCR product insert within a plasmid before it is sent for sequencing. In some instances when running a restriction digest on a gel zero bands were present, including the band where the plasmid should be. It was concluded that between the PCR screen to determine the presence of a PCR product insert and the restriction digest, DNase became introduced. Prior to the restriction digest plasmid isolates were run on a gel resulting in extremely blurry bands, suggesting DNase may have already been introduced before the restriction digest and had become introduced during the plasmid extraction step. A new plasmid extraction kit was used and seemed to resolve this problem.

Although these issues prevented all work being completed in the set timeframe this investigation has paved the way for future AMP studies in *S. lalandi* and *G. australis*. Two genes have been fully sequenced from *S. lalandi*, and many others identified in the transcriptome of both species.

4.5 Future Recommendations

4.5.1 Characterisation of AMPs

A wider range of AMPs in the immune systems of *S. lalandi* and *G. australis* could be further characterised using the same approaches adopted in this research. The transcriptomes available for *S. lalandi* and *G. australis* could be used to identify other AMPs using a variety of different techniques such as manual database searching, particularly when it comes to the *G. australis* transcriptome. The AMPs already identified in each transcriptome also need to be further characterised to determine their specific sequence using RACE-PCR. Greater transcriptomic work could also focus on characterising the expression of various AMPs and a range of studies have shown the use of RNA-seq for comparing gene expression during infection, vaccination and throughout development (Qian*, et al.*, 2014).

Sequencing the genomes of these species is a possibility with the cost being more affordable in eukaryotic organisms. Understanding how these genes are organised in the genome may provide insights into their exact identity. Also information gained from having a genome may increase the assortment of AMPs found as the transcriptomes used relied on the AMPs showing constitutive expression and some may be inducible. An example is β-defensin found in mice which has been shown to be induced in the epithelia of organs following *Pseudomonas aeruginosa* infection in the airway (Bals *et al.*, 1999). Therefore, this inducible expression may explain why no β-defensin sequences were able to be isolated from the *S. lalandi* and *G. australis* transcriptome.

From the scope of information this research has reached, there is a significant amount of work that can be further compiled. The work characterising the *G. australis* defensin-like peptide needs to be refined through alterations of PCR parameters in order to increase primer specificity. The next step for hepcidin and moronecidin would be recombinant protein production which can be carried out using protein expression vectors. A specific gene can be introduced into a vector that is placed into a target cell which is stimulated to synthesise proteins. The protein can then be isolated and purified in order for functional studies to be

carried out. Bioactivity studies to see what organisms the *S. lalandi* hepcidin and moronecidin show microbicidal activity against could then be analysed. Bioactivity of these proteins may be limited as folding of the proteins can be functionally important and some AMPs are known to stay in a linear formation until contact with a pathogen associated membrane protein (PAMP), at which time they fold into their active structure (Lai & Gallo, 2009). Other AMPs may need to have their activation analysed as they may need folding into a certain structure before bioactivity assays can occur (Aoki & Ueda, 2013). However, bioactivity studies could focus on looking at activity against pathogens affecting aquaculture success and antibiotic resistant bacteria such as methicillin resistant *Pseudomonas aeruginosa* (MRSA) and vancomycin resistant *Enterococcus faecalis* (VRE). Other areas for future research include looking at how particular AMPs could be used as therapeutic agents. For example, carrying out a red blood cell screen looking at haemolytic activity and attraction to zwittertonic cells. These types of assays are important as AMPs that show haemolytic activity may need chemical modification before use in therapeutics (Bragadeeswaran *et al.*, 2011). Overall, the possibility of future works from this research are extensive and have crucial and important applications.

4.5.2 Application of AMPs

The aim of this work was to characterise AMP genes in jawed (*S. lalandi*) and jawless (*G. australis*) vertebrates in order to gain insight into the immune systems of these species. Understanding the AMPs that are present and their expression in fish is important for this and with future bioactivity studies have applications in biomedicine, aquaculture and understanding and fighting diseases in fish.

4.5.2.1 Biomedicine

It is estimated that routine operations will become deadly within 20 years as the ability to fight conventional infections is lost due to the rise in antibiotic resistant microbes (Mazel & Davies, 1999; Buonocore, 2011). Therefore, much research has been focused on discovery and isolation of alternative compounds to supersede traditional antibiotics (Marshall & Arenas, 2003). AMPs have been

identified as promising candidates for biomedical uses in order to combat the issue of antimicrobial resistance (Wiesner & Vilcinskas, 2010; Buonocore, 2011). As fish live in a microbe dense environment and are exposed to many organisms that terrestrial animals are not, the identification and characterisation of fish AMPs may provide a unique contribution to this area of biomedicine (Masso-Silva & Diamond, 2014). Moronecidin and piscidins are of significant interest as they are a fish specific family of AMPs and could be very effective against terrestrial microbes. A study looking at the antimicrobial activity of moronecidin has shown antibacterial action against methicillin resistant MRSA and VRE (Lauth, *et al.*, 2002). Thus highlighting AMPs can be used as a natural antibiotic to combat the issue of antibiotic resistance. Other actions of fish AMPs has also been investigated, one such example is the use of tilipa hepcidin in mice. Hepcidin was shown to have a bacteriostatic effect acting as an antimicrobial and immunomodulatory agent against the bacterial pathogen *Vibrio vulnificus* (Pan *et al.*, 2012). Overall, understanding AMPs and their function in non-mammalian species will be of vital importance in biomedicine where they have the potential as a therapeutic replacement option for traditional antibiotics.

4.5.2.2 Aquaculture

Aquaculture is becoming increasingly necessary in the developed world because of both increasing population size and decreasing wild fish stocks from recreational and commercial fishing (Tidwell & Allan, 2001; Noga, *et al.*, 2011). Current obstacles in aquaculture are the high mortality rate in young fish and the continued disease and abnormalities that can occur during development (Gjedrem *et al.*, 2012). Fishes rely heavily on their innate immune system to fight pathogen invasion during the first stages of life as their adaptive immune system is inactive until they are completely developed (Buonocore, 2011). AMPs are a major component of the fishes innate immune defence system protecting them from pathogenic infections (Falco, *et al.*, 2012). Therefore, due to the impact on fish immune systems AMP levels could be useful to determine, maintain, or improve fish health in aquaculture (Falco *et al.*, 2009; Cabello *et al.*, 2013).

S. lalandi are found mainly in waters surrounding New Zealand (Aotearoa) and Australia, preferring temperate and sub-tropical waters, 18-24°C (Stewart Fielder, 2013). *S. lalandi* are excellent candidates for introduction into New Zealand aquaculture due to their fast growth rates, large size (reaching 2.5m long and a maximum weight of 96.8kg), and high value in cuisine as a traditional food source for Māori (Miller, *et al.*, 2011). Japan currently has the largest aquaculture industry for various *Seriola spp*. where they make up to 57% of all farmed aquaculture (139000 tonnes per annum) (Stewart Fielder, 2013). *S. lalandi* aquaculture in Australia began in 2001 and has grown consistently with aquaculture of *S. lalandi* in New Zealand presently under development (Miller, *et al.*, 2011). Australia currently produces 3000-4000 tonnes of *S. lalandi* per annum (Miller, *et al.*, 2011). The growth of aquaculture systems in Australia has been constrained by high mortality rates, disease and growth deformities in farmed *S. lalandi* (Moran et al., 2007). There is no large scale production of *S. lalandi* in New Zealand aquaculture due to the large costs required for infrastructure and the current limitations with disease prevalence in farmed fish (Miller, *et al.*, 2011). Within New Zealand and Australia, *S. lalandi* are not included in any quota management system and as such have no conservation status, therefore, to ensure sustainable aquaculture of *S. lalandi* hatchery production in New Zealand and Australia is used (Stewart Fielder, 2013). Overall, aquaculture is a promising field for global food production but growth in this industry has been constrained by high mortality rate in juvenile fishes and adult disease and deformities (Miller, *et al.*, 2011). Therefore, it is vital to develop tools that will increase survival and quality of hatchlings (Stewart Fielder, 2013). Greater success of species in aquaculture is understanding fish physiology which will provide insights into development, health, growth, and reproduction. Due to the impact AMPs have on the fish immune system, they have been proposed as a therapeutic target for up-regulation, they have possible uses in vaccine development and could be used as a measure of overall fish health. AMP use has experimentally been shown to protect fish, where in coho salmon following infection with *Vibrio anguillarum* fish began receiving cecropin, an AMP isolated from insects. Mortality rates in infected coho salmon decreased from 58% to 13% (Jia *et al.*, 2000). Therefore, these results highlight the potential for AMPs to protect fish against infections and using what is naturally found in fish needs to be investigated. Stress in

aquaculture is another major issue contributing to fish mortality and can lead to down regulation of AMP expression, therefore, being able to measure AMP levels in farmed fish could be a good measure of health status (Buonocore, 2011). Also targeting AMPs for over expression in farmed fish could lead to enhanced disease resistance improving the efficiency of traditional treatments against pathogens (Noga, *et al.*, 2011) and immunostimulants such as bacterial peptidoglycan have been added to fish diets in order to increase AMP expression (Falco, *et al.*, 2012). Overall, understanding aspects of *S. lalandi* immunity is vital for successful introduction of this species into New Zealand aquaculture.

4.5.2.3 Disease

Much like farmed fish, wild fish stocks can also be detrimentally affected by disease and can be a significant issue in the wild especially when disease or parasites from an introduced species start to negatively affect native species (Masso-Silva & Diamond, 2014). Disease in the wild can be due to parasites, bacteria, viruses, fungi and water molds. In some cases disease in wild fish stocks can be a major cause of mass die offs (Poulin *et al.*, 2011). A recent example is *G. australis* lamprey or piharau an ancient primitive fish with a single species inhabiting Aotearoa (New Zealand). Since October 2011 significantly high numbers of piharau have been found dead in Aotearoa rivers and research into this has shown individuals that are dying are exhibiting red skin markings. Due to this the disease is now referred to as lamprey reddening syndrome (LRS) (Mitland, *et al.*, 2014). The cause of LRS is not yet known but it thought to be associated with a bacterial or viral infection. Piharau are an important taonga species for Māori communities and due to this syndrome are drastically declining in numbers in the wild (Mitland, *et al.*, 2014). Therefore, it is of significant importance to find a way to treat and mitigate this disease. Little is known about piharau and their immune systems. Isolating and characterising AMPs in these organisms will provide a greater understanding of their immune system and possible mechanisms to treat this fatal disease.

4.6 Conclusion

Overall, understanding aspects of fish immunity is vital for economical and successful fish farming, while also providing possible therapeutic benefits associated with AMP research in biomedicine and disease in wild fish stocks. The aim of this work was to identify yellowtail *S. lalandi* and *G. australis* AMPs involved in innate immunity and to use molecular techniques to fully sequence and quantify their expression in adult individuals. Using RNA-Seq transcriptomic libraries several *S. lalandi* and *G. australis* potential AMP genes were found. Using molecular approaches hepcidin and moronecidin were successfully isolated and sequenced from *S. lalandi* tissues. Expression of moronecidin and hepcidin was also analysed where hepcidin showed strong expression in liver tissues and moronecidin showed high expression in the spleen and gill tissues. In conclusion, this study provides preliminary data on AMP genes found in *S. lalandi* and *G. australis*, thus permitting the development of future research applications.

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Appendices

Appendix I - Ethics Statement

Approval from the Animal Ethics Committee or the Biological Safety Committee at the University of Waikato was not required for this research project. Tissues samples had previously been collected, therefore, were taken from long term cold storage for molecular analysis.

Appendix II - HSNO Declaration

The University of Waikato received Hazardous Substances and New Organisms (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 antimicrobial peptide genes, hepcidin and moronecidin 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. cloni* 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 (http://www.epa.govt.nz/search-databases/Pages/applications-details.aspx?appID=APP201152).

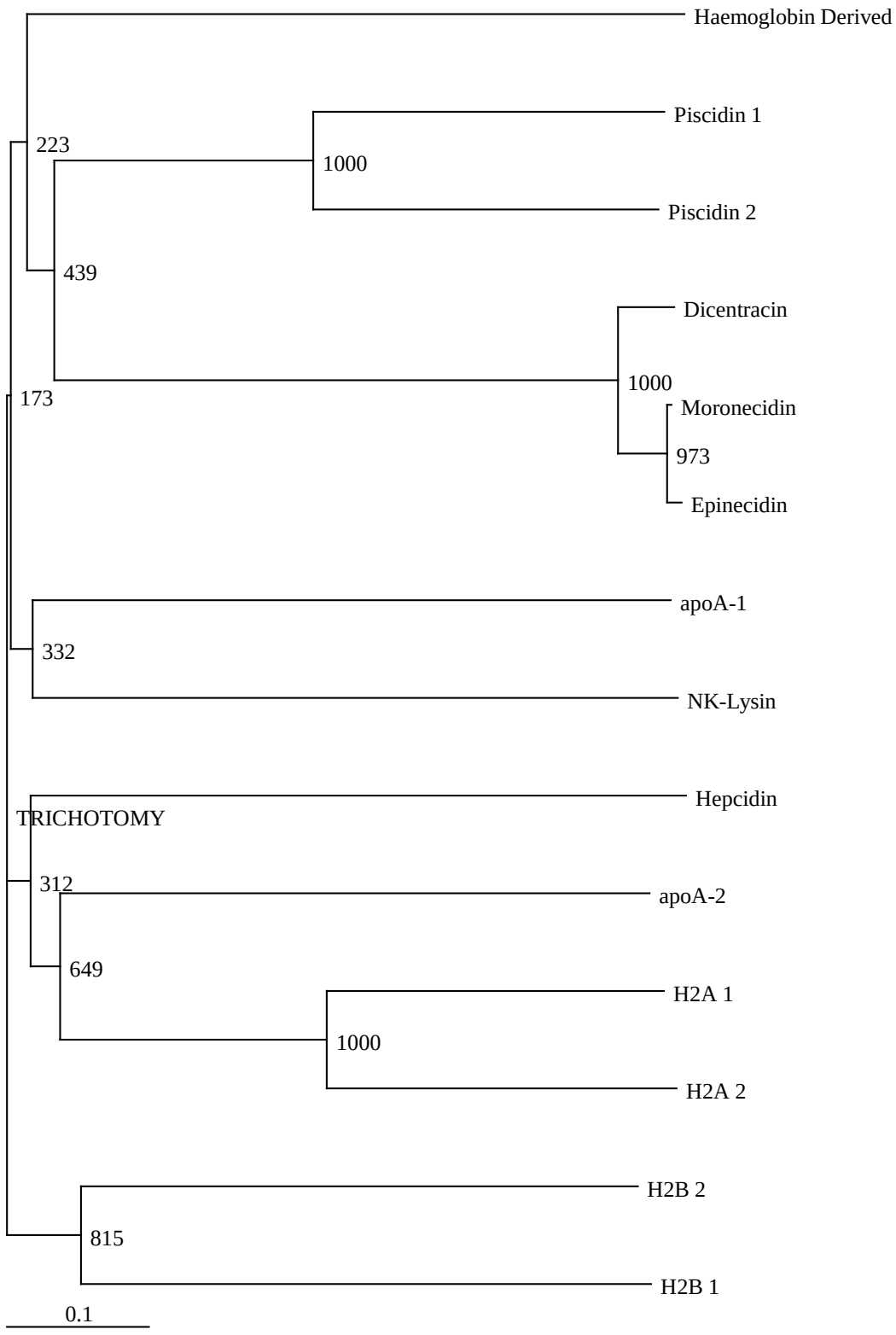
Appendix III - Accession Numbers

Sequences used in the generation of the phylogenetic tree in the section 3.1.

apoA-1 Common Carp *Cyprinus carpio* (Accession number, CAC34942.1)
apoA-2 Rainbow Trout *Oncorhynchus mykiss* (Accession number, NP_001154920.1)
AsCath-1 Atlantic Salmon *Salmo salar* (Accession number, NP_001117045.1)
AsCath-2 Atlantic Salmon *Salmo salar* (Accession number, NP_001117058.1)
acCATH Arctic Char *Salvelinus alpines* (Accession number, ACE96052.1)
codCATH-1 Atlantic Cod *Gadus morhua* (Accession number, ACE96051.1)
CATH1-SALTR Brown Trout *Salmo truttafario* (Accession number, ABW16872.1)
CATH-THYTH Grayling *Thymallus thymallus* (Accession number, CAQ60112.1)
Dicentracin European Bass *Dicentrarchus labrax* (Accession number, P59906.1)
Epinecidin-1 Grouper *Epinephelus coioides* (Accession number, AAQ57624.1)
fuBD1 Fugu *Takifugu rubripes* (Accession number, CAJ57646.1)
Gramminstin (Pp1) Soapfish *Pogonoperca punctata* (Accession number, BAF37109.1)
HbβP-1 Channel Catfish *Ictalurus punctatus* (Accession number, NP_001187115.1)
HbβP-2 Channel Catfish *Ictalurus Punctatus* (Accession number, AHA82590.1)
HbβP-3 Channel Catfish *Ictalurus punctatus* (Accession number, AHA82591.1)
Hepcidin Turbot *Scophthalmus maximus* (Accession number, AAX92670.1)
Hepcidin Red Sea Bream *Chrysophrys major* (Accession number, AAR28076.1)
Hepcidin Zebrafish *Danio rerio* (Accession number, P61516.1)
Hepcidin Striped Bass *Morone saxatilis x Morone chrysops* (Accession number, P82951.1)
OM-hep1 Medaka *Oryzias latipes* (Accession number, ADM83600.1)
OM-hep2 Medaka *Oryzias latipes* (Accession number, ADM23869.1)
HFIAP-1 Hagfish *Myxine glutinous* (Accession number, AAQ04687.1)
HFIAP-3 Hagfish *Myxine glutinous* (Accession number, AAQ04688.1)
Hipposin Zebrafish *Daino rerio* (Accession number, NP_001083033.1)
HLP1 Zebrafish *Daino rerio* (Accession number, XP_001923636.1)
JF-1 Japenese Flounder *Paralichthys olivaceus* (Accession number, AAT01564.1)
JF-2 Japanese Flounder *Paralichthys olivaceus* (Accession number, AAT01563.1)
LEAP2 Grass Carp *Ctenopharyngodon idella* (Accession number, ACR54299.1)
LEAP2 Winter Flounder *Paralichthys olivaceus* (Accession number, ACB97648.1)
LEAP2 Medaka *Oryzias latipes* (Accession number, XP_004080006.1)
LEAP2 Channel Catfish *Ictalurus punctatus* (Accession number, AAX45792.1)
LEAP-2A Rainbow Trout *Oncorhynchus mykiss* (Accession number, AAS49157.1)
LEAP-2A Carp *Cyprinus carpio* (Accession number, AGK89728.1)
LEAP-2A Zebrafish *Danio rerio* (Accession number, NP_001122249.1)
LEAP-2B Rainbow Trout *Oncorhynchus mykiss* (Accession number, NP_001117937.1)
LEAP-2B Carp *Cyprinus carpio* (Accession number, AGK89729.1)
LEAP-2B Zebrafish *Danio rerio* (Accession number, XP_003200926.1)
MgCATH29 Atlantic Hagfish *Myxine glutinosa* (Accession number, AAQ04688.1)
MgCATH37 Atlantic Hagfish *Myxine glutinosa* (Accession number, AAQ04687.1)
NK-lysin Channel Catfish *Ictalurus punctatus* (Accession number, NP_001187137.1)
OgBD1 Orange Spotted Grouper *Epinephelus coioides* (Accession number, AFA41485.1)
OmBD1 Rainbow Trout *Oncorhynchus mykiss* (Accession number, ABR68250.1)
OncorhyncinII Rainbow Trout *Oncorhynchus mykiss* (Accession number, P06350.2)
Piscidin-1 Striped Bass *Morone saxatilis* (Accession number, Q8UUG0.1)
Piscidin-4 Striped Bass *Morone saxatilis* (Accession number, ADP37959.1)
PleurocidinWinter Flounder *Peluronectes americanus* ID, AAK52842.1)
rtCATH-1 Rainbow Trout *Oncorhynchus mykiss* (Accession number, AAT67998.1)
rtCATH-2 Rainbow Trout *Oncorhynchus mykiss* (Accession number, AAT44539.1)
Sal-1 Atlantic Salmon *Salmo salar* (Accession number, Q801Y3.1)
Sal-2 Atlantic Salmon *Salmo salar* (Accession number, NP_001134321.1)
SAMPH1 Atlantic Salmon *Salmo salar* (Accession number, P84408.3)
TH1-5 Tilapia *Oreochromis mossambicus* (Accession number, XP_003453514.1)
TH2-3 Tilapia *Oreochromis mossambicus* (Accession number, XP_003450578.1)
tnBD1 Pufferfish *Tetraodon migroviridis* (Accession number, CAJ57644.1)
tnBD2 Pufferfish *Tetraodon migroviridis* (Accession number, CAG00590.1)
ZFDB1 Zebrafish *Danio rerio* (Accession number, AJG06055.1)

ZFDB2 Zebrafish *Danio rerio* (Accession number, NP_001075023.1)
ZFDB3 Zebrafish *Danio rerio* (Accession number, NP_001075024.1)
Piscidin-1 Atlantic Cod *Gadus morhua* (Accession number, ACS91329.1)
Moronecidin Chinese Perch *Siniperca chuatsi* (Accession number, AAV65044.1)
Dicentracine European Sea Bass *Dicentrarchus labrax* (Accession number, AAP58960.1)
Moronecidin Sand Bass *Morone chrysops* (Accession number, AAL57318.1)
Moronecidin Stripped Bass *Morone saxatilis* (Accession number, AAL57319.1)
Piscidin-Like Hong Kong Grouper *Epinephelus akaara* (Accession number, ACE78290.1)
Piscidin-Like Orange Spotted Grouper *Epinephelus coioides* (Accession number, ACE78291.1)
Piscidin-Like Yellow Croaker *Larimichthys crocea* (Accession number, ACE78289.1)
Graduscidin-1 Atlantic Cod *Gadus morhua* (Accession number, ADK63423)
Graduscidin-2 Atlantic Cod *Gadus morhua* (Accession number, ADK63424)
Piscidin-2 Atlantic Cod *Gadus morhua* (Accession number, ADU34222)
Piscidin-2β Atlantic Cod *Gadus morhua* (Accession number, ADU34223)
Moronecidin Black Cod *Anoplopoma fimbria* (Accession number, C3KH06-1)
Moronecidin Mandarin Fish *Siniperca chuatsi* (Accession number, Q2VWH5-1)
CATH1_SALFO Brook Trout *Salvelinus fontinalis* (Accession number, B8ZHC5-1)
CATH2_SALFO Brook Trout *Salvelinus fontinalis* (Accession number, B8ZHC6-1)
aCATH Ayu *Olecoglossus altivelis* (Accession number, CBV36822.1)
omDB-2 Rainbow Trout *Oncorhynchus mykiss* (Accession number, NP_001182097.1)
omBD-3 Rainbow Trout *Oncorhynchus mykiss* (Accession number, CBB12548.1)
omBD-4 Rainbow Trout *Oncorhynchus mykiss* (Accession number, NP_001182098.1)
saBD Gilthead Seabream *Sparus aurata* (Accession number, FM158209)
ScBD Mandarin Fish *Siniperca chuatsi* (Accession number, ACO88907.1)
Defensin Medaka *Oryzias latipes* (Accession number, NP_001153910.1)
Hepcidin1 Gilthead Seabream *Sparus aurata* (Accession number, ABV07929.1)
Gramminstin-like Rainbow Trout *Oncorhynchus mykiss* (Accession number, CDQ73614.1)
apoA-1 Silver Carp *Hypophthalmichthys molitrix* (Accession number, ADF97611.1)
apoA-1 Mud Carp *Cirrhinus molitorella* (Accession number, ACY82518.1)
apoA-1 Spotted Barbel *Hemibarbus mylodon* (Accession number, ACI15889.1)
apoA-2 Ayu *Plecoglossus altivelis* (Accession number, CBD77421.1)
apoA-2 Yellow Perch *Perca flavescens* (Accession number, ABW06868.1)
apoa-2 Barramundi *Lates calcarifer* (Accession number, ABV66070.1)
Cathelicidin Rainbow Trout *Oncorhynchus mykiss* (Accession number, NP_001117952.1)
H1 Tetra *Astyanax altiparanae* (Accession number, AHW46398.1)
H1 Zebrafish *Danio rerio* (Accession number, XP_009301186.1)
H1 Brown Trout *Salmo trutta* (Accession number, P02254.1)
NK-Lysin Tetra *Astyanax mexicanus* (Accession number, XP_007253844.1)
NK-Lysin Zebrafish *Danio rerio* (Accession number, XP_009291114.1)
NK-Lysin Guppy *Poecilia reticulate* (Accession number, XP_008398114.1)
NK-Lysin Pufferfish *Takifugu rubripes* (Accession number, XP_003962755.1)
H2A Tounge Fish *Cynoglossus semilaevis* (Accession number, XP_008308956.1)
H2A Southern Platyfish *Xiphophorus maculatus* (Accession number, XP_005812306.1)
H2A Guppy *Poecilia reticulate* (Accession number, XP_008401837.1)
H2B RainbowTrout *Oncorhynchus mykiss* (Accession number, P69069.2)
H2B Tetra *Astyanax mexicanus* (Accession number, XP_007238182.1)
Hbß Catfish *Silurus asotus* (Accession number, O13163.2)
Hbß Loach *Paramisgurnus dabryanus* (Accession number, AAM93251.1)
LCRP Sea Lamprey *Petromyzon marinus* (Accession number, Q10996.1)

Appendix IV – *S. lalandi* Phylogenetic Tree



Supplementary Figure 1: Phylogenetic tree showing all identified *S. lalandi* sequences from the RNA-seq transcriptomic library search.

Appendix V - *S. lalandi* Alignments

High Density Lipoprotein apoA-1 Alignment

```
apoA-1 Snakehead Murrel         MKFVALAVALLLAVGCQAASLQADAPSQLAHIKAAIDVYANQVKDSFKNA
apoA-1 Cobia                    MKFVALALALLLAVGSQAASLQSDAPSQLAHIRAAMDVYLNQVKDSANRA
Potential apoA-I S. lalandi     --------------------------------------------------


apoA-1 Snakehead Murrel         LNSLDDTEHQELKQRLSQRVDEFHAQLKALQGSVSPITDSVVSTLADATA
apoA-1 Cobia                    LDQLDDTEYRQLKQSLSQRLDIMYNQIKAMQGAVSPITDNVVSTIADATA
Potential apoA-I S. lalandi     -------------------------------------QTD-----------
                                                                     **


apoA-1 Snakehead Murrel         DFRASLTKDIETLKADLEPKRAKLREVINEHLQEYRIQLEPIVKKYYDKH
apoA-1 Cobia                    DLRASLITDIETLKLELEPKRIALRDVVNKHIEEYRELMQPIFNEYYTKH
Potential apoA-I S. lalandi     ---AWLVX---------EX-------VINKHIDEYRTQMQPIISEYYSKH
                                   * *           *        *:*:*::***  ::**..:** **


apoA-1 Snakehead Murrel         TADMEALKVRLEPVVEELRAKVATNVEETKAALMPILEAIRTKVHARLEN
apoA-1 Cobia                    TSEMEALRLKLEPVLEDLRQKVAVNLEETKAAVTPIVEQVRSKLSERLEA
Potential apoA-I S. lalandi     TADMEALRVKLEPVVEELRQKVATNLDETKTALMPIVEAVRNKLSERLET
                                *::****:::****:*:** ***.*::***:*: **:* :*.*:  ***


apoA-1 Snakehead Murrel         LKELVSPYVEEYKDQLKQAYSQVRSIDSQEVNARSIDSQEVNALREKIAP
apoA-1 Cobia                    LKELASPYVEEYKDQMKQVYSQAQNIN----------TEDLTNMKEKILP
Potential apoA-I S. lalandi     LKDMASPYVEEYKDQLKQAYSQAQXXX----------XX-----------
                                **::.**********:**.***.:


apoA-1 Snakehead Murrel         LVEDIKVKLHEIFEAVAATVTKS
apoA-1 Cobia                    LAEEIKVKLQSIFEIIAAAVTKN
Potential apoA-I S. lalandi     ------------------------
```

Supplementary Figure 2: Potential apoA-I protein sequence obtained from the *S. lalandi* transcriptomic library aligned against a cobia (*Rachycentron canadum*; Accession number, ACV50420.1) and snakehead murrel (*Channa striata*; Accession number, AIL82447.1) apoA-I protein.

High Desnity Lipoprotein apoA-II Alignment

```
apoA-II Rainbow Trout           MNGKLALALVLALQVSVCLCQ--VPEPDKELVEKYEAMKSVFYKRLMNAY
apoA-II Ayu                     MSGKYLLAVFLALQVSMSLCQLEIPQPDQELVDKYENMKSIFYKRLLNAY
Potential apoA-II S. lalandi    --------------TLLARCS----KP-----------------LLLSCX
                                              . :. *.    :*                 *:..


apoA-II Rainbow Trout           SKVQAAVGPMTENLGQG-HGQAAKDYIEELQGNPKFLSAVKIGTGLAQEA
apoA-II Ayu                     NKMQAAVAPVADSLGEG-RSQAAKDYIEDLQTKPQFQAAVKVATGLAGEA
Potential apoA-II S. lalandi    ------------KIGESERGQTARTYLEELQGKPELQAAVKVASGLGEEA
                                          .:*:. :.*:*: *:*:** :*:: :***:.:**. **


apoA-II Rainbow Trout           APLVDKARMAGLGLYGHYVRPHVGTYLDEAITSIKVYLDKVLPAEE
apoA-II Ayu                     APLVDKARMAGLGLYGKYLRPHVGTYLDDAINNIKVYLDQYMPAE-
Potential apoA-II S. lalandi    APPVGQGPYSSSGSVRTLPAPPCWR-LPERRHRPRQGLPGQIPAR-
                                ** *.:.  :. *       *     * :     :  *   :**.
```

Supplementary Figure 3: Potential apoA-II protein sequence obtained from the *S. lalandi* transcriptomic library aligned against a rainbow trout (*Oncorhynchus mykiss*; Accession number, NP_001154920.1) and ayu (*Plecoglossus altivelis*; Accession number, CBD77421.1) apoA-II protein.

Piscidin Alignment

```
Piscidin Striped Bass              MKCVMIFLVLTLVVLMAEPGEGFFRHL---FRGAKAIFRGARQGWRAHK-
Piscidin Orange Spotted Grouper    MKFVMVFLVLSLVVLMAEPGEGFLRHIKSFWKGAKAIFRGARQGWREHR-
Potential Piscidin 2 S. lalandi    MKFVMIFLVLSLVVLMAEPGECFFKHLKTIWSGAKAIFRGARAGWRGHRN
Potential Piscidin 1 S. lalandi    -----SFSCCRWSSSWLNXGECFFKHLKTIWSGAKAIFRGARAGWRGHRK
                                         *          : ** *::*:   : ********** *** *:

Piscidin Striped Bass              ----VVSRYRNR-------DVPETDN--------------NQEEPYNQR-
Piscidin Orange Spotted Grouper    ----ALSKQRKM-------DQGGGGNEVD-----------NGTPPYWQK-
Potential Piscidin 2 S. lalandi    SRRMAAIRYRNAXAN-CMRYQQGGYNRLAGTACNLPMHFCTLSQPFCVSF
Potential Piscidin 1 S. lalandi    LTQNGCDKVQKWXWANCMRYQQGGYNQKS--------AALMGSEPVHFKE
                                          : ::              *                 *

Piscidin Striped Bass              ---------------
Piscidin Orange Spotted Grouper    ---------------
Potential Piscidin 2 S. lalandi    CVLSIPPWHL-----
Potential Piscidin 1 S. lalandi    FELQLSALQFCLSPP
```

Supplementary Figure 4: Potential piscidin protein sequence obtained from the S. lalandi transcriptomic library aligned against a orange spotted grouper (*Epinephelus coioides*; Accession number, AKA60777.1) and striped bass (*Morone saxatilis*; Accession number, ADP37959.1) piscidin protein.

Epinecidin Alignment

```
Potential Epinecidin S. lalandi    AKAXAKAQSSGRMKFIALFLVLSLVVLMAEPGEGFFHHILSGIFHVGKMI
Epinecidin Orange Spotted Grouper  ------------MRCIALFLVLSLVVLMAEPGEGFIFHIIKGLFHAGKMI
                                             *: ********************:.**:.*:**.****

Potential Epinecidin S. lalandi    HGAIQRRRHGMTELE---QEQFDRDRADFAVLLWPQL
Epinecidin Orange Spotted Grouper  HGLVTRRRHGVEELQDLDQRAFEREKA-FA-------
                                   ** : *****: **:   *. *:*::* **
```

Supplementary Figure 5: Potential epinecidin protein sequence obtained from the *S. lalandi* transcriptomic library aligned against a orange spotted grouper (*Epineohelus coioides*; Accession number, AAQ57624.1) epinecidin protein.

Histone Derived H2A Alignment

```
H2A Guppy                          --MSGRGKTGGKAR---AKAKTR--SSRAGLQFPVGRVHRLLR-KGNYAE
H2A Tounguefish                    --MSGRGKTGGKAR---AKAKTR--SSRAGLQFPVGRVHRLLR-KGNYAQ
Potential H2A 1 S. lalandi         LKMSGRGKKAVPKP---KSSVSR--SSRAGVTFPVGRIHRLLR-RGQYAK
Potential H2A 2 S. lalandi         AKAKTKVCVGGPRRXXVAKAVSXXXSQRAGLQFPVGRIHRHLKTRTTSHG
                                      . :   .        .: :   *.***: *****:** *: :

H2A Guppy                          RVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAVRNDE
H2A Tounguefish                    RVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAVRNDE
Potential H2A 1 S. lalandi         RVGSGSAVYLSAVLEYLCAEILELAGNASRDNKKHRIAPRHILLAVKNDE
Potential H2A 2 S. lalandi         RVGATAAVYSAAILEYLTAEVLELAGNASKDLKVKRITPX----------
                                   ***: :.** :*:**** **:*******::* *  ** *

H2A Guppy                          ELNKLLGGVTIAQGGVLPNIQAVLLPKKTEKAAKTK
H2A Tounguefish                    ELNKLLGGVTIAQGGVLPNIQAVLLPKKTEKAAKTK
Potential H2A 1 S. lalandi         ELNKLLAGVTISEGGVIXXXXXXLLPQK--------
Potential H2A 2 S. lalandi         ---------SLAAG----------HPW---------
                                           ::: *          *
```

Supplementary Figure 6: Potential H2A protein sequence obtained from the *S. lalandi* transcriptomic library aligned against a guppy (*Poecilia reticulate*; Accession number, XP_008401837.1) and toungefish (*Cynoglossus semilaevis*; Accession number, XP_008308956.1) H2A protein.

Histone Derived H2B Alignment

```
H2B Tetra                   ---------------------------MPEPAKAAP--KKGSKKAVTKTA
H2B Zebrafish               ---------------------------MPEPAKAAP--KKGSKKAVTKTA
H2B Rainbow Trout           ---------------------------MPEPAKSAP--KKGSKKAVTKTA
Potential H2B S. lalandi    GQGGXXXRXXXXXLXSSCSTXXXTEEKMPDPAKSAPAPKKGSKKAVTKTQ
                                                       **:***:**  ***********

H2B Tetra                   GKGGKKRRKTRK-ESYAIYVYKVLKQVHPDTGISSKAMGIMNSFVNDIFE
H2B Zebrafish               GKGGKKRKRTRK-ESYAIYVYKVLKQVHPDTGISSKAMGIMNSFVNDIFE
H2B Rainbow Trout           GKGGKKRKRSRK-ESYAIYVYKVLKQVHPDTGISSKAMGIMNSFVNDIFE
Potential H2B S. lalandi    XXARQEAPQEQEXESYAIYVYKVLKQVHPDTGISSKAMGIMNSFVNDIFE
                              . ::  : :: ************************************

H2B Tetra                   RIAGESSRLAHYNKRSTITSREIQTAVRLLLPGELAKHA-VSEGTKAVTK
H2B Zebrafish               RIAGEASRLAHYNKRSTITSREIQTAVRLLLPGELAKHA-VSEGTKAVTK
H2B Rainbow Trout           RIAGESSRLAHYNKRSTITSREIQTAVRLLLPGELAKHA-VSEGTKAVTK
Potential H2B S. lalandi    RIX-RGSVTAGALQQEIHHHLQGDPDRRPPAAARRAGRARRVRGHQGLTK
                            **  ..*  *   ::.     : :.  *   ... * :*   .* :.:**

H2B Tetra                   YTSSK
H2B Zebrafish               YTSSK
H2B Rainbow Trout           YTSSK
Potential H2B S. lalandi    YTSSK
                            *****
```

Supplementary Figure 7: Potential H2B protein sequence obtained from the *S. lalandi* transcriptomic library aligned against a rainbow trout (*Oncorhynchus mykiss*; Accession umber, P69069.2), tetra (*Astyanax mexicanus*; Accession number, XP_007253844.1) and zebrafish (*Danio rerio*; Accession number, NP_001013481) H2B protein.

Haemoglobin Derived Alignment

```
Potential HbβP S. lalandi     -GXXEDAERAAIISLWGKIDVGEIGPQALTRLLIVYGQGERHFTTFGNVS
HbβP Japanese Amberjack       MVDWTDAERAAITSLWGKIDVGEIGPQALTRLLIVYPWTQRHFTTFGNVS
HbβP Mandarin Fish            MVKWTDAERSAITSLWGSIDVGEIGPQALSRLLIVYPWTQRHFSSFGNIS
                                   ****:** ****.***********:******   :***::***:*

Potential HbβP S. lalandi     TNAAILGNPKVAQHGKTVMGGLENAVKNLDDIKNTYAKLSRMHSEKLHVD
HbβP Japanese Amberjack       TNAAILGNPKVAQHGKTVMGGLENAVKNLDDIKNTYAKLSRMHSEKLHVD
HbβP Mandarin Fish            TNAAILGNPKVAHHGKVVMGGLENAVKNMDDIKNAYAKLSVMHSEKLHVD
                              ************:***.***********:*****:***** *********

Potential HbβP S. lalandi     PDNFRALAECISVCVAAKFGKQVFTADVQEAWQKFLSVVVSALGRQXHXG
HbβP Japanese Amberjack       PDNFRALAECISVCVAAKFGKQAFTADVQEAWQKFLSAVVSALGRQYH--
HbβP Mandarin Fish            PDNFRLLAECISVCVAAKFGPSVFTADAQEAWQKFLAVVVSALGKQ----
                              ***** ************** ..****.********:.******:*

Potential HbβP S. lalandi     SXDAVHPSK
HbβP Japanese Amberjack       ---------
HbβP Mandarin Fish            ---------
```

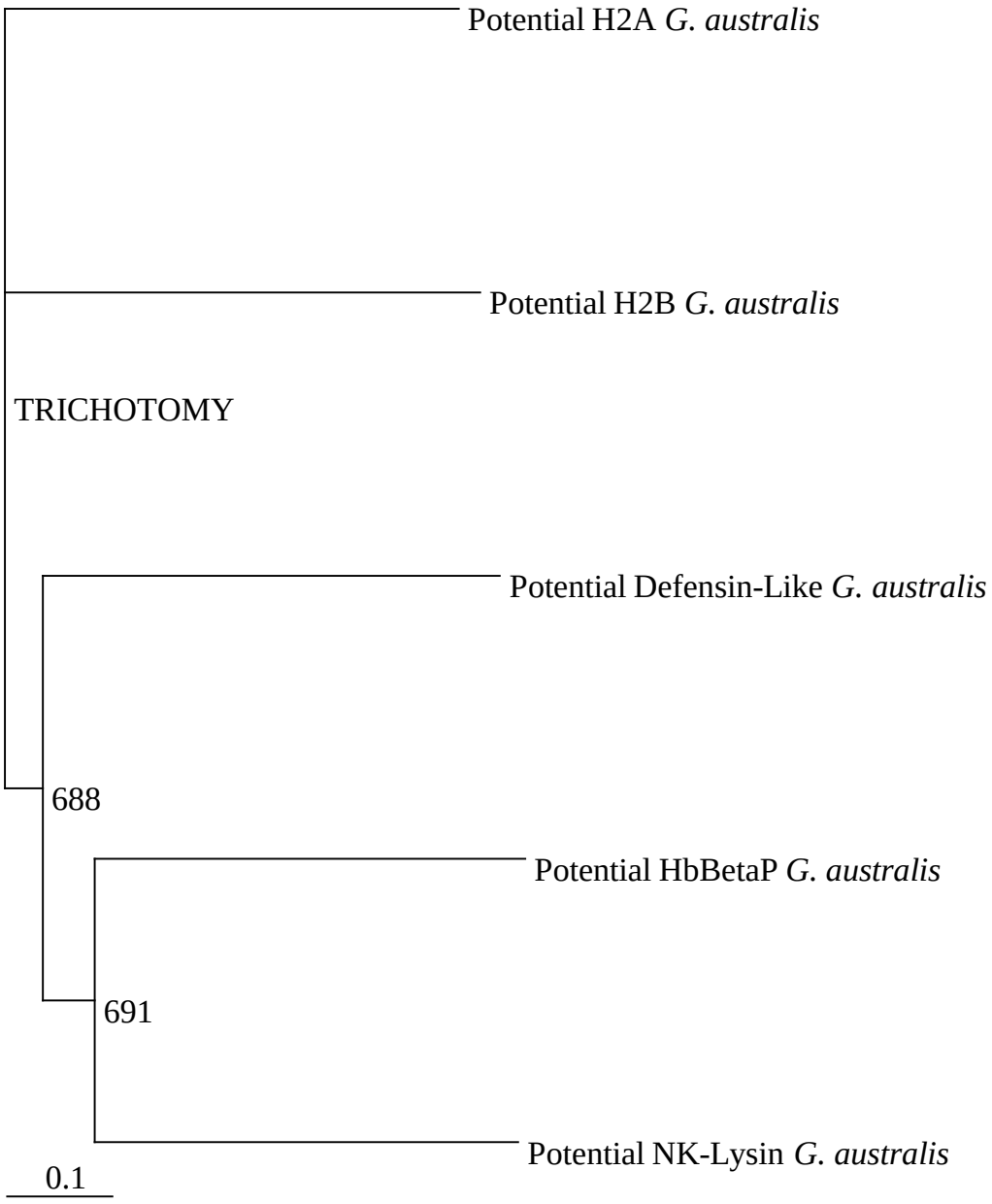
Supplementary Figure 8: Potential haemoglobin derived protein sequence obtained from the *S. lalandi* transcriptomic library aligned against a Japenese amberjack (*Seriola quinqueradiata*; Accession number, Q9PVM1.3) and mandarin fish (*Siniperca chuatsi*; Accession number, AAY79276.1) haemoglobin derived protein.

NK-Lysin Alignment

```
Potential NK-lysin S. lalandi     --------------------------------------------------
NK-Lysin Pufferfish               ------MATSSILLLCILVTCSVWTVQARNLKV-STDDDDEDQDELAIEA
NK-lysin Channel Catfish          MFWNLLVASFFIGSACAMHMEYLRVDSAEELLDGSLDSTDEDEDLAMSET


Potential NK-lysin S. lalandi     --------------KXIKKIIGKNTTAEEVKSKLNTVCNEIGLLKDLCRK
NK-Lysin Pufferfish               GRLPGVCWACKWALKKVKIIIGNNSNSEAIKAKLMSICNQIGLLKSLCRK
NK-lysin Channel Catfish          QLLPGACWACQWAMKKVKKQLGNNPTVDIIKAQLKKVCNSIGFLRGLCKK
                                               * :*  :*:*.. : :*::* .:**.**:*:.**:*

Potential NK-lysin S. lalandi     FVKKHLGELIEELTTSDDVRMICVNTKACKPKELSHLIFYPXXXDXXXXX
NK-Lysin Pufferfish               FVTKHLGVLIEELTTSDDVRTICVNVKACKPKELEELFQSGFSSQLEMNE
NK-lysin Channel Catfish          MINKYLDTLVEELSTTDDPTTICGNLGICKSLSMLELFQAFPQHHKQI--
                                  ::.*:*. *:***:*:**   ** *   **. .: .*:      .

Potential NK-lysin S. lalandi     XXXXPC
NK-Lysin Pufferfish               YA----
NK-lysin Channel Catfish          ------
```

Supplementary Figure 9: Potential NK-lysin protein sequence obtained from the *S. lalandi* transcriptomic library aligned against a channel catfish (*Ictalurus punctatus*; Accession number, NP_001187137.1) and pufferfish (*Takifugu rubripes*; Accession number, XP_003962755.1) NK-lysin protein.

Appendix VI - *G. australis* Phylogenetic Tree



Supplementary Figure 10: Phylogenetic tree showing all identified *G. australis* sequences from the RNA-seq transcriptomic library search.

Appendix VII - *G. australis* Alignments

Haemoglobin Derived Alignment

```
Potential HbβP G. australis          ------------TNKPMRKSGCG-------HPG----EVLHHHPPLLQA
Haemoglobin Chain A River Lamprey    PIVDSGSVAPLSAAEKTKIRSAWAPVYSNYETSGVDILVKFFTSTPAAQE
                                               ::*.  :*. .        .*      :.  .*  *

Potential HbβP G. australis          FFPKFKGMTSAEDLKKSADVRWHAERIINAVNDAVASMDDTEKMSMKLTQ
Haemoglobin Chain A River Lamprey    FFPKFKGMTSADQLKKSADVRWHAERIINAVNDAVASMDDTEKMSMKLRD
                                     ***********::*********************************** :

Potential HbβP G. australis          LSTKHANSFQVDPQYFKV------------------------------
Haemoglobin Chain A River Lamprey    LSGKHAKSFQVDPQYFKVLAAVIADTVAAGDAGFEKLMSMICILLRSAY
                                     ** ***:***********
```

Supplementary Figure 11: Potential haemoglobin derived protein sequence obtained from the *G. australis* transcriptomic library aligned against a river lamprey (*Lametra fluviatilis*; Accession number, P02207.2) haemoglobin derived chain A protein.

Histone Derived H2A Alignment

```
Potential H2A G. australis           ---TPSTCGRS-------TTRXGX---VGRVHRLLRKGNYAERVGAGAPV
H2A Zebrafish                        MSGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPV
                                          .* *::       ::* *    **********************

Potential H2A G. australis           YLAAVLEYLSAEILELAGNAARDNKKTRIIPRHLQLAVRNDEELNKLMGG
H2A Zebrafish                        YLAAVLEYLTAEILELAGNAARDNKKTRIIPRHLQLAVRNDEELNKLLGG
                                     *********:**************************************:**

Potential H2A G. australis           VTIAQGGVLPNIQAVLLPKKTSQAXXXNXXXXXXXPIHNGSFRAT
H2A Zebrafish                        VTIAQGGVLPNIQAVLLPKKTGQAAASSGKSGKKGSSQSQEY---
                                     **********************.**   .       . :. .:
```

Supplementary Figure 12: Potential H2A protein sequence obtained from the *G. australis* transcriptomic library aligned against a zebrafish (*Danio rerio*; Accession number, NP_957367.1) H2A protein.

Histone Derived H2B Alignment

```
Potential H2B G. australis           ILPRQRALGRPRLPPPKRAQRRL-PRLPAKTQRSDATAERRATPFTSTR-
H2B African Clawed Frog              -MP---ELGKSALAPKKGSKKAV-TKAQKKDGKKRKRSRKESYSVYVYKV
                                      :*    **:. *.* * ::: : .:   *  :.   :.:.: ..   :

Potential H2B G. australis           -SRFTPTPXISSKAMSIMNSFVNDIFERIASEASRLAHYNKRSTRTX---
H2B African Clawed Frog              -LKQVHPDTGISSKAMGIMNSFVNDIFERIAGEASRLAHYNKRSTITSREI
                                      .:. * . ******.**************.************* *

Potential H2B G. australis           -----------------SRGXXAIRYRPSSRPK
H2B African Clawed Frog              -QTAVRLLLPGELAKHAVSEGTKAVTKYTSAK--
                                                     *.*  *:   .*::
```

Supplementary Figure 13: Potential H2B sequence obtained from the *G. australis* transcriptomic library aligned against a African clawed frog (*Xenopus laevis*; Accession number, NP_001086753.1) H2B protein.

NK-Lysin Alignment

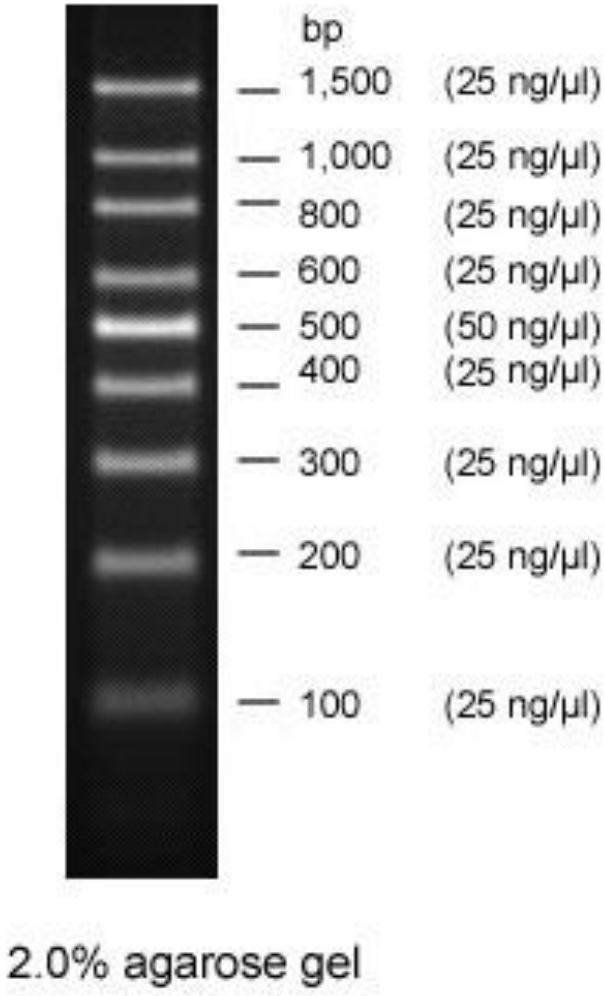
```
Potential NK-Lysin G. australis     ------------------------------------------------
Saposin Common Canary               MARPLILGLLSLLGLLAAAVASPVLWQKECAKGPEVWCQSIRTASQCGAL

Potential NK-Lysin G. australis     ----------------------------------------VKAYLDSACS
Saposin Common Canary               KHCQQNVWNKPAVSSIPCDLCKELVTVAGKILKDNGTEDEIRSYLEKTCE
                                                                            :::**:.:*.

Potential NK-Lysin G. australis     MLPDPGLVSQCRELVENYLDTVIDMLKQEMD-PSVVCTALQVCKTQQSV-
Saposin Common Canary               FLPDPGLVSECKEIVDSYLPTIMDMIKEELDKPEVVCSALALCHSLQKHL
                                    :********:*:*:*:.** *::**:*:*:* *.***:** :*:: *.

Potential NK-Lysin G. australis     ------------------------------------------------
Saposin Common Canary               AAMKLQKQLQTNKIPELDFSELASPFMANVPLLLYPQDKPKQKPKASGDV

Potential NK-Lysin G. australis     ------------------------------------------------
Saposin Common Canary               CQDCIQLVTDVQEAVKTNSSFVKSLVAHAKEECDRLGPGMSDMCKSYISE

Potential NK-Lysin G. australis     ------------------------------------------------
Saposin Common Canary               YSDLAIQMMMHMKDQQPKDICAMVGFCSSVKSVPLQPLVPAQVVHEVKVE

Potential NK-Lysin G. australis     ------------------------------------------------
Saposin Common Canary               IVEKAAVQEKTFSLCEICETMVKEVTGLLESNKTEEEIVHEMEVVCHLFP

Potential NK-Lysin G. australis     ------------------------------------------------
Saposin Common Canary               GSVKDQCKDFIEVYGQAVIDMLLEATNPEAVCAMLKCCAASKLPQQPVVV

Potential NK-Lysin G. australis     ------------------------------------------------
Saposin Common Canary               KPAGGFCDICKMVVAYADKELEKNATTAEIEALLEKVCHFLPESVSEQCV

Potential NK-Lysin G. australis     ------------------------------------------------
Saposin Common Canary               QFVEQYEPVVVQLLAEVMDPTFVCTKLGVCESAKEPLLGNDACVWGPGYW

Potential NK-Lysin G. australis     ----------------------
Saposin Common Canary               CKNMDTAAQCNAVDHCKRHVWN
```

Supplementary Figure 14: Potential NK-lysin protein sequence obtained from the *G. australis* transcriptomic library aligned against a common canary (*Serinus canaria*; Accession number, XP_009097109.1) saposin protein.

Appendix VIII - GenScript Ladder

Genscript 100bp DNA ladder

http://www.genscript.com/molecule/M102O-100_bp_DNA_Ladder.html



Appendix IX - Real-Time PCR Raw Data

	B-actin	GAPDH	Geometric Mean	Moronecidin	Hepcidin
Gill 1	18.66	18.91	18.78	15.5	33.99
Gill 1	18.6	18.99	18.79	15.44	33.46
Gill 2	18.47	18.77	18.62	15.8	35.78
Gill 2	18.47	18.68	18.57	15.76	33.82
Gill 3	18.37	18.54	18.45	15.81	34.49
Gill 3	18.35	18.54	18.44	15.81	33.87
Liver 1	25.16	22.38	23.73	24.4	34.53
Liver 1	25.07	22.44	23.72	23.84	31.15
Liver 2	24.61	21.95	23.24	24.65	32.35
Liver 2	24.68	22.06	23.33	24.71	32.3
Liver 3	25	22.47	23.70	24.44	33.57
Liver 3	25.06	22.36	23.67	24.37	32.5
Spleen 1	18.26	19.29	18.77	15.3	38.67
Spleen 1	18.25	19.34	18.79	15.36	37.84
Spleen 2	18.2	19.37	18.78	15.37	36.95
Spleen 2	18.22	19.26	18.73	15.25	36.87
Spleen 3	17.97	18.96	18.46	15.37	37.39
Spleen 3	17.98	18.73	18.35	15.2	36.96

Appendix X - Real-Time PCR Data Analysis

Gill Tissue

	B-actin	GAPDH	Geometric mean	Moronecidin	Tag-HK	Mean	**RE**	Hepcidin	Tag-HK	Mean	**RE**
Gill 1	18.66	18.91	18.78	15.5	3.28	3.32	**9.98**	33.99	-15.21	-14.94	**0.000032**
Gill 1	18.6	18.99	18.79	15.44	3.35			33.46	-14.67		
Gill 2	18.47	18.77	18.62	15.8	2.82	2.82	**7.05**	35.78	-17.16	-16.20	**0.000013**
Gill 2	18.47	18.68	18.57	15.76	2.81			33.82	-15.25		
Gill 3	18.37	18.54	18.45	15.81	2.64	2.64	**6.23**	34.49	-16.04	-15.73	**0.000018**
Gill 3	18.35	18.54	18.44	15.81	2.63			33.87	-15.43		
						Average	**7.75**				**0.000021**
						SE	**1.14**				**0.000006**

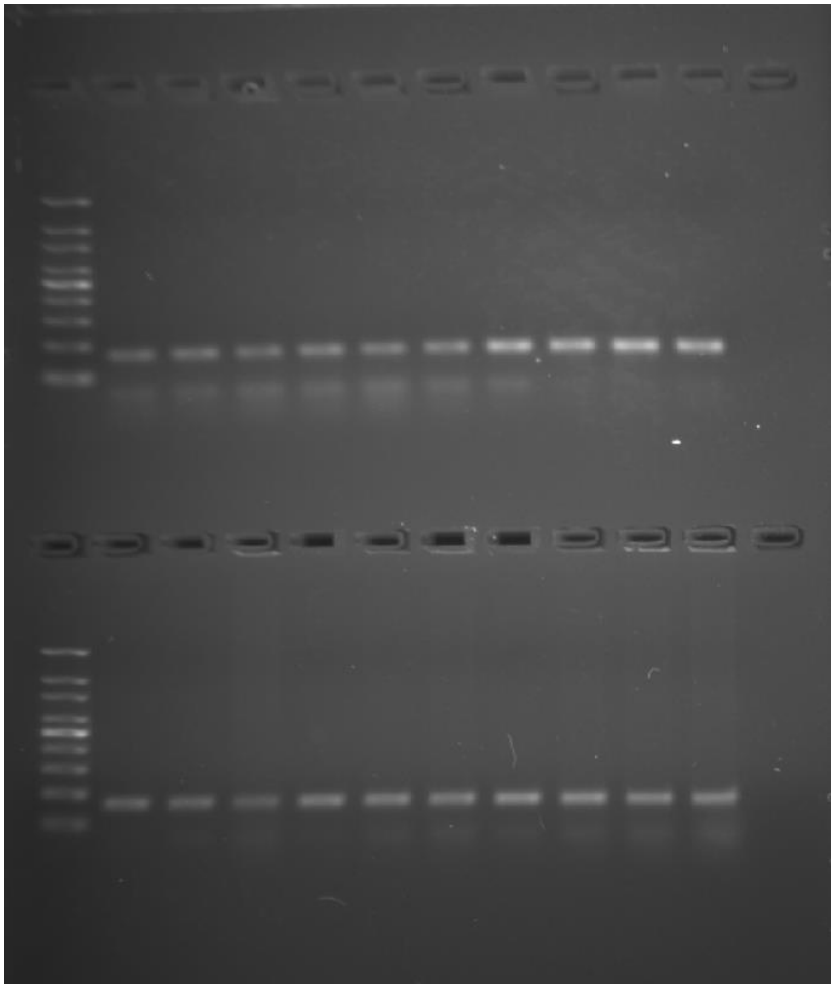
Liver Tissue

	B-actin	GAPDH	Geometric mean	Moronecidin	Tag-HK	Mean	RE	Hepcidin	Tag-HK	Mean	**RE**
Liver 1	25.16	22.38	23.73	24.4	-0.67	-0.40	0.76	34.53	-10.80	-9.12	**0.001802**
Liver 1	25.07	22.44	23.72	23.84	-0.12			31.15	-7.43		
Liver 2	24.61	21.95	23.24	24.65	-1.41	-1.39	0.38	32.35	-9.11	-9.04	**0.001903**
Liver 2	24.68	22.06	23.33	24.71	-1.38			32.3	-8.97		
Liver 3	25	22.47	23.70	24.44	-0.74	-0.72	0.61	33.57	-9.87	-9.35	**0.001534**
Liver 3	25.06	22.36	23.67	24.37	-0.70			32.5	-8.83		
						Average	**0.58**				**0.001746**
						SE	**0.11**				**0.000110**

Spleen Tissue

	B-actin	GAPDH	Geometric mean	Moronecidin	Tag-HK	Mean	RE	Hepcidin	Tag-HK	Mean	**RE**
Spleen 1	18.26	19.29	18.77	15.3	3.47	3.45	10.91	38.67	-19.90	-19.48	**0.000001**
Spleen 1	18.25	19.34	18.79	15.36	3.43			37.84	-19.05		
Spleen 2	18.2	19.37	18.78	15.37	3.41	3.44	10.89	36.95	-18.17	-18.16	**0.000003**
Spleen 2	18.22	19.26	18.73	15.25	3.48			36.87	-18.14		
Spleen 3	17.97	18.96	18.46	15.37	3.09	3.12	8.69	37.39	-18.93	-18.77	**0.000002**
Spleen 3	17.98	18.73	18.35	15.2	3.15			36.96	-18.61		
						Average	**10.16**				**0.000002**
						SE	**0.74**				**0.000001**

Appendix XI - PCR Screen



Supplementary Figure 15: Gel image showing a PCR screen on ten transformed *E. cloni* colonies, none of them show the presence of a genetic insert.