THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Immune defence mechanisms of barramundi (*Lates calcarifer*) peripheral blood against Streptococci

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BSc. (hons)

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2016

The School of Biological Science

Abstract

IMMUNE DEFENCE MECHANISMS OF BARRAMUNDI (*Lates calcarifer*) PERIPHERAL BLOOD AGAINST STREPTOCOCCI

Aquaculture now supplies more than half of the fish used for human consumption and is a major contributor to global food security. As aquaculture growth must be sustained to continue to supply increasing population-driven demand for food fish, efficiency must be increased. In spite of major inroads in disease control through mass vaccination of farm fish, bacterial diseases continue to cause substantial losses, particularly in tropical and warm-temperate regions where aquaculture is expanding fastest. To develop new vaccines, and improve existing vaccines for effective disease control in these regions, an improved understanding of the mechanisms of pathogen immune evasion and dissemination within the host may identify new vaccine targets. *Streptococcus agalactiae* and *Streptococcus iniae* are significant pathogens of warm and temperate farmed and wild fish. Both have a broad host range, express a polysaccharide capsule as a major virulence determinant and cause similar pathologies characterized by rapid sepsis, followed by meningitis and death. *S. iniae* causes significant mortality in barramundi, *Lates calcarifer*. Interestingly, *S. agalactiae* ST261, although causing mortality in wild fish along the Queensland coast, does not appear to cause mortality in barramundi farmed in close proximity. This difference provides a basis for comparison, in order to explore the requirements for blood colonization and sepsis by *S. iniae* in barramundi, and to increase our understanding of sepsis in fish.

S. iniae grew rapidly in barramundi blood, doubling in less than 30 minutes in a whole blood bactericidal assay. In contrast, *S. agalactiae* was unable to multiply. Moreover, *E. coli* DH5α was completely killed in barramundi blood during the same incubation period, suggesting

that antibacterial humoral and cellular immune defences were functional within the blood bactericidal model. A capsular defective strain of *S. iniae* also survived in the blood-bactericidal assay, however the rapid proliferation was reduced, suggesting that while the capsule is important for bacterial colonisation it is not the only means used to evade blood-borne defences. As gram-positive pathogens are particularly susceptible to lysozyme due to the dominance of muramic acids in the cell wall, the role of lysozyme was investigated. Lysozyme levels in barramundi serum and plasma were undetectable in a lysis assay using *Micrococcus lysodeitikus*, indicating very low levels of circulating lysozyme in healthy barramundi.

The first responder immune cells in blood are neutrophils and these are critical in the prevention of sepsis; for example, granulocytopenia (circulating granulocyte deficiency) is associated with increased susceptibility to *Escherichia coli* K1 and *Klebsiella pneumoniae* sepsis in neonates, and this can be partially repaired by injection of granulocyte colony stimulating factor (G-CSF) to increase neutrophil numbers (Deshmukh *et al.*, 2014). This critical role in the prevention of sepsis implies that pathogens that cause sepsis must therefore be capable of evading or subverting neutrophilic response. As neutrophils are short-lived and previous work on fish neutrophils has focused on populations derived from hematopoietic tissues (head-kidney), cells that are likely to be immature in their development, a novel procedure for effective enrichment of mature neutrophils from barramundi peripheral blood was devised. Use of a dextran layer during Percoll density gradient separation effectively removed reticulocytes and erythrocytes from barramundi blood and enriched neutrophils to around 40-50% in the resulting cell suspensions. These mature enriched neutrophils did not release reactive oxygen species (ROS) or

myeloperoxidase (MPO) in response either Streptococcus species tested at an MOI of 1 but showed functionality by response to PMA and CaI positive control. Production of neutrophil extracellular traps (NETs) was investigated in plate-based DNA release assays. There was a low level of DNA release in response to both capsulated and acapsular strains of *S. iniae* and *S. agalactiae*, but microbial nucleases did not appear to play a role in evasion of NETs or neutrophils. Moreover, *S. iniae* and *S. agalactiae* did not kill or induce apoptosis in barramundi peripheral blood neutrophils. However, both capsular and acapsular strains of *S. iniae*, but not *S. agalactiae*, bound to mature peripheral blood neutrophils in an actin-independent manner but were not phagocytosed. This suggests that *S. iniae* may recruit neutrophils, but block activation and subsequent phagocytosis. It may be that *S. iniae* co-opts the innate ability of neutrophils to pass through tight junctions in order to cross the blood brain barrier and cause meningitis. Future work should focus on the mechanisms by which this key bactericidal process in peripheral blood neutrophils is blocked by *S. iniae*, as the factors involved in neutrophil interaction are potentially targets for vaccination against *S. iniae* in farmed fish.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

A reliable method for enrichment of neutrophils from peripheral blood in barramundi (*Lates calcarifer*).

Masterman KA, Barnes AC.

Fish Shellfish Immunol. 2016 Sep 15;58:174-176. doi: 10.1016/j.fsi.2016.09.028

PMID: 27640156

Publications included in this thesis

No publications included.

Contributions by others to the thesis

No contributions by others.

Statement of parts of the thesis submitted to qualify for the award of another degree

None.

Acknowledgements

Thank you to the Australian Postgraduate Association for my scholarship, which made it financially possible for me to do this PhD at all.

Thank you to Andy and the Barnes lab for the help, support, coffees and comradery. Special big thanks to Emma, a great office buddy and Jerome, always happy to help, all the time. Much appreciation to Emma and Sheridan for thesis editing support.

In memorial to Jared Purton, I wish I could have shared this bumpy journey and all its travails with you, sure could have done with bouncing ideas off you.

Biggest hugs and thanks of all to Andrew, and my parents, for all the support and encouragement along the way. Couldn't have done it with Andrew, who has just been a fabulous dive buddy, sounding board and who is great at talking me down when I'm going loopy. You make my life happier and easier and I appreciate it all.

Between submitting my thesis for assessment and receiving the comments back, Andrew died on a tragic scuba diving accident. My loss is enormous, and I dedicate this thesis to him as it simply wouldn't have been completed without him.

Keywords

Cell biology, innate immunity, neutrophil, aquaculture, *Streptococci*, teleost, vaccines

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 060601, Biochemistry and Cell Biology, 80%

ANZSRC code: 060605, Microbiology, 20%

Fields of Research (FoR) Classification

FoR code: 0601 Biochemistry and Cell Biology, 80%

FoR code: 0605 Microbiology, 20%

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List of Abbreviations used in the thesis

CL	Chemiluminescence
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetraacetic Acid, disodium salt
FBS	Foetal bovine serum
FITC	Fluorescein Isothiocyanate Isomer I
GAS	Group A *Streptococcus*
GBS	Group B *Streptococcus*
HBSS	Hanks' Balanced Salt Solution
HK	Head-Kidney
LPS	Lipopolysaccharide
MOI	Multiplicity of Infection
OD	Optical Density
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PMA	Phorbol Myristate Acetate
PTG	Peptidoglycan
QLD	Queensland
ROS	Reactive Oxygen Species
UQ	The University of Queensland

Streptococcal pathogenesis in barramundi (*Lates calcarifer*): the host innate immune response and how *Streptococci* circumvent them.

CHAPTER ONE: General background introduction

1.1 Aquaculture, food security and sustainability

Globally, the demand for seafood extends beyond that which can be sustainably harvested from wild fisheries. According to the Food and Agriculture Organization of the United Nations (FAO), in the last 30 years, wild caught fisheries increased production ~26% and in parallel aquaculture production increased 92% (FAO, 2014). Globally, finfish, molluscs and crustaceans represents about 17% of animal protein supply. While terrestrial livestock production provides the majority of consumptive protein, issues with greenhouse gases and pollution of water and soil, coupled with soil compaction, increased salinity and reduced fertility continue to make long-term sustainability, at current levels of intensity, questionable. Intensive housing practices and feed production has also driven emerging zoonotic virus and diseases, H5N1 influenza from poultry (Monne *et al.*, 2015), H1N1 influenza from swine (Gray *et al.*, 2007) and Bovine spongiform encephalopathy from cattle (Marbaix *et al.*, 2016). Due to these outbreaks, consumer awareness continues to increase regarding farming practices, combined with the ongoing pressures for space for housing, rather than farming, the aquatic environment continues to provide an accessible solution.

Additional environmental issues with industrialised terrestrial farming include bio-accumulation of pesticides such as organophosphates, carbamates and triazines/ triazoles, which may wash into coastal communities and have been reported to cause thyroid disruption in rodents, birds, amphibians and fish (Rattner, 2009, Brar *et al.*, 2010). This can result in abnormal thyroid activity, which may cause developmental and neurological problems (Boas *et al.*, 2012, Yamauchi *et al.*, 2002). Replacement of potentially harmful agrichemicals (pesticides, insecticides, nematicides,

herbicides and fungicides) is ongoing; however, poor long term monitoring and low socio-economic circumstances may result in population-level effects. A well-known example, which notably affected avian species, is dichlorodiphenyltrichloroethane (DDT), which has been banned since 1972; though USA Environment and Protection Agency (EPA) reported in 1989 the persistent organic has a half-life of 2 – 15 years. DDT has also been detected in fish species, though below maximum levels recommended for consumption (Bagumire *et al.*, 2008). Together, terrestrial farming concerns regarding disease, air, soil and water pollution and environmental degradation further increase pressure on wild caught fish, and aquaculture supply, to provide growing amounts of consumptive protein.

As the global population continues to expand and drives an increase in food production, nutrient sources are under constant pressure. Fish and seafood are low in saturated fats and cholesterol, and are one of the best sources of omega-3 fatty acids, which are important for metabolism, and which mammals cannot synthesis. Aquaculture fills a supply niche, providing sustainably controlled, more cost-effective protein alternatives, projected to reach 80 million tonnes by 2050, to maintain current consumption levels (FAO, 2014). Up to 2006, the average annual growth rate was around 7%, and it has long been on course to overtake wild caught fisheries as a source of seafood (Troell *et al.*, 2014).

While the need for feed increases, aquaculture poses some environmental issues, including ocean pen discharge affecting the local marine environments (Government, 2014). Discharges may include tank particulate, faecal matter, nutrients, feedstock, as well as antibiotics and chemicals. For example, over-nitrification through inadequate waste-water management may change the biodiversity of the benthos (Edgar *et al.*, 2005) and consequently affect other fisheries through food web alteration. Recently in Tasmania, Australia, abalone fisheries claimed that expansion of the salmonid aquaculture industry would adversely affected their yield and promotes disease. In response, The Department of Primary Industries, Parks, Water and Environment (DPIPWE), commissioned a review of the report, and surmised the claims were not supported (Buxton, Council,

2014). It should be noted that according to the Tasmanian Salmonid Growers Association, the industry is almost worth $550 million AUD annually, and all reports pertaining to environmental change should be carefully examined for impartial, unbiased data and conclusions. To address this problem, technological solutions, such as fully contained recirculating aquaculture, and integrated multi-trophic systems, are being adopted, where polyculture of lower trophic organisms such as sea scallops (*Placopecten magellanicus*) and salmon (Parsons *et al.*, 2002), scampi (*Macrobrachium rosenbergii*) and carp (Adhikari *et al.*, 2012) or macroalgae and bivalves are being adopted with increasing frequency (Zhou *et al.*, 2006, Nunes *et al.*, 2003). Polyculture can provide an efficient, cheap and self-contained method to purify over-nitrified water and control algae blooms (Li *et al.*, 2015).

Fish farming may relieve the pressure of land clearance and associated costs, and so is attractive to developing countries, particularly in Africa and Latin America. However, more than 84% of global aquaculture production currently comes from Asia (FAO, 2014). The most commonly aquacultured species of fish are carp, catfish, salmon, striped bass, trout, tilapia and sturgeon. Heavily farmed shellfish include shrimp, oyster, mussels and clams. The most expensive production cost of aquaculture is feedstock, with common ingredients including fishmeal, fish oil, rice, soybean, corn and wheat, which need to be cultivated and / or harvested (Péron *et al.*, 2010). Moreover, grain based feeds are also prone to global price fluctuation, making business profit projections difficult (Rana *et al.*, 2009). Recently, Commonwealth Scientific and Industrial Research Organisation (CSIRO) developed a fishmeal-free prawn feed. The microbial biomass based patented technology claims to boost productivity, producing “bigger, healthier, faster-growing prawns” and has been sold to Ridley Corporation, who aims to develop the product further (Glencross *et al.*, 2014, Glencross *et al.*, 2015). Attention has also focused on a limited water flow biofloc, involving microbial recycling of nutrients that may be utilised as a food supply (Najdegerami *et al.*, 2016, Avnimelech, 1999). It is imperative that science and technology continue to be employed, in order to develop more sustainable

alternatives for aquaculture feed than that made from other fish, or from intensively farmed grains (Naylor *et al.*, 2000).

Another major factor that hinders growth of the aquaculture industry is disease. Increased density of fish stock in tanks may increase their stress, and correspondingly disease prevalence, an issue for open cage aquaculture (Bondad-Reantaso *et al.*, 2005, Castillo-Vargasmachuca *et al.*, 2012). Closed system aquaculture, while controlling the output into the environment, still faces the issues of stress and disease development. Indeed, losses due to disease are estimated to be in the region of 30% of total production globally. In addition to direct losses, antibiotics and pesticides used to control disease may pollute the marine environment and promote antibiotic resistance in bacteria. Antibiotics may have long reaching consequences in driving resistance in microbes, as bio-accumulation in sediment may maintain resistance genes in the environment and promote accumulation and transfer of multiple resistances (Nygaard *et al.*, 1992). Resistance may become an issue for marine bacterial zoonotic diseases, as well as for further treatment of farmed fish. Bacteria resistant to oxytetracycline have been isolated from wild fish, which was attributed to the use of the antibiotics in proximal aquaculture pens (Miranda *et al.*, 2013, Bjorklund *et al.*, 1990).

Vaccination is the most effective form of bacterial disease control in finfish aquaculture (Sommerset *et al.*, 2005) and is vital to maintaining aquaculture productivity. However, there are several bacterial diseases for which vaccination is not 100% effective, or for which effective vaccines have not yet been developed or deployed. Bacterial evolution and consequent escape mutants may therefore result in the need for repeat vaccination, decreasing yield, increasing costs, fish stress and disease susceptibility, and requiring further antibiotic use. Vaccination needs to be cost-effective, ideally a one-off dose, which can protect fish against multiple diseases for the duration of the farming time. This has been achieved over the last two decades with the use of oil emulsion injectable vaccines, universally adopted in salmonid aquaculture (Kuzyk *et al.*, 2001, Press *et al.*, 1995). The common bacterial infections in the salmon industry that have been largely controlled by

vaccination, including vibrosis (*Listonella anguillarum*), cold-water vibrosis (*Allivibrio salmonicida*) (Gravningen *et al.*, 2008), furunculosis (*Aeromonas salmonicida*) (Smith *et al.*, 2000), and bacterial kidney disease (*Renibacterium salmoninarum*) (Sommerset *et al.*, 2005, Salonius *et al.*, 2005). The majority of diseases resulting in salmon aquaculture losses today are from viruses and protozoa. The major pathogens affecting productivity are Infectious Salmon Anaemia (ISA Virus; family *Orthomyxoviridae*), Pancreas Disease (salmonid alphavirus) and Amoebic Gill Disease, caused by the parasite *Neoparamoeba perurans* (Pettersen *et al*., 2015, Weston *et al*., 1999, Thorud *et al*., 1998).

In Australia, aquaculture is a relatively small industry but is growing steadily and has adopted and developed high standards of environmental practice. The farming industry is dominated by pearl oyster, which accounted for around 83% ($61 million) of total aquaculture production by value in 2013 – 14, corresponding to the majority of the mariculture in Western Australia (Government, 2014). In terms of fish farming, Atlantic salmon (*Salmo salar*) farmed in Tasmania is currently worth $300 million AUD and predicted to grown to $500 million AUD by 2020.

Barramundi are currently farmed at a relatively small scale in seawater raceways, freshwater recirculating systems and fresh and brackish water ponds. There are many small scale producers, predominantly in Queensland, but there are also several large scale producers including a recently established marine farm at Cone Bay, in Western Australia, that has a license to expand to 30,000 tonnes production. This will more than double the entire current production market for barramundi in Australia, decreasing the dependence on imported product and allowing tighter monitoring of production conditions. Indeed, barramundi consumed in Australia are primarily obtained from aquaculture, of which ~60% is imported from neighbouring countries such as Viet Nam, Thailand and Indonesia, where they are also native, and farmed on a much larger scale. Therefore, there is substantial scope for increasing local production, where environmental impact can be controlled and disease minimised.

The major disease affecting barramundi during grow-out is a bacterial sepsis caused by *Streptococcus iniae*, a Lancefield untypeable, gram-positive coccus. Currently, disease is prevented by autogenous (custom-made) vaccination (there is no licensed generic vaccine for *S. iniae* in Australia). Although killed autogenous vaccines tend to provide over 90% protection against the strain within the vaccine (Aviles *et al.*, 2013) there are occasional failures resulting from selection of novel serotypes (Millard *et al.*, 2012, Bachrach *et al.*, 2001, Eyngor *et al.*, 2008). The mechanisms, via which pathogenic streptococcal species can cause fatal bacterial sepsis in teleosts, even when the innate and acquired immune defences are in place, are not well understood.

The sentinel innate cells play a pivotal role as the first response barrier against microbes, and by excluding and clearing of infectious agents, they can halt disease progress. The breakdown of the front-line immune defences may be host-mediated, with cells unable to recognise, engulf or degrade pathogens. Alternatively, it may be pathogen driven, with factors produced by the infecting microorganism to neutralise, abrogate or hide itself. This research will seek to define these mechanisms, both from the fish immunity angle, as well as the bacterial side, of immune escape. *Streptococci* are essentially blood-borne pathogens causing systemic sepsis in fish; my investigation will centre on the mechanisms required for bacterial survival and proliferation in blood.

1.2 Fish pathogenic Streptococci

Bacteria cause more than half of all infectious diseases in aquaculture, affecting fresh, salt and euryhaline fish, while the remainder are caused by viruses, parasites and fungi (Pillay *et al.*, 2005). The genus *Streptococcus* comprises of chain-forming gram-positive bacteria subdivided into broad species groups, defined by their ability of the bacteria to oxidise or lyse red blood cells (RBC). α-haemolytic species oxidise RBC, causing the colonies to appear green on blood agar, β-haemolytic species lyse RBC, forming a clear area around the colony and γ-haemolytic do neither. Blood contains over 4000 components including cells, clotting factors, sugars, lipids, vitamins, minerals, hormones,

enzymes, and other proteins (Sjoholm, 1975). This rich environment allows rapid proliferation and dissemination of *Streptococcus* bacteria.

Streptococcal disease in fish was first reported in 1966, though the species was not identified (Robinson *et al.*, 1966). Our species of interest*, S. iniae,* is poorly defined via standard forms of identification and is often misidentified as *S. uberis* or *S. dysgalactiae* subsp. *equisimilis. S. iniae* is prone to misidentified, due to variation in haemolysis between aerobic and anaerobic culture conditions. Simple phlebotomy and blood analysis is a method often used to identify diseases, for example probable liver disease via an increase in serum transaminases (Johnston, 1999), but screening of changes in blood parameters has failed to define streptococcal disease in fish (Chen *et al.*, 2004). Isolation of bacterial genomic DNA and polymerase chain reaction (PCR) for species specific genes remains a rapid, consistent and relatively easy form of identification (Kawata *et al.*, 2004, Mata *et al.*, 2004)

For comparison, *Streptococcus agalactiae* has not been found to be fatal in barramundi, but it has been identified as the causative agent in sepsis and mortality of Queensland Grouper (*Epinephelus lanceolatus*) (Bowater *et al.*, 2012). Both of these bacteria have polysaccharide capsules reported to deter phagocytosis and increase virulence (Locke *et al.*, 2007a). While barramundi are not generally susceptible to disease from *S. agalactiae*, it is likely that they come in contact with the bacteria due to overlap of the farm and wild marine environments, and interchange of water and aquatic organisms therein along the north Queensland coast where these wild outbreaks have occurred.

I contend that by comparing the interactions of these two species with the barramundi innate immune system, the critical mechanisms of pathogenesis of *S. iniae* and the weaknesses in innate defences that it exploits may be more readily identified. As *S. agalactiae* does not appear to cause sepsis or disease in barramundi, I hypothesize that *S. iniae* has specific routes of immune escape that enable proliferation and dissemination, which may result in death of the fish.

1.2.1 Streptococcus iniae

First isolated and identified in 1976 (Pier *et al.*, 1976), *S. iniae* is a leading pathogen in farmed fish and has been isolated in dozens of warm water species. It may cause mass mortality (as high as 50%) in a wide range of wild and farmed species of fish, with symptoms including lesions, meningoencephalitis, and septicaemia (Weinstein *et al.*, 1997, Francis *et al.*, 2014). Other bacteria which may also cause septicaemia in fish include *Aeromonas sobria*, *A. caviae*, *A. hydrophila*, *A. salmonicida*, *Edwardsiella ictaluri*, *Hafnia alvei*, *Photobacterium damselae* subsp. piscicida, *Piscirickettsia salmonis*, *Pseudomonas fluorescens*, *P. plecoglossicida*, *Serratia marcescens*, *S. difficilis* (*S. agalactiae*), *Vibrio alginolyticus*, *V. cholerae* (non-Ol), *V. ordalii* and *V. salmonicida* (Austin *et al.*, 2007). Zoonotic infections have been reported primarily in fish handlers, and infections may manifest as inflammation, bacterial sepsis and toxic shock (Weinstein *et al.*, 1997).

Early vaccines used formalin killed bacteria and were able to elicit antibodies production for six months in rainbow trout (Eldar *et al.*, 1997). The bacteria have since been isolated from wild fish, which have been proposed as a vector for transmission to cage aquacultured fish (Zlotkin *et al.*, 1998). However, West Australian losses in 2006 were observed from cages in fresh water, so marine transmission could not have been a factor. The purported bacterial reservoir was mud and silt (Creeper *et al.*, 2006). Temperature stress has been identified as an immune suppressant in tilapia (Ndong *et al.*, 2007), leading to increased susceptibility to *S. iniae,* as well as water inversion, algal blooms (Creeper *et al.*, 2006) and cage mate aggression (Faisal *et al.*, 1989).

There are several theories about how the bacterium enters the host; the likely pathways are gastrointestinal, nares and / or skin barrier breech (Bromage *et al.*, 2002, Francis *et al.*, 2014). Identification of *S. iniae* can be difficult as it does not express any of the 18 Lancefield antigens most commonly used for streptococcal identification (Lancefield, 1933), and may display misleading or misinterpreted alpha or beta-haemolysis dependent on incubation conditions (temperature, humidity, moisture content of agar). Due to these variable factors, commercial bacterial

identification systems often fail to identify *S. iniae* (Roach *et al.*, 2006). Molecular fragment length polymorphisms (MFLPs) can be used to identify and cluster isolates (Kvitt *et al.*, 2004).

Subdivisions into type I or II further divided the species, based on the ability of the bacteria to break down arginine for use as a source of carbon and energy. Arginine is broken down by the enzyme arginine dihydrolase (AD) and serotype I was classified as AD positive and serotype II as AD negative (Zlotkin *et al.*, 1998). Comparing type I and II in anti-sera agglutination assays, anti-serum raised against AD positive isolates did not cross react strongly with AD negative isolates (Barnes *et al.*, 2003) allowing inference of geographical isolate evolution. However, the AD reaction was shown to be an artefact of the assay methodology employed, rather than lack of enzyme production by the AD negative isolates (Barnes *et al.*, 2003).

The identification of antigenic differences through cross-agglutination led to an understanding of why vaccine failure occurred, and stimulated research into capsular immunogenicity and evolutionary modification of streptococcal species (Aviles *et al.*, 2013, Millard *et al.*, 2012, Bachrach *et al.*, 2001, Eyngor *et al.*, 2008).Typing methods to identify *S. iniae* need to be stringent, as epitope mutations often enable evasion from vaccination derived antibodies (Eyngor *et al.*, 2008, Millard *et al.*, 2012). Identifying stable epitopes is imperative to offer cross-protective measures in aquacultured fish susceptible to repeated *S. iniae* outbreaks.

With the Australian barramundi industry currently under-supplying demand, it is vital to protect barramundi against such epitope escape, thereby maximising long-term health and productivity within the industry. Vaccinations are an environmentally safer option than antibiotics, which can drive antibiotic resistance in and between bacterial species via plasmid transmission (Leclercq *et al.*, 1988). There are currently no antibiotics registered for use in aquaculture in Australia; however, several are used off label when prescribed by veterinarians. Use of antibiotics can be expensive and, given that barramundi develop from fingerlings to plate sized in less than 18 months, they are a non-ideal short term solution to bacterial disease.

Since *S. iniae* is able to proliferate in blood, the question arises as to why neutrophils, the primary immune sentinel cell, do not recognise, engulf and digest the bacteria, restricting early spread. The bacterial resistance may be driven by physical barriers, such as a capsule, or by the production of soluble factors that neutralise microbial identification, engulfment or digestion. Bacterial nucleases have been identified as one such factor, produced by both *Streptococcus* and *Staphylococcus* species (Beiter *et al.*, 2006, Berends *et al.*, 2010, Derre-Bobillot *et al.*, 2013). Although nucleases may provide some level of bacterial escape, secreted or intracellular targets can prove to be complicated vaccine targets if antibody access is limited.

Group A Streptococcus (GAS) vaccines (for humans) often target the stable M protein in a multivalent approach (Kotloff *et al.*, 2004), but this approach was unsuccessful against *S. iniae* (Aviles *et al.*, 2013). A surface immunogenic protein (SIP) was identified as cross-serotype protective in Group B Streptococcus (GBS) (Brodeur *et al.*, 2000, Maione *et al.*, 2005). Whilst cross-serotype protective vaccines have been developed in humans and other animals, work is still required in fish.

Vaccines that are cross-protective amongst species within a genus have been attempted; a formalin-killed *S. agalactiae* vaccine was protective in tilapia (*Oreochromis niloticus*), however a subsequent challenge with *S. iniae* showed that the vaccine offered no cross-protection (Evans *et al.*, 2004). Stable, serotype cross-reactive vaccines are vital for protection against streptococcal infections in fish, where rapid evolution of new serotypes that can lead to vaccine escape and to further unwanted use of antibiotics.

1.2.2 Streptococcus agalactiae

A gastrointestinal bacterium often found living commensally in humans; *S. agalactiae* is β-haemolytic and expresses Lancefield Group B carbohydrate, a peptidoglycan-anchored antigen, defining this species as a Group B *Streptococcus* (GBS). GBS infection or septicaemia may occur when the bacteria switches from a commensal to a virulent pathogenic state. The driving factors behind

this switch are unknown and multi-locus sequence typing (MLST) has not identified common factors of invasive versus non-invasive GBS infections (Davies *et al.*, 2004, Singh *et al.*, 2012). *S. agalactiae* can cause mastitis in cows, meningoencephalitis in fish, and neonatal sepsis and meningitis in humans (Wessels, 1991, Glaser *et al.*, 2002, Yang *et al.*, 2013, Amal *et al.*, 2013). *S. agalactiae* may be separated into ten serotypes, defined by the antigenic nature of the polysaccharide capsule (Glaser *et al.*, 2002, Sheppard *et al.*, 2016). Among the ten serotypes, only types Ia, Ib, II, III and V cause invasive human disease (Glaser *et al.*, 2002, Tettelin *et al.*, 2002, Bellais *et al.*, 2012).

S. agalactiae has been isolated during fish mortalities (serotypes 1a, 1b and III), and identification of the bacteria (serotype III, ST7) in human sewage was thought to indicate an anthroponotic transmission route (Delannoy *et al.*, 2013, Jafar *et al.*, 2008, Suanyuk *et al.*, 2008, Zhang *et al.*, 2013). *S. agalactiae* has been found to adversely infect many wild and farmed fish in both marine and freshwater environments (Robinson *et al.*, 1966, Evans *et al.*, 2006). The bacterium is responsible for high mortalities in aquaculture farms, resulting in large economic losses (Duremdez *et al.*, 2004, Al-Marzouk *et al.*, 2005, Mian *et al.*, 2009). Several reports propose enhanced virulence as being related to increased temperatures, causing mass mortalities in mullet (*Liza klunzingeri*) and tilapia (*Tilapia sp*.) (Al-Marzouk *et al.,* Amal *et al.*, 2010). Another study has suggested that bioaccumulation of heavy metals may cause immunosuppression, enhancing bacterial pathogenesis in Queensland Grouper (Bowater *et al.*, 2012), and mortality caused by GBS is also increased in grouper when ammonia levels are elevated in the water (Delamare-Debouteville *et al.*, 2015).

Multiple underlying factors may all contribute to higher levels of stress, increasing susceptibility. While the exact transmission vector, or natural source, remains under investigation, they are of great concern since multiple deaths have occurred on the Great Barrier Reef, and a cross-species outbreak could cause catastrophic wild fish losses.

Consumption transmission of GBS between fish has been observed, with mortalities of predators ingesting infected prey, both in marine (Evans *et al.*, 2006) and terrestrial environments (Hetzel *et*

al., 2003). This cross-species transmission has the potential to infect many animals, therefore it is important to determine the dominant conserved epitopes that immune cells respond to, and target these for vaccines. By examining GBS genomes isolated from several species, and identifying a panel of conserved antigenic proteins, there is potential to develop a cross-reactive vaccine (Pereira *et al.*, 2010). However, an effective cross-reactive, long term protective vaccine against *S. agalactiae* has not yet been established. Serotype replacement is an issue with streptococcal species (Miller *et al.*, 2011, Mulholland *et al.*, 2012, Millard *et al.*, 2012), so multivalent vaccines of polysaccharide-protein conjugates, which combine epitopes from the most virulent strains, are often most effective (Madhi *et al.*, 2013).

1.2.3 Virulence factors

Like most bacterial pathogens responsible for meningitis, including Meningococcus (*Neisseria meningitis*), Pneumococcus (*Streptococcus pneumoniae*), group B *Streptococcus*, *Listeria monocytogenes* and *Haemophilus influenza*, the major virulence factor in *S. iniae* and *S. agalactiae* is the polysaccharide capsule (Rubens *et al.*, 1987, Moxon *et al.*, 1990). The capsule of *Streptococcus* species is one of several important virulence factors identified in relation to fish infection (Locke *et al.*, 2008). Other noteworthy virulence factors include the M-like protein and streptolysin S (Fuller *et al.*, 2002, Baiano *et al.*, 2008, Locke *et al.*, 2008). Moreover, the enzyme phosphoglucomutase was found to be critical to capsular production and targeted insertional mutagenesis; disruption of the gene caused a decrease in capsule thickness and rigidity of the cell wall (Buchanan *et al.*, 2005). This rendered the mutant more susceptible to anti-microbial peptide attack and blood clearance, making it of interest for vaccine development.

Other proteins have also been assessed as vaccine candidates including *S. iniae* C5a peptidase in channel catfish (*Ictalurus punctatus*), however, protection was considered moderate (Jiang *et al.*, 2015). The immunogenic capsule is composed of high molecular weight saccharide polymers (Tavares-Dias *et al.*, 2009). Synthesis of the *S. iniae* capsule is controlled and accomplished by a suite

of 21 genes (Miller *et al.*, 2005, Lowe *et al.*, 2007). In *S. agalactiae*, the 17 gene polycistronic capsule-operon is continually targeted as the primary vaccine candidate (Yamamoto *et al.*, 1999). Acapsular strains are often still able to cause disease, albeit with significantly altered pathology and reduced morbidity (Millard *et al.*, 2012, Turner *et al.*, 2015, Doran *et al.*, 2003), demonstrating some capsular redundancy in bacterial pathogenesis.

The study of virulence factors in fish streptococcal disease often requires *in vitro* models to break down complex multi-facetted mechanisms and to determine, at a more simple level, what is actually going on. Studies utilising epithelial monolayers divulged that *S. iniae* was able to cross epithelial layers *in vitro* without damaging the cells, despite escaping the phagolysosome and existing in the cytoplasm of epithelial cells (Eyngor *et al.*, 2007). This invasion and transcytosis process was not associated with cellular or tissue structural damage, indicating that bacterial translocation through intact skin to initiate infection may well be a route of entry into the host. GBS mammalian studies also found *S. agalactiae* could grow on, and translocate through, chorion cell culture, indicating that outermost membrane invasion may be a prequel to neonatal GBS infections (Winram *et al.*, 1998, Valentin-Weigand *et al.*, 1997). The ability to move into and between skin layers without triggering an immune response, or causing tissue damage, may well indicate how streptococcal species are so successful at rapid dissemination and disease.

The binding of *S. iniae* to epithelial cells was thought to be mediated by two genes which were identified as homologues of other *Streptococcus* surface anchoring genes (Baiano *et al.*, 2008, Locke *et al.*, 2008, Cheng *et al.*, 2002). Allelic replacement determined M-like protein (simA), but not C5a peptidase (scpI), contributed to adherence of the bacteria to fish epithelial cells (Locke *et al.*, 2008). However, when a challenge model assessed *S. iniae* simA as a vaccine target in barramundi, there was no cross-protection against other farmed fish capsular serotypes (Aviles *et al.*, 2013).

Necrotic tissue destruction caused by streptococcal species is often mediated via pore-forming cytotoxins, in *S. iniae* the specific cytolysin is Streptolysin S (Fuller *et al.*, 2002). However, like many

of the virulence factors identified so far, the binding, translocation, dissemination and disease outcome does not appear to be fully mediated by any one strategy. While Streptolysin S was necessary for tissue damage, it did not contribute to bacterial colonisation or resistance to phagocytosis (Fuller *et al.*, 2002, Locke *et al.*, 2007b).

Despite high variability, the capsule continues to be the major target of research. The assessment of the multi-gene loci and associated enzymes, recently uncovered a capsular associated virulence target coined "*pdi*", the polysaccharide deacetylase of *S. iniae* (Milani *et al.*, 2010). The function of the *pdi* is to remove of acetyl groups from lysine amino acids, altering the structure. Like in many other studies, allelic exchange to abrogate gene function displayed that some bacterial strategies were blunted, whereas others remained unaffected. The loss of *pdi* negatively impacted virulence by decreasing resistance to lysozyme, and reducing bacterial adherence and invasion of epithelial cells. There was no difference observed between the deletion-mutant and wild-type regarding resistance to oxidative killing and resistance to anti-microbial peptides. Factors which are the most important to target for vaccines remain to be defined, but with the increase in bioinformatics tools and genome sequencing, working backwards may well prove a better strategy.

Construction of pan-genomes allowed core gene maps to be constructed across many serotypes (Tettelin *et al.*, 2005), and can direct vaccine targets to the ~80% genes shared by all strains of the species. Targets for *S. agalactiae* vaccines may include virulence factors that assist bacterial cell adhesion (C5a peptidase ScpB, surface immunogenic protein (SiP), pili), tissue invasion (via invasins), resistance to phagocytosis (capsule), and others factors vital to the progress of the bacteria to the blood-brain barrier, which the bacteria cross before causing septic meningitis (Brodeur *et al.*, 2000, Cheng *et al.*, 2002, Nuccitelli *et al.*, 2011). The science of genome-based reverse vaccinology has assisted in determining if these virulence factors may actually make good vaccine candidates. Via bioinformatics screening, bacterial genomes may be examined for potential B cell binding epitopes, and a selection of candidate proteins expressed in *Escherichia coli,* tested for antibody generation

and protection in murine models. This process identified and resulted in the generation of a successful broad strain vaccine for *Meningococcus* group B (MenB) caused by *Neisseria meningitidis* (Seib *et al.*, 2010).

This reverse vaccine screening method for discovery of B cell epitopes has also been utilised for Group B *Streptococcus* (GBS). GBS causes significant health issues to newborn babies, with 0.1 - 0.4% of newborns contracting GBS disease during birth. These infections can cause life-threatening disease including septicaemia, pneumonia and meningitis. Due to this public health concern, the capsular operon of GBS has been extensively studied (Rubens *et al.*, 1987, Toniolo *et al.*, 2015). A four protein vaccine was able to elicit some protection, but not against all strains tested (Maione *et al.*, 2005). While reverse vaccinology offers fast track epitope discovery, a sustained, long term immune memory must be confirmed in the host. Finding highly conserved bacterial antigens, to generate broad immunological coverage against GBS, generates vast interest, given it is the major cause of neonatal GBS disease (CDC, 2000).

1.2.4 Capsule and acapsular mutants

The capsule of streptococcal species is a major virulence factor contributing to colonisation and host evasion of streptococcal species. The outer polysaccharide capsule is a polymer constructed of high molecular weight branched oligosaccharide subunits. N-acetylneuraminic acid (Neu5Ac or sialic acid), is at the outer end of polysaccharide side chains and is critical to the virulence of the capsule (Kean *et al.*, 1966). Sialic acids are derivatives of the monosaccharide neuraminic acid, and must be activated before they can be incorporated into the capsular polymer. Capsular sialic acid is thought to assist microbes evade immune strategies of detection and clearance (Severi *et al.*, 2007). The exact genes and gene products involved in capsular construction remain inconclusive, with redundancy and possible gene duplicates making single gene knockouts ambiguous.

The operon genes have been characterised as regulation and transport (cps A-D) subunit assembly and polymerisation (cpsE-J), sialyl-transferase (cpsK), transport (cpsL) and sialic acid synthesis (neu A-D) (Chaffin *et al.*, 2000). Information on capsular genes and their conservation is important when designing vaccines targeting any one or more of these genes. Through construction of multi-strain pan-genomes, conserved targets may be selected to ensure vaccine coverage and strain cross-reactivity are increased (Medini *et al.*, 2005). Even with as few as six infectious neonatal strains, only an 80% homology was seen, indicating a high level of unique genes within the species (Tettelin *et al.*, 2005, Tettelin *et al.*, 2006). This genomic plasticity has allowed *Streptococcus* to create an advantageous broad range of genetic redundancies, facilitating immune escape (Janulczyk *et al.*, 2010). With increasing interest in reverse vaccinology to identify lymphocyte epitopes from pan genomes, it is this redundancy that can be overridden by targeting core, stable, immunogenic epitopes, which may not have been so easily identified without whole genome sequencing technology.

Through extensive genomic mutation screening of *S. iniae* strains, a pattern of highly mutable genes was observed (Millard *et al.*, 2012). Three amino acid sequence variants were found within *cpsG*, directly associated with vaccine escape, and eight amino acid sequence variants were observed within the highly variable *cpsE*. However, allelic replacement of *cpsE* or c*psG* did not result in a capsular deficient phenotypic susceptible to host immune cell clearance (Unpublished data). This indicates that neither gene is an absolute requirement for capsular biosynthesis. One large fragment deletion mutant encompassing *cpsF* - *cpsM* resulted in a truncated cpsF protein and an attenuated strain of *S. iniae,* indicating a loss of a something vital to the capsular construction and / or virulence (Lowe *et al.*, 2007). Upon further allelic exchange deletion studies (*cpsA* and *cpsY*), it was concluded the large deletion mutant had significant non-capsular genetic differences compared to the wild type, so the loss of virulence could not be defined solely by capsular gene differences. There may be multiple factors involved in streptococcal pathogenesis; even comparison of commensal and

disease-associated strains has not defined the exact nature of what makes one strain lethal and another attenuated (Fuller *et al.*, 2001).

1.3 Teleost Immune development

In order to better understand infectious disease processes, investigation of interactions between pathogens and the host immune system can be very informative. In fish, this can be quite difficult due to the differences in cellular immunity between teleost fish and mammals, in which there are many well developed tools and models available. With so much research focused on mammals, parallels and similarities are sought, though evolutionary divergence occurred 400 million years ago (Carroll, 1988) and the oldest fossil of a ray-finned fish has been dated as being 420 million years old (Alroy, 2013).

In mammals, immune cells are derived from haematopoietic stem cells created in bone marrow, seeding and developing B cells and seeding the thymus to generate T cells (Seita *et al.*, 2010). Blood cell lineages may be termed erythroid, myeloid or lymphoid. Cells which engulf and present antigens are termed Antigen Presenting Cells (APCs) and these include myeloid lineage professional APC dendritic cells (DCs) and macrophages (Mp) (Guermonprez *et al.*, 2002). Also within the myeloid lineage are granulocytic cells that exist as three cell types (in mammals), the neutrophil, eosinophil and basophil, which act as phagocytes and may release granules to kill invaders and abnormal cells, or stimulate the immune system (Geering *et al.*, 2013). These detection and destruction events primarily occur in the secondary lymphoid organs, where antigens are subsequently presented to lymphocytes. In mammals, these secondary sites are the lymph nodes, spleen and mucosal associated lymphoid tissues (van de Pavert *et al.*, 2010).

Contrastingly, fish bones are not hollow and are described as having a ‘woody interior’; they do not contain bone marrow and pluripotent stem cells. The equivalent to bone marrow in bony fish is the anterior kidney, which seeds lymphoid and myeloid cells. Teleost granulocytes are believed to

develop and mature in the kidney, spleen and mucosa, whereas mammalian granulocytes populate the blood (Catton, 1951). The presence of teleost granulocytes varies by fish species and inconsistency of nomenclature based on morphology, ontogeny, and function confounds classification (Ainsworth, 1992). Nonetheless, neutrophils and macrophages form part of the first line of immunological defence in both mammals and teleosts, and these sentinel cells are involved in homeostasis, detection and elimination of abnormal cells and pathogens (Janeway *et al.*, 2001b).

1.3.1 The innate immune system

Teleosts rely heavily on physical barriers including the gills and scales to protect them from pathogens in their immersion environment (Ellis, 2001). Moreover, skin mucus contains lectins, lysozymes, complement proteins, antimicrobial peptides (AMPs) and immunoglobulins to deter pathogenic infiltration (Suzuki *et al.*, 2003, Rombout *et al.*, 1993, Aranishi *et al.*, 1997, Boshra *et al.*, 2006, Fasulo *et al.*, 1993). AMPs are potent, broad spectrum antibiotics which can kill microbes and modulate the immune response. Innate cells, including granulocytes and phagocytic cells, are thought to be the source of AMPs, though localisation, isolation and confirmation is difficult. Some classes of AMPs including the defensins and cathelicidins have been reported to activate both the innate and acquired immune systems, highlighting their potency (Nagaoka *et al.*, 2000, Douglas *et al.*, 2003).

The organs of importance for immune cellular development in teleosts are similar to mammals, being the thymus and spleen, with the addition of the kidney which is sub-divided as anterior (or head-kidney) and the middle kidney (Zapata *et al.*, 1995). The primary site of haematopoiesis differs, in that the teleost anterior kidney is the equivalent of the bone marrow in mammals, seeding both lymphoid and myeloid lineage cells (Catton, 1951). Cells have been identified as analogous to T and B cells, macrophages and granulocytes (Rombout *et al.*, 1997, Trede *et al.*, 1998). Teleost equivalent T cells appear to have sub-populations which specialise in recognition and responses to specific antigens and both T cells and macrophage are needed to co-stimulate B cells to produce antibodies

(dos Santos *et al.*, 2000). The Major Histocompatibility Complex (MHC) and T cell receptor (TCR) have been identified in teleosts and are considered the most primitive versions of these molecules (van Erp *et al.*, 1996, Tian *et al.*, 2013, Nakanishi *et al.*, 2002, Rodrigues *et al.*, 1995). Reagents to isolate or identify these cells and receptors are sparse for lower vertebrates, therefore molecular biological techniques are often utilised.

Innate cells of the myeloid lineage, granulocytes and phagocytes, are vital to the clearance of a diverse range of pathogens via recognition through an array of specific receptors, though these cells have no capacity to form long term memory. Activation of innate immune cells drives release of effector molecules, including the complement cascade, cytokines, chemokines, superoxides, nitric oxides, peroxidases and AMPs (Campos-Perez *et al.*, 2000, Palic *et al.*, 2005). Cellular immunity studies in fish have identified non-specific cytotoxic cells in a variety of fish species including rainbow trout (*Oncorhynchus mykiss*) (Greenlee *et al.*, 1991), common carp (*C.carpio*) (Hinuma *et al.*, 1980), damsel fish (*Dascyllus albisella*) (McKinney *et al.*, 1994) and tilapia (*Oreochromis* spp.) (Jaso-Friedmann *et al.*, 2000, Faisal *et al.*, 1989).

As the first responders to pathogenic signals in multiple teleost species, granulocytes, specifically neutrophils, are vital to the initiation of immunological clearance. Further understanding of how teleost immune cells interact with potential invading microbes, will lead to identification of potential weaknesses, or areas microbes exploit, and therefore, eventually, to more effective vaccines.

1.3.2 The complement system

The complement system is a team of 35 soluble and membrane bound innate immune proteins that sequentially complex, cleave and activate to result in a Membrane Attack Complex (MAC), creating a pore in the surface of an invading microorganism, causing lysis of the target. The complex pathways contain many individual components that may additionally stimulate phagocytic cells, opsonise microbes, stimulate chemotaxis and inflammation, and enhance the binding of antibodies. There are

several pathways which may be activated by different means, and use different components to reach a common convergence point of C5 cleavage via C5 convertase, to release C5b catalysing the formation of the MAC (Janeway *et al.*, 2001a).

In teleosts, it has been discovered that multiple isoforms of components may exist that do not exist in mammals. The C3 component, common to the classical, alternative and lectin pathways, exhibits isoform diversity, hypothesised to expand the host immune recognition repertoire both in trout (*O. mykiss)* (Sunyer *et al.*, 1996) and gilt-head bream (*Sparus aurata*) (Sunyer *et al.*, 1997). While the three complement pathways have been found in many species of fish, this defence seems only to be available to fishes more evolutionarily advanced than jawless fishes such as hagfish (Fujii *et al.*, 1992) and lamprey (Nonaka *et al.*, 1984), which have a C3 homologue but the remainder of the pathway is incomplete.

The classic complement pathway (CCP) is triggered by antibody binding to cell surface ligands, or acute phase proteins which are released in response to inflammation. Additionally, the CCP may be triggered by bacteria, virus and virus-infected cells (Spiller *et al.*, 1998, Alberti *et al.*, 1993, Merino *et al.*, 1998). In a similar fashion, the alternative complement pathway (ACP) possesses many of the same components but is activated by bacteria, fungi, viruses, or tumour cells independently of antibody binding. The lectin binding pathway is also independent of antibody, and is triggered by a complex of mannose binding lectin (MBL) and mannose-binding lectin-associated proteases 1 and 2 (MASP-1 and -2), which are serine proteases.

In teleost, many of these components or homologues have been identified and phylogeny studies suggest C3, C4 and C5 originate from a common ancestral gene, also present in some invertebrates (Al-Sharif *et al.*, 1998). Classic, alternative and lectin binding pathway constituents vary between fish species, but whether components are absent or have not been sought is unknown, given the number of teleost species used as animal models. The common carp (*C. carpio)* has been extensively examined and contains most units of the three pathways (Nakao *et al.*, 2001, Nakao *et al.*, 2006).

The latter components of the complement cascade, which coordinate the formation of the MAC, have been identified in many fish including rainbow trout (*O. mykiss*) (Tomlinson *et al.*, 1993) common carp (*C. carpio*) (Nakao *et al.*, 1996), Japanese flounder (*Paralichthys olivaceus*) (Katagiri *et al.*, 1999) and pufferfish (*Takifugu rubripes*) (Yeo *et al.*, 1997). The complement pathways are an important part of the innate defence, designed to both enhance the innate and acquired response, as well as directly kill microorganisms. Common assays for complement activity include cellular haemolysis (using serum), which demonstrate an intact lytic pathway and complement derived opsonisation, enhancing in turn the phagocytic activity of macrophages. Heat inactivation destroys complement activity, so both heat and non-heat treated serum may be used to determine if lytic activity is complement-driven.

1.3.3 Neutrophils

Neutrophils, the most common granulocyte, are the first responders to acute inflammatory signals and respond via chemotaxis to the cytokine interleukin-8 and complement component C5a (Holland *et al.*, 2004, Huber *et al.*, 1991). Once neutrophils have homed into a site of infection, they defend via phagocytosis, degranulation and release of antimicrobials or via production of Neutrophil Extracellular Traps (NETs). NETs have been identified to be involved in bacterial capture and clearance in a suicidal lytic process. A live cell NET form, involving only mitochondrial DNA, has also been reported (Yousefi *et al.*, 2009). The self-destruction process, which differs from apoptosis (Fuchs *et al.*, 2007), is made up of a web of extracellular DNA cast out like a net to capture and opsonise microbes for immune clearance (Wartha *et al.*, 2008). NETs were first identified via neutrophils from rabbit and human 2004 (Brinkmann *et al.*, 2004) and have since been located and recorded in humans (Guimaraes-Costa *et al.*, 2009, Urban *et al.*, 2006), mice (Berends *et al.*, 2010), cats (Wardini *et al.*, 2010), fish (Palic *et al.*, 2005) and cows (Grinberg *et al.*, 2008).

Since NETs are composed of DNA, histones, elastase, calprotectin and lactoferrin, these extracellular web components may be specifically targeted for degradation (Urban *et al.*, 2009). Bacteria have

been reported to produce nucleases to degrade NETs and escape entrapment (Alghamdi *et al.*, 2005, Buchanan *et al.*, 2006, Beiter *et al.*, 2006, Berends *et al.*, 2010, Seper *et al.*, 2013, Derre-Bobillot *et al.*, 2013). Nucleases work by cleaving the phosphodiester bonds between the nucleotides, the building blocks of nucleic acids, breaking apart the strands. The enzyme may work as an exonuclease, cleaving from the end of the polynucleotide chain, or an endonuclease, cleaving within the chain. Determining the ability of microbes to escape NETs via enzymatic degradation may be useful information when developing vaccine targets. Subsequent neutralisation of bacterially produced nucleases may serve to amplify the effect of NET production and immune clearance.

Neutrophils are stimulated via chemotactic signals to move towards microbes and the maturation status of neutrophils is important, as immature cells lack the receptors to cast NETs (Akashi *et al.*, 2000). Myeloperoxidase (MPO), a peroxidase enzyme predominantly found in azurophilic granules in neutrophils, has been found to be required for NET production. MPO catalyses the production of reactive oxygen species (ROS), via the breakdown of hydrogen peroxide (H_2O_2) (Metzler *et al.*, 2011). MPO is not only released by neutrophils for microbial degradation, but can also bind to cell surface integrins CD11b and CD18 (MAC-1 complex) to activate the polymorphs (Bochev *et al.*, 1993). An inherited deficiency in MPO can lead to increased susceptibility to microbial infection, however the lack of MPO seems most critical only when other immune defences are overwhelmed (Rydell-Tormanen *et al.*, 2006, Caruso *et al.*, 2002). In humans, NET-dependent inhibition of *Candida albicans* is the key form of early defence, and individuals with MPO-deficient neutrophils lack the ability to control such infections efficiently (Metzler *et al.*, 2011).

While most agree both NADPH oxidase and MPO are required for NET release (Kirchner *et al.*, 2012, Parker *et al.*, 2012) the necessity of ROS is variable as ROS-independent mechanisms of have been reported (Gabriel *et al.*, 2010, Menegazzi *et al.*, 1992). Bacteria may combat the effects of ROS degradation with mucoidal exopolysaccharides, which can also inhibit neutrophil chemotaxis and interfere with the neutrophil derived ROS (Carulli *et al.*, 1995). An extracellular polysaccharide

capsule, or a positive surface charge, may also render bacteria resistant to NET mediated killing (Wartha *et al.*, 2007), highlighting the importance of the capsule to immune escape.

1.3.4 Neutrophil function

Engagement of neutrophils is initiated by release of chemoattractants, which draw neutrophils to the site of microbial inflammation. Mature cells move through the bloodstream and into the tissue where required. Interleukin-8 (IL-8) is a prominent neutrophil chemoattractant in humans (Huber *et al.*, 1991) and endothelial cells rapidly produce this cytokine upon *in vitro* stimulation with LPS, IL-1, or TNF-alpha (Strieter *et al.*, 1988, Strieter *et al.*, 1989, Huber *et al.*, 1991). Inaugural tissue activation is regulated by lectin adhesion molecule-1 (LECAM-1), which is subsequently shed once neutrophils are engaged (von Andrian *et al.*, 1991, Huber *et al.*, 1991). A secondary tier of glycoprotein receptors promotes downregulation of endothelial cell-to-cell contact components, including vascular endothelial cadherin, alpha-catenin, beta-catenin, and gamma-catenin (Del Maschio *et al.*, 1996). This architectural rearrangement allows neutrophils to pass through cell barriers, towards an inflammatory target. IL-8, also known as CXCL8 or Neutrophil Activating Peptide-1 (NAP-1), continues to guide neutrophils through the endothelial matrix, towards the site of inflammation. Mammalian IL-8 expression has been linked to TLR binding, and has been found to be more than just a neutrophil chemokine; it is also involved in activation of basophils, eosinophils and resting T cells (Baggiolini *et al.*, 1989, Peveri *et al.*, 1988). IL-8 may also induce respiratory burst and degranulation, and has been identified as a primary pro-inflammatory mediator of psoriasis and gingivitis (Bruch-Gerharz *et al.*, 1996, Kim *et al.*, 2012). It is of note that mice and rats lack an IL-8 homolog, murine KC (CXCL1), MIP-2 (CXCL2) and LIX (CXCL5-6) are considered functional homologues, and activate neutrophils similarly (Hol *et al.*, 2010).

A fish analogue of IL-8 was first identified in flounder (*Paralichthys olivaceous*), where upregulation was detected in head-kidney and spleen of *in vivo* LPS injected fish and *in vitro* in stimulated leukocytes (Lee *et al.*, 2001a). Further research identified an IL-8 homologue in rainbow trout

(*Oncorhynchus mykiss*) although it was not inducible by LPS (Laing *et al.*, 2002). Subsequently, homologues were identified in haddock (*Melanogrammus aeglefinus*) (Corripio-Miyar *et al.*, 2007), Japanese sea perch (*Lateolabrax japonicus*) (Qiu *et al.*, 2009), Carp (*Cyprinus carpio* L) (Huising *et al.*, 2003) and many other fish. Further work in carp uncovered two IL-8 like chemokines (Abdelkhalek *et al.*, 2009), and the transcript were linked to separate chromosomes (van der Aa *et al.*, 2010). The two isoforms possess different induction requirements and kinetics, one found in all teleost (CXCL8-l1) and the other (CXCL8-l2) only in carp and zebrafish (*Danio rerio*) (van der Aa *et al.*, 2010, Chen *et al.*, 2013). Cell cultures of carp neutrophils, using zymosan-activated carp serum as a positive control, were matched for chemotactic activity by both CXCL8-l1 and CXCL8-l2, at 200 ng / ml.

Once neutrophils have travelled to the region of microbial interaction, they may undergo oxidative burst, the rapid production of reactive oxygen species (ROS). ROS, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are produced from the rapid metabolism of oxygen upon activation, and can be used to degrade microbes. ROS are an important part of innate immunity, as they non-specifically degrade microbial DNA, damage lipids, enzymes and proteins, which may cause membrane disruption. The production of ROS by neutrophils is highest in acute / early stimulated cells, and the neutrophils' ability to produce ROS drops in the pro-resolving / resolution phase (Havixbeck *et al.*, 2016). Base oxidation of nucleotides, particularly guanine which is a low-potential base, by unstable oxygen, derived anions promotes microbial mutations (Vieira *et al.*, 1990). Many ROS are produced as natural products of cellular metabolism, predominantly for intercellular and intracellular signalling (Boveris *et al.*, 1972). However, catalase and superoxide dismutase neutralise the damage by converting ROS into oxygen and water. Humans with chronic granulomatous may have deficiencies in generating ROS (Deffert *et al.*, 2014), and are more susceptible to infection by a broad range of microbes including *Salmonella enterica*, *Staphylococcus aureus*, *Serratia marcescens*, and *Aspergillus* spp. (Winkelstein *et al.*, 2000). Generation of ROS involves the NOX family NADPH oxidases (Leto *et al.*, 2009), which are proteins that transfer electrons across biological membranes.

The membrane associated NADPH-dependent oxidase complex (NOX2 is only found in neutrophils, other forms exist), donates an electron, therefore reducing oxygen to superoxide anion, hydrogen peroxide, and other free radicals, including hydroxyl radicals (OH^-), singlet oxygen (O_1) or hypochlorous acid (HClO) (Nauseef, 2004). The NADPH oxidase complex exists as a preassembled intracellular complex, associated with the cytoskeleton in unstimulated endothelial cells, and produces a low basal level of ROS (Li *et al.*, 2002). For phagocytic cells, which burst only upon stimulation at a much higher level, recruitment of Rac, a small GTPase, to the phagocyte membrane, triggers induction of NADPH oxidase assembly (Diekmann *et al.*, 1994, Gorzalczany *et al.*, 2000, Clark *et al.*, 1990). Rac is an enzyme that can bind and hydrolyse the purine nucleoside guanosine triphosphate (GTP). The NADPH complex, in addition to Rac, is made up of two membrane bound and three cytosol phagocytic oxidase (phox) components. Initiation of NADPH oxidase activity occurs almost simultaneously with granule degranulation, with a lag of only around 20 seconds (Segal *et al.*, 1980). The complex utilises oxygen to produce the degradative radicals, which are very unstable due to their charge, and seek a more stable configuration, making them strong cidal and cytotoxic agents (Rada *et al.*, 2004).

Hand in hand with ROS production, neutrophils release a unique peroxidase termed myeloperoxidase (MPO), the most abundant protein in neutrophils, making up to 5% of their dry weight (Schultz *et al.*, 1962). Originally MPO was termed verdoperoxiase, as it was found to make pus green (Agner, 1947). The enzyme converts hydrogen peroxide, to hypochlorous acid (HOCl), and by-products chloride anion (Cl^-) during respiratory burst. MPO synthesis begins in the promyelocyte stage of neutrophil development, through to the start of the myelocyte phase. Human monocytes may also contain small amounts of MPO (Nichols *et al.*, 1973). The cationic, tetrameric, glycosylated enzyme is predominantly found in lysosomal or azurophilic granules, and is released into the exogenous surroundings, or into the lysosome, upon degranulation. The lysosomal hemoprotein has

been found to able to degrade bacteria, fungi, viruses and malignant cells, in an oxygen-dependent manner (Nguyen *et al.*, 1997, Klebanoff *et al.*, 1992, Yamamoto *et al.*, 1991, Clark *et al.*, 1981).

While degradative enzymes are vital to host-mediated immunity against microbial invaders, there is potential for self-damage, due to their powerful oxidative potential when controls malfunction. Excess release of MPO (and other ROS), by neutrophils, can lead to tissue damage at sites of inflammation, exacerbating conditions such as human atherosclerosis (Daugherty, 1994) and vasculitis. Small vessel vasculitis is a chronic auto-inflammatory disease mediated by MPO-DNA complexes, and auto-antibodies generated against them (Kessenbrock *et al.*, 2009). Enhanced expression of an upstream transcription factor-binding site from the *MPO* operon has also been linked to acute promyelocytic leukemia, measured by increased levels of MPO mRNA and expression (Reynolds *et al.*, 1997). It would follow that production of such potent degradative products is under fairly rigorous control (Lacy, 2006, Sato *et al.*, 2013).

Detection systems for MPO include the cytochemical stain 3,3'-Diaminobenzidine (DAB), which is two part substrate system, visualised as dark brown peroxidase precipitate in the cytoplasm of fixed cells (Fahimi *et al.*, 1973). This staining system has been frequently used on cytospin slides to confirm presence of MPO in neutrophil granules. There are also MPO monoclonal antibodies available, which have an advantage over the DAB liquid substrate system, of being able to recognize MPO in inactive precursor form (Pulli *et al.*, 2013).

Further mechanisms of microbial containment and degradation utilised by neutrophils include casting out DNA to form extracellular traps (ETs), in a lytic or non-lytic manner, using chromosomal and mitochondrial DNA respectively (Brinkmann *et al.*, 2004, Fuchs *et al.*, 2007). The lytic process involves chromatin (macro-complex of DNA, RNA and proteins), which may be categorised into tightly bound heterochromatin or gene rich transcriptionally active, less condensed, euchromatin. Heterochromatin may be further subdivided into the repetitive structural chromatin termed as constitutive, and non-repetitive silenced genes which are facultative (discretionally transcribed).

Condensed heterochromatin may be released by mechanisms such as histone deacetylation, which is an important process in gene regulation (Kishimoto *et al.*, 2006, Bannister *et al.*, 2011). Histones are the primary protein of chromatin, and are responsible for the tightly wound, condensed structure of chromatin. Facultative heterochromatin may be activated simple by the loss of the tightly condensed structure, which triggers the release of repressive histones. One of the first indicators of ET formation is the loss of distinct differences between the heterochromatin and the euchromatin. As the membranes (both nuclear and vesicle, but not plasma) break down, the chromatin unwinds, and the ET components decondense, and combine in the cytoplasm. Neutrophil elastase (NE) released by the cell remain bound to ETs, and may directly target the outer membrane protein A (OmpA) of gram negative bacteria (Belaaouaj *et al.*, 2000) or other bacterial virulence factors (Weinrauch *et al.*, 2002). NE is a serprocidin, which are serine proteases with microbicidal activity, and others include proteinase-3 and cathepsin G. All three are produced by neutrophils, and found in early granules, primarily the azurophil granules. Upon neutrophil activation, azurophil granules undergo exocytosis and mix with other NET components. The combination of DNA, NE, cathepsin G and histones cumulatively form a sticky web like matrix, to trap and degrade microbes, including bacteria, fungi and yeast.

Anti-microbial peptides (AMPs) are important immune component of NETs, produced to specifically degrade microorganisms, often stored pre-made but inactive within polymorphonuclear leukocytic granules. Phagocytosis of pathogens, combines the phagosome with intracellular lysosomes, forming a toxic environment inside the phagolysosome (Nordenfelt *et al.*, 2011). The microbe is killed by a combination of reactive oxygen species, antibacterial proteins and proteolytic enzymes. AMPs are normally small, <30 kDa, and display cationic and amphipathic properties (containing both hydrophobic and hydrophilic regions), and kill bacteria by disrupting the lipid cell membrane, though some may also target protein and DNA synthesis (Patrzykat *et al.*, 2002, Park *et al.*, 1998a, Otvos *et al.*, 2000). Cathelicidins are a prominent family of AMPs (aka CAMPs) in mammals, which have also

been identified in fish, including hagfish (Uzzell *et al.*, 2003), rainbow trout (*O. mykiss*) (Zhang *et al.*, 2015), Atlantic cod (*Gadus morhua*), Arctic charr (*Salvelinus alpinus*) (Maier *et al.*, 2008a) and Atlantic salmon (*Salmo salar*) (Chang *et al.*, 2006). Cathelicidins are stored in neutrophilic granules as inactive precursors, which are activated via cleavage by NE. This may occur via granule release, or fusion of azurophil and large granules. Inhibitors of NE abrogate antimicrobial activity of CAMPs (Cole *et al.*, 2001, Scocchi *et al.*, 1992). This highlights the importance of NET components, both to directly capture microbes, as well as indirectly, to activate other antimicrobials and innate immune pathways.

As well as CAMPs, other AMPs have been identified in fish including hepcidin (Douglas *et al.*, 2003), defensins (Jin *et al.*, 2010), moronecidin (Bae *et al.*, 2014), misgurin (Park *et al.*, 1997), parasin (Cho *et al.*, 2002), piscidin (Lin *et al.*, 2012) and daxin (Oren *et al.*, 1996). Hepcidins in mammals are heavily involved in regulation and homeostasis of iron metabolism (Kulaksiz *et al.*, 2005). Similar hepcidin-like molecules have been identified in barramundi (Barnes *et al.*, 2011), gilthead seabream (*Sparus aurata* L.), Japanese flounder (*Paralichthys olivaceus*) (Hirono *et al.*, 2005), tilapia (*Oreochromis mossambicus*) (Huang *et al.*, 2007) and common snowtrout (*Schizothorax richardsonii*). The roles of these molecules are specifically as AMPs (Shike *et al.*, 2004, Pereiro *et al.*, 2012). Upregulation of AMP mRNA may be observed in response to stimulation, with a wide variety of Pathogen Associated Molecular Patterns (PAMPs), although DNA or endotoxin contamination of stimuli, such as LPS, may cloud results (Maier *et al.*, 2008b). In rainbow trout, stimulation with pathogenic *Aeromonas salmonicida* caused upregulation of CAMP rtCath1 mRNA simultaneously, the effect on rtCath2 differed, being produced constitutively (Chang *et al.*, 2006). Differential expression of cathelicidins in fish has been linked to toll like receptors (TLRs), PI3-kinase pathways and inflammatory pathways, however, this is dependent on the stimuli used (Broekman *et al.*, 2013, Bridle *et al.*, 2011).

While AMPs promote antimicrobial activity at cutaneous and mucosal interfaces, bacteria may counter the effect of AMPs via the sub capsular M protein (*Streptococcus*), or even by exopolysaccharide factors (*Pseudomonas aeruginosa*) (Cole *et al.*, 2001, Lauth *et al.*, 2009, Foschiatti *et al.*, 2009). The mechanism of action of CAMP inhibitors, is often to nullify NE, which activates CAMPs. While NE drives CAMP activation, it may also cause inflammatory destruction. NE inhibitors (NEI) have been developed to dampen down neutrophil driven tissue inflammation. Chronic obstructive pulmonary disease (COPD), in humans, is driven by NE degradation of extracellular matrix and proteins, destroying lung parenchyma (Kuna *et al.*, 2012, Stevens *et al.*, 2011). Other human diseases, which affiliate NE and inflammatory tissue damage, include cystic fibrosis, rheumatoid arthritis, pulmonary emphysema and asthma (Lee *et al.*, 2001b, Doring, 1999, Tremblay *et al.*, 2003). While AMPs and inflammation strike a delicate balance between microbial defence and inflammatory disease, human deficiencies in neutrophils and NETs experience greater and more virulent bacterial and fungal infections (Metzler *et al.*, 2011, Roongpoovapatr *et al.*, 2010).

Neutrophil extracellular traps (NETs) were first described in 2004 by Brinkman *et al.* (Brinkmann *et al.*, 2004) and subsequently have been described in other cell types. These include macrophages, mast cells and other cells generally termed phagocytes (Chow *et al.*, 2010, von Kockritz-Blickwede *et al.*, 2008). Focusing on NETs, which are the best characterised, these structures can be produced in a lytic manner, using nuclear DNA as the NET backbone, or in a process which does not kill the cell, using mitochondrial DNA. The combination of DNA, NE, cathepsin G and histones create a sticky web to capture and break down microbes. With a broad range of NET stimulants described, a single common causative factor of NETosis is still to be identified.

While most agree, both NADPH oxidase and MPO are required for NET release (Kirchner *et al.*, 2012, Parker *et al.*, 2012), the necessity of ROS is variable, as ROS-independent mechanisms of have been reported (Gabriel *et al.*, 2010, Menegazzi *et al.*, 1992). Bacteria may combat the effects of ROS degradation with mucoidal exopolysaccharides, which can inhibit neutrophil chemotaxis and

interfere with the neutrophil derived ROS (Carulli *et al.*, 1995). An extracellular polysaccharide capsule, or a positive surface charge, may also render them resistant to NET killing (Wartha *et al.*, 2007).

The most commonly used stimulant for NET production is Phorbol-12-myristate 13-acetate (PMA). PMA acts by mimicking a cellular signal that activates several families of protein kinase C (PKC) phosphorylative enzymes, resulting in a signal cascade and eventual NET release (Neeli *et al.*, 2013). Formation of PMA-stimulated NETs is dependent upon nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a membrane bound enzyme complex. This reactive anion can go on to produce a suite of ROS, which can directly degrade pathogens in a phagolysosome, or stimulate NET production (Fuchs *et al.*, 2007). Myeloperoxidase has been found to be required for NET production, as it catalyses ROS production from hydrogen peroxide (H_2O_2) (Metzler *et al.*, 2011).

An alternative NET stimulating pathway, independent of PMA and associated PKC isoforms, has also been identified. Calcium ionophore (CaI) is an antibiotic, which allows divalent cations to be transported across the cell membrane, and is a potent stimulant of NETs (Wang *et al.*, 2009). CaI induces histone deimination, converting the positively charged amino acid arginine to a neutral citrulline. This post translational modification therefore allows alternative folding to occur, releasing the helical DNA structure and associated proteins. This indicates there are several pathways leading to NETosis, and that these pathways are dependent on the stimuli.

Pathogen activation of NETs has identified a range of stimulating bacteria (*Streptococcus spp*, *Aeromonas hydrophila*, *Vibrio cholera, Staphylococcus aureus*) (de Buhr *et al.*, 2014, Brogden *et al.*, 2014, Seper *et al.*, 2013, Malachowa *et al.*, 2013), protozoa (*Besnoitia besnoiti*, *Leishmania spp*) (Munoz Caro *et al.*, 2014, Guimaraes-Costa *et al.*, 2014), mould (*Aspergillus fumigatus*) (Katzenback *et al.*, 2009) and yeast (*Candida albican*) (Urban *et al.*, 2006). Immune components such as IgA, an important mucosal antibody, also trigger NET formation, which has been attributed to ROS production when opsonised particles are engulfed (Aleyd *et al.*, 2014). Complement C5a in

conjunction with cytokines, such as Interferon Alpha (IFN-α) or Granulocyte-macrophage colony-stimulating factor (GM-CSF), may also activate NETs, though these NETs are of a mitochondrial DNA source (Yousefi *et al.*, 2009).

Microbes are recognised by Pattern Recognition Receptors (PRRs) on host cells, detecting Pathogen, Danger or Microbial Associated Molecular Patterns (PAMPs, DAMPs or MAMPs respectively), which are quite simply, molecular motifs specific to microbes. These are not associated with host self, and are recognised as foreign by host cellular receptors. Well known PAMPs include peptidoglycan found in bacterial cell walls, lipopolysaccharide (LPS) found in gram negative bacteria, lipoteichoic acid (LTA), specific to the cell wall of gram positives and flagellin derived from bacterial flagella. Other PAMPs exist for identifying DNA, RNA, fungi, protozoa and cancer proteins.

Recognition of microbes and control of the infection by neutrophils can involve phagocytosis, as well as production of exogenous degradative products. Within the host cell, multiple toxic vesicles create a harsh environment. Control of phagocytosis is receptor mediated in mammals, primarily through integrins like MAC-1 and Fc receptors, which enhance microbial intake. Neutrophil engulfment occurs via cytoskeletal rearrangement (Bengtsson *et al.*, 1993), the plasma membrane extends around the target, enveloping the microbe, which buds off inside the cytoplasm as a phagosome. The phagosome merges, in a calcium dependent manner (Jaconi *et al.*, 1990), with an intracellular lysosome, full of digestive enzymes and granules. It is within this phagolysosome that the microbe is degraded (Goetz *et al.*, 1987). These same granules may fuse with the host cell membrane and degranulate into the surrounding environment, if exogenous release is required (Tapper *et al.*, 2002).

Location of vesicle production is dependent on the maturation status of the neutrophil. At the early promyelocyte stage, vesicles form via cis-Golgi (closest to nuclear membrane) bud off, whereas later on, they are formed via the trans-Golgi (closest to cell membrane) bud off forming specific granules (Bainton *et al.*, 1966). Fusion of granules or endosomal compartments with the phagosome, during

maturation, is believed to be dependent on calcium (Bengtsson *et al.*, 1993), and protein kinases (Mohn *et al.*, 1995). In mammals, Rab5a GTPase seems to drive phagolysosome maturation (Perskvist *et al.*, 2002). Granules may be stored in intracellular vesicles or made in a *de novo* (fresh, as needed) process upon receptor stimulation (Mahdy *et al.*, 2006, Bulow *et al.*, 2002).

In mammals, four categories of neutrophilic granules have been identified. Azurophilic (or primary) granules contain MPO, an important peroxidase enzyme which produces reactive oxygen species during respiratory burst. The secondary (or specific) granules contain lactoferrin, a non-haem iron binding transferrin protein, which binds and moves iron in the blood. Lactoferrin is resistant to proteolysis and tolerant of a broad range of pHs, which allows it to function inside acidic vesicles within neutrophils. It has been found to have anti-microbial and anti-inflammatory properties, and interacts with DNA, RNA, polysaccharides and heparin (Bennett *et al.*, 1978). Lactoferrin has not been specifically identified in teleost neutrophils, but has been assessed as an immunostimulant to assist innate immunity. In gilthead seabream (*S. auratus L*.), the addition of lactoferrin during *in vitro* cell assays improved respiratory burst and innate cytotoxic activity (Esteban *et al.*, 1998). Similarly, orally administered (bovine) lactoferrin improved thermal tolerance in Japanese flounder (*P.olivaceus*) (Yokoyama *et al.*, 2006). Whether fish are able to produce any lactoferrin remains unclear; it may be a matter of detection methods used.

A poorly characterised family of immune lectins that can bind to lactoferrin, called intelectins, were first identified in African clawed frog *Xenopus laevi* (Roberson *et al*., 1982). The purported role of intelectins is innate stimulation and opsonisation for phagocytic enhancement (Tsuji *et al.*, 2001, Russell *et al.*, 2008). It has been proposed from zebrafish (*D. rerio*) genomic analysis that intelectins may evolutionarily belong to four separate clades in mammal, frog, fish, and lancet. Phylogenetic trees suggest intelectin genes have undergone lineage specific gene conversion or duplication (Yan *et al.*, 2013). The presence and ability of intelectin to bind to lactoferrrin would suggest coexistence, so the lack of definition and detection in teleost remains unconfirmed.

Tertiary granules of mammals are identified as containing gelatinase and secretory vesicles, which express albumin and alkaline phosphatase. Confirming presence of serum gelatinases and other proteolytic enzymes is highly variability in teleost families, but generally metalloproteases and serine proteases were found to be the major gelatinolytic proteases in fish (Lodemel *et al.*, 2004, Praveen *et al.*, 2004). Research using the Japanese killifish (*Oryzias latipes*), found the expression of gelatinases to be localised to ovary, rather than immune organs. Many gelatinolytic proteases were also identified in fish seminal fluid, which was unexpected, as fish have no accessory glands, from which many proteases come from in mammals (Kowalski *et al.*, 2004). This suggests an alternative or additional role for the proteolytic enzymes usually involved in gelatin hydrolysis into polypeptides, peptides, and amino acids. The secretory vesicles within the tertiary granules contain high amount of albumin, which is a major plasma protein in vertebrates, and is involved in chaperone transport of fatty acids, bilirubin, steroids, amino acids, and copper (Gorin *et al.*, 1981, Schoentgen *et al.*, 1986). Albumin has been identified in the blood several fish species including Atlantic salmon (*S. salar*) and rainbow trout (*O. mykiss),* though this may not be neutrophil associated (Byrnes *et al.*, 1990, Gong *et al.*, 1998). The source of cDNA from each study was liver, and was not linked to a specific cell type. In the Australian lungfish (*Neoceratodus forsteri*), plasma albumin was found to be more closely related to tetrapods than to teleost albumin. The diversity of teleost albumin may reflect specific roles linked to osmotic and transport functions, dependent on the environmental conditions of the host (Sepulveda *et al.*, 2004). There is no literature on the state of albumin in fish neutrophil vesicles, and it is thought to be entirely absent from some species. Instead high density lipoproteins function as the transporter of free fatty acids in serum (De Smet *et al.*, 1998).

Lastly, alkaline phosphatase is an enzyme found in bacteria through to mammals. The enzyme is responsible for dephosphorylating nucleotides, proteins, and alkaloids via hydrolysis. In teleost, the enzyme was found in digestive organs, and thought to have a role in metabolism (Sastry, 1975b, Sastry, 1975a) or wound healing. The enzyme was thought to be located in the lysosomes of

phagocytes, but this was not confirmed (Iger *et al.*, 1990). It has not been located in the tertiary granules neutrophils in fish.

While host cells fight pathogens with digestive exogenous and endogenous enzymes, sticky DNA webs and intracellular capture, bacteria have developed counter measures to block, neutralise and even kill the immune cells, which attempt to contain them. Unlike NETosis, apoptosis is a highly regulated and controlled process often not driven by pathogenic signal, first formally described in 1972 (Kerr *et al.*, 1972). Apoptosis is programmed cell death, triggered by two different pathways, the intrinsic (mitochondria-mediated) pathway, and extrinsic (receptor-mediated) pathway. Intracellular biochemical signals cause morphological cell changes, leading to cell death. These changes may include blebbing (swelling and bubbling of membranes), cell shrinkage, nuclear fragmentation (activated endogenous endonucleases cleave DNA), chromatin condensation (strands condense), and chromosomal DNA fragmentation (Kerr *et al.*, 1972). Apoptosis is a rapid process, and cellular fragments may be released in discrete vesicles, termed apoptotic bodies, which express a phosphatidylserine receptor as a signal for phagocytosis (Li *et al.*, 2003). This receptor medicated clean-up of dead cells avoids activation of inflammatory pathways.

Similarly, autophagy is a non-pathogen driven, stress-related, cell clearance mechanism, which promotes the degradation and recycling of cellular components. Autophagy isolates cytoplasmic components within a membrane, forming an autophagosome, which then fuses with a lysosome containing degradative enzymes (Lawrence *et al.*, 1992, Marzella *et al.*, 1981). Macroautophagy is the primary form of autophagy, and degrades unused proteins and damaged organelles (Bergamini *et al.*, 2004). Microautophagy involves direct engulfment of cellular organelles into a lysosome (Kunz *et al.*, 2004), and chaperone-mediated autophagy requires a chaperone protein complex, which may then bind, and be ingested by a lysosome (Kaushik *et al.*, 2006). Both apoptosis, and autophagy, minimise immune pathway activation, and are required for normal tissue growth and specialisation.

Contrastingly, necrosis is driven by factors external to cells such as trauma, toxins or infection resulting in self-destruction, perpetuated by lytic enzymes. This autolytic process occurs when various receptors are activated, which causes loss of cell membrane integrity, and an uncontrolled release of cellular contents. There are six forms of necrosis; coagulative, liquefactive, gangrenous, caseous, fat and fibrinoid necrosis (Kumar *et al.*, 2014). Necrosis can also be caused by components of the complement system, and activated cells such as natural killer cells and macrophages (Blom *et al.*, 1999, Arantes *et al.*, 2000). Necrotic and apoptotic neutrophils are also reported to release α-defensins, which serve to suppress pro-inflammatory cellular and cytokine activation, thus limiting tissue damage during an inflammatory response (Miles *et al.*, 2009). Necrotic neutrophils have also been reported to stimulate pro-inflammatory cytokines, via macrophage activation (Cavassani *et al.*, 2008). The role of necrosis and signalling pathways may be a flexible, situation specific process, determined by multiple factors.

Although neutrophil death often results from NET production, the web facilitates capture of invaders, as well as triggering a greater immune response. Escape from NETs, therefore, may be vital to bacterial survival. DNA, the major component of NETs, may be damaged by a number of stressors, including ROS, radiation, UV light, and carcinogens. Cells contain molecules designed to repair DNA, targeting damage, and are able to repair damage regions via nucleotide excision, performing mismatch repair or fixing double strand breakage (Mu *et al.*, 1995). These nuclease enzymes can cleave phosphodiester bonds in nucleic acids. These bonds are the linkages between the 3' carbon atom of one sugar molecule, and the 5' carbon atom of another. DNA contains deoxyribose and RNA has ribose. Originally termed a polynucleotidases, or nucleodepolymerase, nucleases were first identified as enzymes capable of restricting the growth of *Escherichia coli* phage (Linn *et al.*, 1968). Nucleases may be involved in degrading foreign nucleic acids, repair, recombination, synthesis, packaging and splicing events. They are classified as exonucleases, working on the ends of nucleic acid strands, or endonucleases, able to work within a molecule. Some

may specialise in single or double stranded nucleic acid, affect DNA or RNA or work in a 3'->5' or 5'->3' direction. Well known nucleases are deoxyribonuclease (DNase) (Salnikow *et al.*, 1970) and ribonuclease (RNase) (Weatherford *et al.*, 1972). Bacterially produced nucleases, such as micrococcal nuclease from *Staphylococcus aureus*, are utilised for research when break-down of DNA is desired (Cuatrecasas *et al.*, 1967, Kim *et al.*, 2014).

1.4 The acquired immune system

Adaptive immunity is specific, learned and includes immunological memory which facilitates rapid expansion of specific cells upon re-exposure to a pathogen. The adaptive immune response includes both humoral and cell-mediated components, primarily mediated by lymphocytes. The thymus is the first organ to develop lymphocytes, followed by the kidney and spleen, but the order by which the organs form and develop cells may vary between freshwater and marine teleosts (Zapata *et al.*, 1997, Josefsson, 1991, Chilmonczyk, 1992). In mammals, T cells either assist B cells to produce antibodies or directly attack and kill abnormal cells, and are identified by cell surface molecules CD4 or CD8 (Clusters of Differentiation receptor nomenclature) as either helper or cytotoxic T cells respectively. Identifying the same markers in fish has been difficult, though CD8alpha cells are able to kill allogenic target cells in rainbow trout (*O. mykiss*) (Fischer *et al.*, 2003), channel catfish (*Ictalurus punctatus*) (Yoshida *et al.*, 1995) and carp (Toda *et al.*, 2009, Toda *et al.*, 2011). The helper T cell marker CD4 has also proven elusive in bony fish, though prototype analogues and CD4REL (RELated) receptors have been identified in sea lamprey (*Petromyzon marinus*) (Pancer *et al.*, 2004), pufferfish (*T. rubripes*) (Suetake *et al.*, 2004) and rainbow trout (*O. mykiss*) (Laing *et al.*, 2006). With a lack of cross-reactive reagents for these evolutionarily primitive versions of mammalian receptors, the adaptive immune system of teleosts still remains to be completely deconstructed.

Similarly, B cells, which develop in the teleost head-kidney rather than bone marrow, show some similarities as well as differences to their counterparts in higher order organisms. B cells in fish produce antibodies, but the classes and structures differ from mammalian immunoglobulins (Ig). The

classes produced in teleosts are IgM, IgD and IgT (or IgZ) and have less capacity for memory response and affinity maturation. The production of the tetramer IgM varies among fish species, with freshwater species producing the immunoglobulins much earlier in larval development than marine species (Magnadottir *et al.*, 2005). The location, and therefore maturity, of B cells may also affect *in vitro* assessment, with mature and immature cells being found in the spleen and head-kidney respectively. With this knowledge, assessment of specificity and longevity of antibody production becomes important in regards to vaccinations against pathogenic antigens. With so much time, effort and cost involved in vaccine production, maximising the immune memory and broad spectrum cross-reactivity is vital to minimising repeat vaccinations.

An important part of the adaptive immunity is antigen processing and presentation of antigenic peptide to lymphocytes, facilitated by antigen presenting cells. The most effective of the APC's are dendritic cells (DC's), but B cells and macrophages are also professional APC's. Mammalian DC's may be derived from the myeloid or lymphoid stem cell progenitor pathways and exist in specific locations, for example Langerhans cells, which are resident skin DCs. With the large array of DCs in mammals, it was not until 2000 that a specific marker was identified (Geijtenbeek *et al.*, 2000). DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) is encoded in humans by the *CD209* gene. DC-SIGN is a surface bound C-type lectin receptor found on macrophages and DCs. In humans, the receptor is found on immature DCs in tissue, mature DCs in lymphoid tissue but not on the cell surface of plasmacytoid DCs, follicular DCs or CD1a+ Langerhans cells (Ling *et al.*, 2000). Regulation of DC-SIGN is thought to be controlled by interleukin-4 (IL-4), a cytokine usually associated with helper T cell differentiation (Nierhaus *et al.*, 2013).

A mouse homologue has also been identified, also closely associated with CD23 (aka FcεRII), a receptor for binding the allergy related antibody IgE (Huang *et al.*, 2008). Identification of putative DC-SIGN orthologues in other species include porcine (Lee *et al.*, 2003a), bovine (Lee *et al.*, 2003b), zebrafish (*Danio rerio*) (Lee *et al.*, 2003c) and rainbow trout (*O. mykiss*) (Bassity *et al.*, 2012).

More recently, another DC specific protein has been identified, which is expressed in all DC subsets (Lieschke *et al.*, 1992, Roilides *et al.*, 1991). Termed DC-SCRIPT (Dendritic cell-specific transcript), the protein was characterised as containing many zinc fingers, important for nuclear localisation and involved in transcriptional regulation (Pasnik *et al.*, 1999). In humans, DC-SCRIPT has been found to regulate IL-10 production, an anti-inflammatory cytokine (Panopoulos *et al.*, 2006). This protein has also been identified in barramundi (*L. calcarifer)*, identifying DCs generated from spleen and head-kidney cell cultures and demonstrating their ability to phagocytose and stimulate T cell proliferation (Zoccola *et al.*, 2015). Understanding these processes of antigen processing and presentation in lower phyla vertebrate is important to determine lymphocyte activation, and by extension, antibody production, which is vital for successful long-term immunity to reoccurring pathogens.

1.4.1 Protective antibodies

B cells bind foreign immunogens via the B cells receptor (BCR), and if that immunogen is also an antigen, clonal expansion and maturation occurs to produce plasma cells which secrete antibody. These antibodies are specific for the epitope portion of the antigen that the immunoglobulin fragment antigen-binding (Fab) arms recognise. Binding of an antibody to an antigen creates an immune complex, which is an enormous danger signal to the immune system. Immune complexes are cleared via phagocytosis, complement mediated coating, protease activity or opsonisation, enhancing identification and degradation. Immune complexes are such immune stimulants that they themselves can cause disease via autoimmune activation, including systemic lupus erythematosus, rheumatoid arthritis and vasculitis (Cochrane *et al.*, 1973). Interestingly, in fish, specific antibodies can be generated at the sites of microbial penetration (skin, intestine, gills) without generating a systemic response (Cain *et al.*, 2000, Jones *et al.*, 1999).

B cells in fish are found most abundantly in the kidney, spleen, and blood and unlike mammalian B cells, do not undergo affinity maturation post-activation in germinal centres within the red pulp of the spleen (which are absent in fish). This suggests that the B cells selected for antibody production

are already of a high affinity, or develop in an alternative manner (Ye *et al.*, 2011). The most common form of immunoglobulin exists in two forms, and the tetrameric IgM is more efficient at binding complement than the monomeric form (Elcombe *et al.*, 1985).

A secondary Ig class was also identified in channel catfish (*Ictalurus punctatu*s), and the homology to mammalian IgD heavy chain gave rise to the theory that it is an ancestral form common to mammals and bony fishes (Wilson *et al.*, 1997). As recently as 2005, a third class of immunoglobulin was identified in rainbow trout (*O. mykiss*). However, IgT has not been identified in all teleost and seems to be produced by a distinct B cell lineage, primarily involved in gut mucosal immunity (Hansen *et al.*, 2005, Zhang *et al.*, 2010). There was no direct evolutionary linkage between mammalian IgA mucosal antibody and teleost IgT, they appear to have developed independently in parallel, but to have functional convergence (Castro *et al.*, 2013, Zhang *et al.*, 2011).

Since fish are ectothermic, they are greatly affected by the surrounding water temperature, and the immune system may be suppressed by lower than optimal range temperatures (Verlhac *et al.*, 1990, Rijkers *et al.*, 1980). This is particularly important for antibody production when vaccinating fish, as suboptimal temperature may delay or diminish the production of protective antibodies (Johnston, 2006, Avtalion, 1969, Avtalion *et al.*, 1973, Magnadottir *et al.*, 1999). Other factors than can affect antibody titre (other than temperature) are fish size (Sanchez *et al.*, 1993) and seasonal water variation (Magnadottir *et al.*, 1999). These factors can be easily controlled in closed circuit aquaculture systems, but should be monitored for optimal results.

1.4.2 Vaccination

Vaccine trials are necessary to determine whether antibodies are produced against a specific target epitope and these antibodies can be assessed *in vitro* for binding, bactericidal activity, opsonisation and complement fixation as well as *in vivo* for neutralisation and protection against reinfection. Parameters such as adjuvant and cohort number for statistical significance have been carefully

considered (Jarp *et al.*, 1997), as well as temperature days for optimal antibody production (van Ginkewl *et al.*, 1985, Rijkers *et al.*, 1980). Fish vaccines against *Streptococci* are used in many farmed fish species (Pasnik *et al.*, 2005b, Pasnik *et al.*, 2005a), however vaccination often fails because of rapid epitope evolution to evade vaccination-generated antibodies (Bachrach *et al.*, 2001, Millard *et al.*, 2012, Eyngor *et al.*, 2008). Selection of the immunogenic target is more than theoretical, as sometimes key virulence factors fail to offer cross-protective antibodies (Aviles *et al.*, 2013). Inability of many current vaccines to confer long-term cross-protection highlights the demand for better understanding of immune system.

Considerations for vaccination include pathogenic target, efficiency of formulation, size / age of fish, immunisation route, cost and labour, as well as longevity of the immune memory. Vaccination may cause some adverse reactions such as lesions and slower growth (Aunsmo *et al.*, 2008, Sørum *et al.*, 2004). Bacterial vaccinations may be made of many different formulations based on live infections (attenuated or genetically modified) or dead vaccines (inactive, subunit or recombinant).

To improve immune efficacy, an immunostimulatory adjuvant is added (Midtlyng *et al.*, 1998). Adjuvants are of particular importance in boosting the immune response towards inactivated pathogens and recombinant antigens (Tafalla *et al.*, 2013), as the immune responses against these antigens are typically weak. Adjuvants must be carefully assessed to minimise side effects and select the most suitable for the fish, route of vaccination and pathogen target (Midtlyng *et al.*, 1996). Dual vaccines have also been developed targeting more than one bacterial pathogen, which were successful during infectious challenge protection in *Tilapia spp* against *S. iniae* and *Vibrio vulnificus* (Shoemaker *et al.*, 2012). Vaccine technology, while having some successes, has a huge capacity for improvement, which will arise once immune pathways, protective immunity and bacterial pathogenesis pathways are better understood.

1.5 Barramundi, Lates calcarifer

Teleostei are the lowest vertebrate having both an innate and acquired immune system and are a large family with over 24,000 species (Nelson, 1994). While many studies have looked at fish blood parameters, there are many models which differ due to environment. Confounding the situation in teleosts is the variation in nomenclature of cell types, a lack of tools for identification and functional assessment and the diversity of models used. While smaller, easily propagated and housed fish such as zebrafish are used for genomic and evolutionary studies, this model is not ideal for the advancement of vaccine immunology for commercially important aquacultured species. Larger models including carp (*Cyprinus carpio*) and goldfish (*Carassius sp*.), have been utilised for haematogenesis and myelopoiesis studies respectively (Kemenade *et al.*, 1994, Huttenhuis *et al.*, 2005, Carradice *et al.*, 2008). The majority of teleost models are freshwater species (including the three models just mentioned), whereas barramundi are able to live in fresh, brackish or salt water. Studies using barramundi may highlight similarities and differences between marine and fresh water environments and relevant immunological studies reported to date. Zebrafish, (*Danio rerio*) have been utilized for *S. iniae* infection studies, and in these freshwater cyprinids, an intramuscular dose of *S. iniae* caused rapid mortality within 2-3 days (Neely *et al.*, 2002). This bacterial pathogenesis was notably absent of inflammation, suggesting early innate cell signals were not initiated, of which neutrophils are a primary contributor. The importance of neutrophils in *S. iniae* infection was also demonstrated in larval zebrafish, where impaired neutrophil function increased host susceptibility, demonstrating the interaction of the bacteria specifically with neutrophils *in vivo (Harvie et al., 2013)*. There are no studies to date bridging this information to the commercially important aquacultured *Lates calcarifer*, where *S. iniae* may cause rapid, catastrophic losses by unknown mechanisms.

Barramundi prefer brackish warm water, and are in the largest order of vertebrates, Perciformes, which encompasses 40% of all the bony fish including sea bass (*Serranidae sp*.), jacks (*Carangidae*

sp.), snappers (*Lutjanidae sp.*), gobies (*Gobiidae sp*.) and damselfish (*Pomacentridae sp*.). The Australian aquaculture industry produces more than 40 commercially farmed species, the most productive of which including salmonoids, prawns, tuna, and edible and pearl production oysters. In 2010-2011, the aquaculture industry was valued at $950 million AUD and produced 75 thousand tonnes of product, as published by the Australian Government using ABARES fishery statistics (Government, 2013).

Barramundi are farmed in all Australian states except Tasmania, with the bulk coming from Northern Queensland, Western Australia and the Northern Territory, where the warmer climate is optimal. The Australian Barramundi Farmers Association reports Australia imports twice as much barramundi from Asia than is supplied by from local aquaculture, with wild catch making up just over 10% of the total intake (Government, 2011).

Since aquaculture conditions often house fish numbers at a higher density than what occurs in the wild, fish may become stressed and this can increase susceptibly to commensal and environmental infections, which are otherwise controlled and eliminated when in full health (Tafalla *et al.*, 2013, FAO, 2014). Understanding the immune system of the barramundi and how it combats bacterial infections is crucial to developing new vaccines and strategies for improving the yield of healthy fish, and maximising aquaculture production with minimal environmental impact. These studies will seek to define the interactions between the innate immune cells from barramundi and the infectious *pathogen S. iniae* and non-infectious *S. agalactiae.*

CHAPTER TWO: Streptococcal sepsis in fish: A preliminary *in vitro* investigation of survival and proliferation of *S. iniae* and *S. agalactiae* in barramundi blood

2.1 Introduction

Streptococcal infections in fish, caused by *Streptococcus iniae* or *Streptococcus agalactiae,* lead to rapid onset of generalised septicaemia and death (Agnew *et al.*, 2007). In acute cases, which form the majority of outbreaks on fish farms, there may be few external symptoms (Agnew *et al.*, 2007, Bowater *et al.*, 2012) and death is rapid, with mortality occurring within 24 - 48 hours post infection in experimental models (Delamare-Debouteville *et al.*, 2015, Aviles *et al.*, 2013, Shoemaker *et al.*, 2001, Pasnik *et al.*, 2005a). In spite of this characteristic rapid, fatal dissemination through the blood, little is known about how streptococcal fish pathogens survive and proliferate in the host circulatory system.

Bacterial sepsis is a systemic immunopathology associated with presence of bacteria and bacterial products in the blood. In humans it is characterised by severe hypotension, organ failure and death, and may precede toxic shock syndrome (Stevens, 1995). The cell walls of both gram-positive and negative bacteria contain toxic components such as peptidoglycan (PGN) and lipopolysaccharide (LPS) respectively. These are potent bacteria-specific signals that can cause bacterial septic shock, a result of combined action from cytokines, complement and coagulation pathways. Sepsis may occur when the host is unable to contain a primary infection and involves the collapse of the circulatory system, multiple organ system failure and death (Van Amersfoort *et al.*, 2003). The role of neutrophils appears to be dichotomous in sepsis. The usual steady state number of neutrophils can be induced to increase during the early stages of infection. In mammals, this is observed with an increasing number of mature neutrophils being released from bone marrow where they form (Furze *et al.*, 2008). The increase in neutrophil can drive inflammatory response, which then in turn, may

have a deleterious impact in neutrophil function. While an increase in phagocytic capacity and microbial clearance may facilitate containment of an infection, a tipping point exists whereby too many neutrophils, producing too many cytokines, overload the system. Neutrophil paralysis then inhibits the innate cells from continuing the migration and clearance process (Alves-Filho *et al.*, 2010). In humans and a murine knock out model, neutrophil paralysis appears to be toll-like receptor 4 (TLR4) dependent (Alves-Filho *et al.*, 2006). Teleost do not have a function TLR pathway (Sepulcre *et al.*, 2009) nor do the neutrophils develop from bone marrow making comparisons difficult. Studies in zebrafish demonstrate neutrophil infiltration to areas of *S. iniae* colonization, and these fish are acutely susceptible to *S. iniae* infection, developing similar generalized septicemia and meningitis. Consequently, the role of neutrophils in streptococcal sepsis in fish is not fully understood. As primary sentinels cells of the circulatory system, their interactions with blood borne pathogens such as *S. iniae* need to be further investigated.

In gram-positive bacteria, such as *Streptococci*, PGN and teichoic acids (sugar alcohol phosphate polymers) are the main causative agents of septic shock (Lappin *et al.*, 2009). Both LPS and PGN can activate the complement cascade, stimulating cytokine release from monocytes (such as TNF-alpha in mammals), which activate polymorphonuclear leucocytes and drive immune cell recruitment. Cellular activation fuels further cytokine production and innate mechanisms, including phagocytosis, NETosis, production of soluble factors including reactive oxygen species and other bactericidal agents (Janeway *et al.*, 2002).

Whilst little is known about sepsis in fish, streptococcal growth and dissemination in the blood of barramundi is thought to be rapid, as a high level of mortality in immunosuppressed fish occurs in less than 24 hours. Healthy fish do not appear to become diseased, however stress can suppress the immune system, leading to increased susceptibility (Barton *et al.*, 1991, Bly *et al.*, 1997). Stress factors that are relevant for aquacultured fish include procedural interference, physical stress (water changes or cage abrasion), chemical stress (from water parameters) and cage mate stress surmised

as biological stress (Sadhu *et al.*, 2014, Ndong *et al.*, 2007, Barton *et al.*, 1991). Given the short time frame involved, clearance is the responsibility of the innate immune system. Indeed, zebrafish (*Danio rerio*) infected with *S. iniae* suffered 90% mortality within 24 hours of infection (Neely *et al.*, 2002). This rapid lethality was found to be, at least in part, dependent upon capsular polysaccharide as an acapsular (*cpsA* deletion) strain was less virulent (Harvie *et al.*, 2013).

During infection, the first responder neutrophils are recruited rapidly to the bacteria, and phagocytosis and killing can occur within 15 mins (Hampton *et al.*, 1994). Exvasculation of mammalian granulocytes and *in vivo* migration assays show diverse ranges of kinetics. These depend on the chemoattractant or inflammatory agent used, but in most cases data is collected after 2 h of exposure. Observed kinetics suggest interaction of neutrophils, with rapidly proliferating *Streptococcus*, must involve neutrophils in current circulation, rather than recent recruits, although these processes may be occurring in parallel. In the context of these studies, we therefore assess circulating peripheral blood neutrophils, as the first responders to Streptococcal infection of the blood in barramundi. While macrophages are also involved in the initial wave of innate defence against bacteria, *S. iniae* was able to exist unharmed within trout macrophages for more than 24 hours (Zlotkin *et al.*, 2003). However, the effectiveness of neutrophil-mediated bactericidal activity has not yet been determined. Assessing the direct bactericidal activity of neutrophils can be performed by combining a known number of bacteria with whole blood (Ison *et al.*, 1999). By including a gentamicin protection component, and using Triton-X to lyse the blood cells, it is possible to determine the number of bacteria remaining external to blood cells, internal, and internal but not digested (Vaudaux *et al.*, 1979). In essence, the assay may determine the capacity of neutrophils to recognise and kill bacteria, and is consequently a useful starting point for investigation of bacterial sepsis in fish and the possible roles of cellular and humoral components.

Of the humoral components encountered by bacteria invading the circulatory system, lysozyme, which is also known as muramidase or N-acetylmuramide glycanhydrolase, plays a pleiotropic role.

As well as directly degrading bacterial walls, lysozyme has also been reported to be involved in promotion of sepsis and toxic shock by generating hydrogen peroxide, which acts on muscle tissue activating the guanylate cyclase pathway, in turn resulting in vasodilation, and finally leading to cardiovascular collapse (Mink *et al.*, 2009). A second mechanism of action determined lysozyme could also act directly on smooth muscle tissue, with the same end result (Gotes *et al.*, 2012). However, lysozyme has also been reported to have anti-inflammatory properties, whereby inorganic polyphosphate mediated pro-inflammation was suppressed in murine and human culture models (Chung *et al.*, 2016).

Lysozyme is a heat sensitive glycosidase, an enzyme which triggers hydrolysis of glycosidal bonds in polysaccharides. Specifically, lysozyme binds to a hexasaccharide in the polysaccharide D ring changing its conformation and in this weakened state, the glycosidic bond is easily broken (McKenzie *et al.*, 1991). This lysozyme-catalysed degradation process is able to break apart sugars such as PGN, the main component of the bacterial wall. Since gram-positive bacteria have a thicker layer of PGN, they are particularly susceptible to lysozyme hydrolysis, which stops growth, invasion and promotes opsonisation (Nash *et al.*, 2006). Though discovered earlier, the first report naming the molecule as lysozyme was communicated in 1922 (Fleming, 1922).

C-type lysozymes are the major lysozymes produced by vertebrates, while G and I types are produced by a subset of organisms (Qasba *et al.*, 1997). Lysozyme types vary in amino acid identity, size and charge (Xue *et al.*, 2004, Nilsen *et al.*, 2001). In mammals, lysozyme is found in milk, tears, on skin and in mucus. The first reports of teleost lysozyme examined Tilapia (*Tilapia mossambica*), Scat (*Scatophagus argus*) and Perch (*Therapon puta*), and there were differences in detection of lysozyme between organs and fluids (Sankaran *et al.*, 1972). Numerous fish have now been studied for lysozyme identification (Saurabh *et al.*, 2008), and there are many differences between species, in both production and location of lysozyme. Lysozyme levels can be influenced by water temperature, stress and infection (Langston, 2002).

In a recent study, an anti-lysozyme antibody was developed using mummichog (*Fundulus heteroclitus*), which identified myeloid cells as the cells responsible for making lysozyme (Marsh *et al.*, 2010). Given the susceptibility of barramundi to gram positive bacteria, assessing the role of c-type lysozymes in serum and plasma was warranted. In humans, lysozyme is produced by granulocytes, monocytes and tissue dendritic cells (Mason *et al.*, 1975). The enzyme is not produced by eosinophils or basophils, and minimally in lymphocytes (Smith *et al.*, 1982). Generally, in fish, lysozyme is also produced by monocytes, macrophages and neutrophils (Hall *et al.*, 2007). Organs or locations that contain the highest levels of lysozyme include kidney, spleen, skin, gills, serum and liver (Lie *et al.*, 1989, Lindsay, 1986), which are sites of possible microbial invasion.

Common assay methods for measuring lysozyme include an inhibition assay with the bacteria *Micrococcus lysodeikticus* (formerly *Micrococcus luteus*). The lysozyme-sensitive bacteria may be used in a solid plate colony plaque assay or liquid broth turbidity assay, whereby bacterial inhibition may be measured with known concentrations of lysozyme to create a standard curve, which in turn can be used to determine the lysozyme concentration of an unknown sample (Mörsky, 1983, Shugar, 1952). One unit of lysozyme will produce a change in optical density of 0.001 per minute at pH 6.24 at 25°C using a suspension of *Micrococcus lysodeikticus*. Clinically, high throughput turbidity assays have been optimised to measure serum and urine lysozyme for diagnosis for human acute myeloid leukaemia (Sexton *et al.*, 1996). Increased lysozyme levels have also been used for indications of mycobacterial tuberculosis infections and chronic infectious granulomatosis, although more specific molecular analyses may be utilised more effectively these days (Near *et al.*, 1992).

In this study we investigated growth of streptococcal fish pathogens in barramundi blood using a newly adapted blood bactericidal assay, and investigated possible roles of neutrophils and lysozyme in bacterial blood survival in barramundi.

2.2 Materials and Methods

2.2.1 Bacterial strains and culturing

S. iniae QMA0248 and *S. iniae* QMA0249 were isolated from infected barramundi at a recirculating aquaculture farm during an acute and chronic outbreak of streptococcosis respectively. Both were isolated from previously vaccinated fish; QMA0248 is capsular whereas QMA0249 is acapsular, with a frameshift mutation in *cpsE* resulting in truncation of the protein and a deletion of the genes *cpsF* through *cpsM* (Millard *et al.*, 2012). *S. agalactiae* QMA0285 and QMA0281 were both isolated from dead wild giant grouper *Epinephelus lanceolatus* (Delamare-Deboutteville *et al.*, 2015). QMA0285 is virulent and capsular (Delamare-Deboutteville *et al.*, 2015) while QMA0281 is acapsular with a deletion from mid-cpsB through to mid cpsE (Fig. 1. Ben Zakour *et al*., unpublished). Strains were stored without sub-culture at -80°C in Todd-Hewitt Broth (THB) containing 20% glycerol. Strains were grown routinely on Columbia agar base containing 2% defibrinated sheep blood at 28°C. For liquid culture, strains were grown in THB under microaerophilic conditions with minimal agitation to prevent settling. For growth kinetics in liquid culture, strains were grown in starter cultures of 10 ml THB overnight to mid-log phase, then 96 well plate wells inoculated with diluted replicate THB starter cultures of pre-early log phase, and the growth was recorded by measuring the optical density (OD) at 600 nm every 30 min for 24 h.

2.2.2 Assay of capsular polysaccharide presence by buoyant density assay

Buoyant density assays were performed essentially as described previously (Sellin *et al.*, 1995, Lowe *et al.*, 2007) optimised previously in our lab for *S. iniae* and *S. agalactiae* using an isopycnic gradient of Percoll (51%), 1.5M NaCl (10%) and water (39%). Briefly, individual colony replicates of each strain were grown overnight in 100 ml THB to late-log phase (unless indicated), the pellet was harvested and washed once in phosphate buffer saline (PBS) before resuspension in the residual volume (~500 µl). Clear 5 ml flow cytometry tubes (Becton Dickenson, Australia) containing 3 ml Percoll solution

(above), were underlaid with a cushion of 250 µl undiluted Percoll to capture high-density cell bands without pelleting. A volume of 400 µl of the bacterial suspension was layered on top of the Percoll gradient, and tubes were centrifuged in an Eppendorf 5810R centrifuge, using a swing out rotor at 3225 x *g* for 90 min at room temperature. All strains were assessed with 3 biological replicate cultures.

2.2.3 Polymerase Chain Reaction (PCR)

To verify identity of the strains under investigation, PCR employing species-specific primers were employed as previously published for both *S. iniae* (Lox1 5'-AAGGGGAAATCGCAAGTGCC-3' (forward) and Lox2 5'ATATCTGATTGGGCCGTCTAA-3' (reverse))(Mata *et al.*, 2004) and *S. agalactiae* (AgaF 5'-AACAGCCTCGTATTTAAAATGATAGATTAAC-3' (forward) and AdyR 5'- TCCTACCATGACACTAATGTGTC-3' (reverse))(Kawata *et al.*, 2004). Briefly, genomic DNA was extracted using the DNEasy blood and tissue kit (Qiagen) with an additional pre-lysis using a lysis buffer comprising 10 µl Tris/HCl pH 8.0, 40 µl 0.5 M EDTA, 24 µl Triton-X100, 200 µl lysozyme (20 mg/ ml stock), 20 µl RNAse A (at 100 µg/ml) and made to 1 ml with 706 µl sterile ultra-pure water. Bacterial pellet was resuspended in 200 µl fresh lysis buffer and incubated at 37°C for 45 - 60 min (Eppendorf Thermomixer, 500 rpm). Subsequently 200 µl Buffer AL and 25 µl proteinase K (from Qiagen kit) were added and tubes inverted 15 - 20 times. Tubes then incubated at 56°C for 1 h (Eppendorf thermomixer 500 rpm) before proceeding with kit instructions.

Amplification was conducted in 50 µl reactions comprising 25 µl MyTaq™ Red Mix (Bioline (Aust) Pty Ltd, Alexandria, Australia), 0.1 µM of each primer, 5-10 ng gDNA template and sterile water to 50 µl. PCR conditions were 95°C for 3 min for initial denaturation (95°C denaturation for 1 min, 57°C annealing for 30 sec, 72°C extension for 1 min) x 30 cycles, final extension 72°C for 10 min, hold at 4°C. Template free controls were employed in each reaction. Amplicons were observed via agarose gel electrophoresis, using a 1% agarose gel in TAE buffer containing HydraGreen. Gels were run at 100V / 110A for 40 min.

2.2.4 Barramundi phlebotomy

Barramundi fingerlings were obtained from Australian Native Fish Enterprises (Kallangur, Australia) at a 20 - 40 mm size and maintained in recirculated, aerated, brackish water (12 - 15 ppt salt), at 28°C, with water parameters checked daily and partial water exchanges performed as required. They were fed with Ridley Native finfish diet (Ridley Aquafeeds Ltd, Narangba, Australia) twice daily, until satiation, with pellet size adjusted to suit the size of the fish. Blood was collected by caudal venipuncture with a syringe and 23G needle following overdose of anaesthetic (Aqui-S) when fish were 60 - 100 grams (fork length 80-100 mm minimum).

2.2.5 Blood bactericidal assay

Streptococcus iniae strains QMA0249 and QMA0248, *Streptococcus agalactiae* strains QMA0281 and QMA0285 and *Escherichia coli* strain DH5α (positive control) were grown in THB (Oxoid, Thermo Fisher Scientific, Australia), picked from a single colony grown overnight on Todd Hewitt Agar (THA) plates. All streptococcal bacterial cultures were grown stationary at 28°C; *E. coli* was gown at 37°C, shaken at 200 rpm. The strains were grown to late log phase and diluted down to a final 100 colony forming units (CFU) as optimised previously using the unconditioned control wells. For *S. iniae* and *E. coli*, this consisted of a 1:100 primary dilution followed by a 1:200 secondary dilution for subsequent use in a plate. *S. agalactiae* was diluted primarily 1:100 and secondarily 1:100. A volume of 50 μl of these dilutions was used in replicates of 6 wells per bacterial strain, per condition.

Barramundi (*L. calcarifer*) 60 - 100 g in weight, were anaesthetised with an overdose of Aqui-S and bled via the caudal vein with a heparinised 23G needle. Immediately 10% v / v citrate-phosphate-dextrose buffer was added to the blood to minimise clotting. Once sufficient blood was harvested, it was diluted 1 : 1 with room temperature phenol red free RPMI-1640 (Life Technologies, Thermo Fisher Scientific, Australia).

For the bactericidal assay, equal volumes of the diluted bacteria and diluted blood were added into a 96 well round bottom plate and incubated with gentle rocking for 30 min at 30°C. Barramundi immune function is optimal at 28 – 30 °C, within the range the fish are found in the wild (26 - 34 °C) (Bromage, 2004). Temperatures below 24°C increase disease susceptibility of wild barramundi (Pusey *et al.*, 2004). Other *in vitro* immune studies with barramundi also maintain all solutions and tissues in this temperature range (Bromage, 2004, Anderson *et al.*, 2004). Controls included a susceptible *E. coli* strain (DH5α, Thermo Fisher Scientific, Australia), as well as bacteria alone and blood alone. Three conditions were assessed to determine intracellular survival, extracellular survival and total survival as follows. Extracellular bacterial survival was assessed by a direct spot streak of the bactericidal assay mixture, in which 100 µl THB was added to each well after the 30 min bactericidal incubation and 25 µl spotted onto THA. To liberate intracellular bacteria, the blood cells were lysed with 100 ml ice cold 0.02% Triton-X (final concentration 0.01%). To assess if the detergent caused any break up of chains and/or clumping, which may increase individual colony count, bacteria in broth, with and without detergent were tested, there was no difference in colony growth number between the two.

Intracellular survival was assessed utilising a gentamicin protection assay. At 30 min post-inoculation, gentamicin (Life Technologies, Thermo Fisher Scientific, Australia) was added at a final concentration of 200 µg / ml and incubated for a further 60 min to kill all extracellular bacteria. After the antibiotic incubation, the blood cells were lysed with 100 µl ice cold 0.02% Triton-X for plating, the 1:1 dilution of antibiotic takes the concentration below the maximum inhibitory concentration (MIC) of gentamicin for these strain. This allowed any intracellular bacteria to grow on THA agar. There were no wash steps, only dilutions, between incubations, to avoid incidental loss of bacteria and/or cells. The minimum inhibitory concentration of gentamicin was determined for the strains used, and the dilution prior to plating should have provided enough of a dilution such that the antibiotic would not inhibit surviving bacterial growth, however a few caveats exist. The diffusion of

the antibiotic across fish cell membranes is unknown, the diffusion of the antibiotic post treatment and once plated on agar is unknown. Without a bacterial control known to survive intracellularly in fish phagocytes, it cannot be discounted that this assay may have some weaknesses.

For all bactericidal mixtures, 25 μl of the 200 μl was spotted onto square THA agar plates via multichannel pipette, and the plate immediately tilted up to allow the liquid to streak down the plate, spacing out the colonies. The liquid was then allowed to absorb into the agar for a minimum of 30 min before inversion and incubation overnight at 28°C. The colonies were counted and it was assumed one colony represented one bacterial clone, or CFU. An average of the six replicates was compared to controls. All assays were repeated on several occasions with blood from at least three fish on each occasion.

2.2.6 Lysozyme assay

The lysozyme sensitive bacteria, *Micrococcus lysodeikticus* (Sigma-Aldrich, ATCC No. 4698 #M0508, Australia), was embedded in Trypticase Soy Agar (TSA) (Oxoid, Thermo Fisher Scientific, Australia), supplemented with 1% NaCl. Holes were punched in the agar with a sterile 4 mm cork-borer, the agar disc removed via tip if not inside punch tube and wells filled to the brim with ~20 μl of test serum, or a titration of lysozyme (chicken egg white, L6876 Sigma-Aldrich, Australia) of a known concentration. The solution was allowed to completely absorb into the agar, 1 h, before inversion and incubation overnight. The zone of inhibition was measured in mm and triplicate plate averages used to create a standard curve of the known concentrations and zone of inhibition.

Additionally, the assay was conducted in Trypticase Soy Broth (TSB) (Oxoid, Thermo Fisher Scientific, Australia) and growth / lysis of micrococcus assessed via optical density at 450 nm. A suspension of *M*. *lysodeikticus* (0.20 mg / ml) was prepared and 50 μl added per well to a 96 well flat bottom plate. A dilution series of lysozyme was used to create a standard curve for which the unknown plasma and serum samples could be measured via their resultant optical density. For serum harvest, no

anticoagulant was added to the freshly drawn blood and it was allowed to sit undisturbed for 3 h at room temperature. Plasma was harvested in the same manner except that anti-coagulant was added. Citrate- Phosphate- Dextrose solution was added at a ratio of 1:10 to inhibit clotting, and it was allowed to sit undisturbed for 3 h. The blood samples were then centrifuged in a microfuge at maximum speed for 3 min before drawing off the serum or plasma, taking care to avoid collection of any of the blood pellet. Barramundi serum and plasma were separated side by side from whole blood, and tested in triplicate technical replicates from five individual fish (biological replicates). Controls included wells containing serum, plasma or buffer with no bacteria.

2.2.7 Statistical analysis

Replicates of total, external and internal were compared to control CFU numbers in a paired ANOVA test, followed by a Dunnett's multiple comparisons test, with GraphPad Prism, version 6.05, and $p < 0.05$ was considered significant.

2.3 Results

2.3.1 Bacterial growth and colony morphology

Both streptococcal species grew on Columbia blood agar plates incubated at 28°C, colony purity and haemolysis was easily assessed within 24 - 36 h. *S. agalactiae* was slightly slower growing the *S. iniae* therefore zones of beta-haemolysis were more easily seen after 48 h incubation. Morphology of the colonies was quite distinct between the capsular and acapsular strains (Fig. 2A, 3A). Acapsular *S. iniae* strain QMA0249 yielded sticky, spreading colonies compared to QMA0248 which grew in smaller, tight, umbonate colonies characteristic of the species (Agnew *et al.*, 2007). The acapsular QMA0249 was poorly haemolytic. Conversely, *S. agalactiae* capsular strain QMA0285 yielded large, sticky colonies and the acapsular QMA0281 small, opaque colonies. The beta haemolysis was moderate for both capsular and acapsular *S. agalactiae* (Fig. 3A).

In THB, *S. iniae* capsular strain QMA0248 grew consistently faster and to a higher optical density than QMA0249 (Fig. 2B). Similarly, the *S. agalactiae*, capsular strain QMA0285 grew faster and to a higher OD_{600} (Fig. 3B). All four streptococcal strains had similar cell morphology, chain length and Gram staining reaction when examined microscopically (not shown).

2.3.2 Buoyant density assay of bacterial capsule

Capsular *S. iniae* QMA0248 resulted in a single, low-density band high in the Percoll layer, following centrifugation on an isopycnic Percoll gradient. Conversely, acapsular QMA0249 resulted in two bands, one that was rather diffuse near the top of the tube, but lower than the band formed by QMA0248, and a clear, high density band that formed at the bottom of the tube on the undiluted Percoll cushion (Fig. 2C, D). To further investigate the formation of the two bands by the acapsular isolate, both mid- and late-log phase cultures of QMA0249 were compared; however there was no difference in banding pattern observed in the density gradient (Fig. 2D).

S. agalactiae virulent capsular QMA0285 resulted in a discrete single band high on the Percoll layer, whilst the acapsular QMA0281 resulted in a single band on top of the cushion at the bottom of the tube (Fig. 3D).

2.3.3 S. iniae proliferates rapidly in barramundi blood

The capsular isolate of *S. iniae* grew much more rapidly in barramundi blood than in Todd-Hewitt broth. During the 30 min incubation period QMA0248 grew between 2 and 4 times faster than in THB (Fig. 4A). Surprisingly, even the non-capsulated *S. iniae* QMA0249 grew to higher cell density in blood than in THB (Fig. 4B), although less so than QMA0248. The non-pathogenic (to barramundi) *S. agalactiae,* capsular and acapsular strains, also resisted killing in barramundi blood but did not grow significantly during the incubation (Fig. 4C & D). In contrast, *E. coli* DH5α, was eliminated within 30 min in barramundi blood from all fish tested (data not shown). Whilst there was some variation between *S. iniae* growth rates between individual fish, the patterns of growth and inhibition were

similar between individuals (Fig. 4). For QMA0248, the sum of external and internal bacteria was less than that of total numbers collected via blood cell lysis.

2.3.4 Lysozyme levels are low in barramundi serum and plasma

The sensitivity of *Micrococcus lysodeikticus* to lysozyme mediated lysis is frequently used to assess lysozyme activity in fluids and organ lysates. The optical density of bacterial suspension decreases as bacteria are lysed. A serial dilution of commercial lysozyme demonstrated the bacterial degradation kinetics were fast and the assay sensitive, down to 0.49 μg / ml (equivalent to <20 units of activity). However, there was no lysozyme activity detected in serum or plasma of five fish assessed in parallel with the standard curve. There were no significant differences between the negative control sample and the serum and plasma samples assessed (Fig. 5).

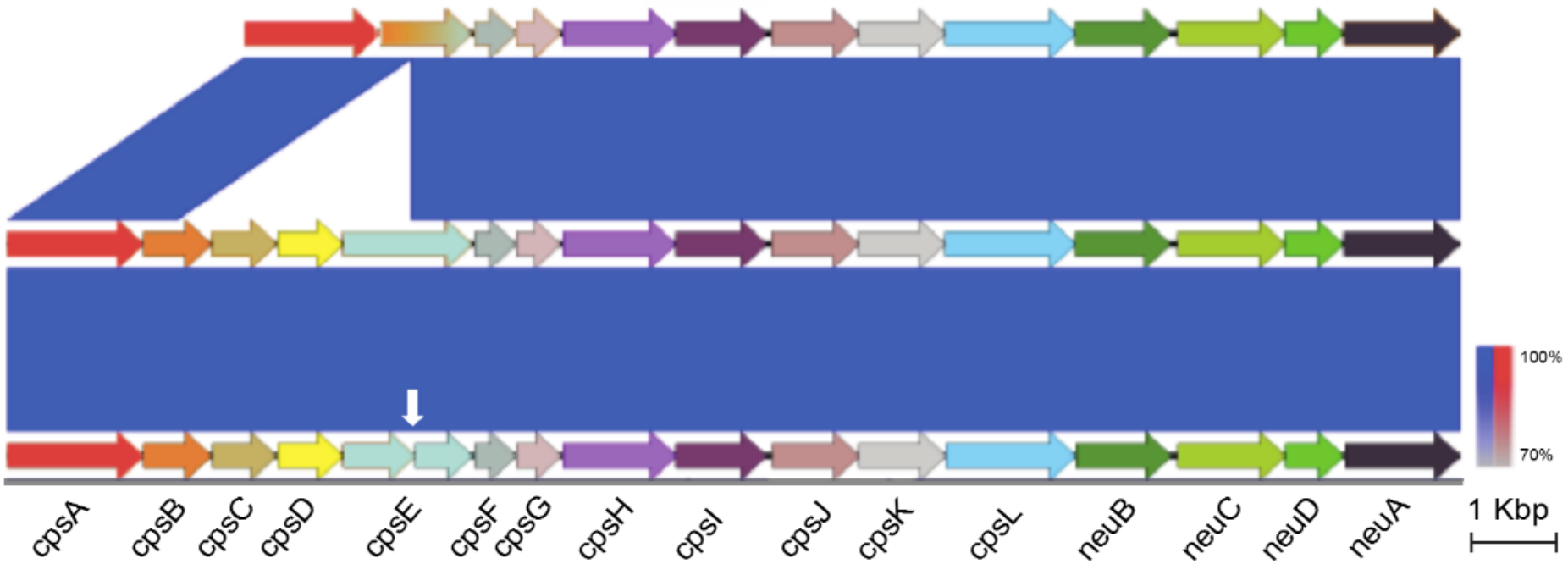

Figure 1. Capsular operon of S. agalactiae isolates from Queensland grouper illustrated with EasyFig. Top QMA0281, middle QMA0285, bottom QMA0368 (Ben Zakour et al., in prep)

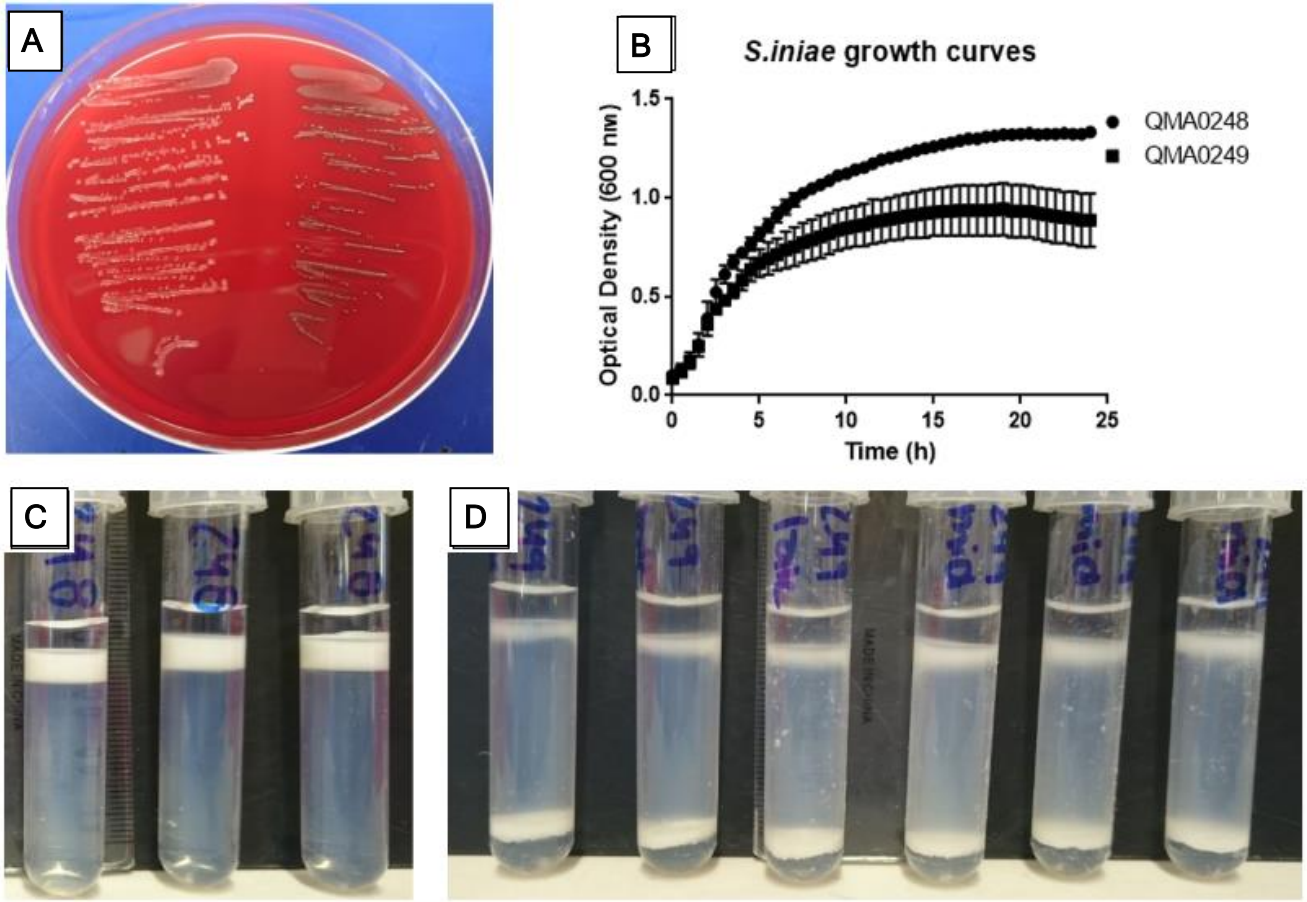

Fig. 2. Growth, hemolysis and capsular polysaccharide expression in S. iniae strains QMA0249 and QMA0248. A) Growth on Columbia agar base containing 2% defibrinated sheep blood after 36 h at 28°C. B) Growth curves over 24 h at 28°C in THB. Points are mean and standard deviations derived from three individual cultures. C) Percoll buoyant density of S. iniae cells from mid-exponential growth phase growth in Todd-Hewitt broth. Three independent cultures of capsular QMA0248 (left), acapsular QMA0249 (centre and left). As acapsular QMA0249 displayed two bands in triplicate independent cultures, QMA0249 was reassessed at both mid (centre) and late (right) log phase to determine capsular expression difference during growth phases, there was no difference.

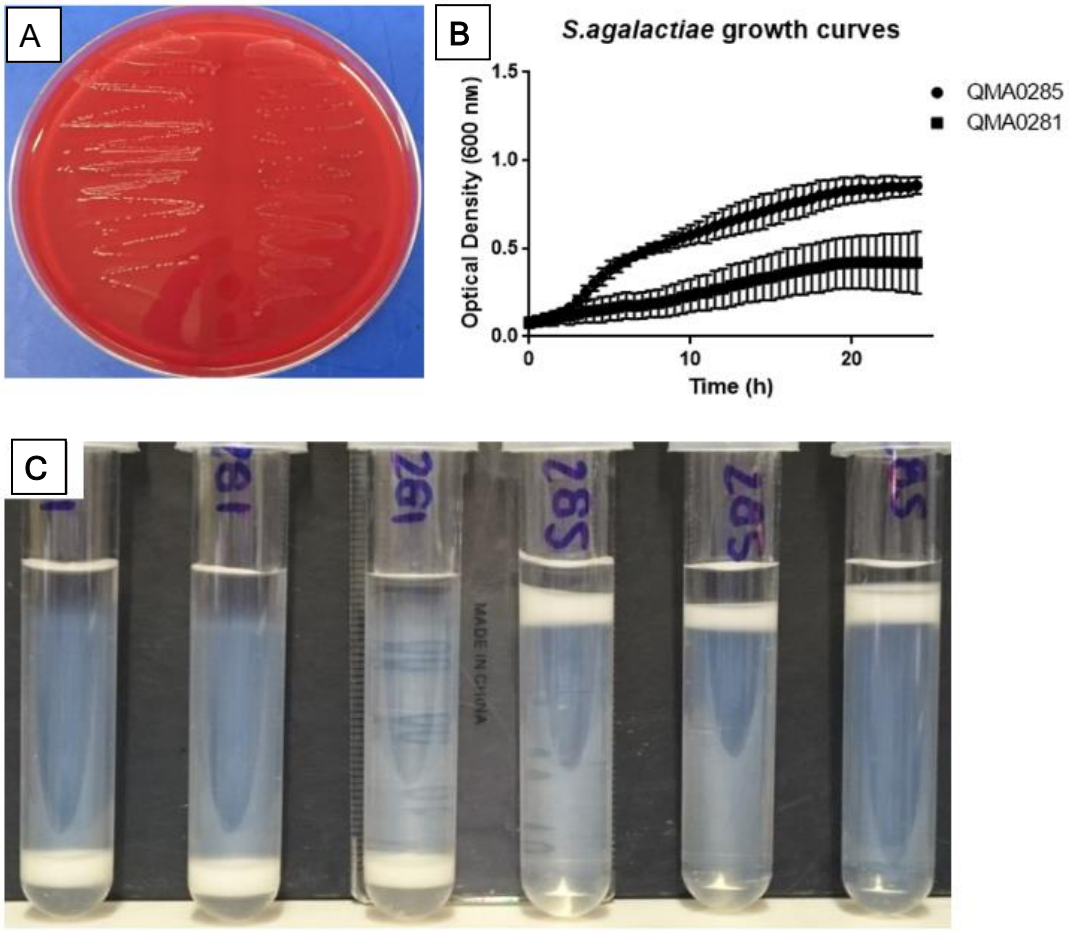

Fig. 3. Growth, hemolysis and capsular polysaccharide expression in S. agalactiae strains QMA0285 and QMA0281. A) Growth on Columbia agar base containing 2% defibrinated sheep blood after 36 h at 28°C. B) Growth curves over 24 h at 28°C in Todd-Hewitt broth (THB). Points are mean and standard deviations derived from three individual cultures. C) Percoll buoyant density of S. agalactiae cells from mid exponential growth phase growth in THB. Three independent cultures of capsular QMA0285 (right), acapsular QMA0281 (left).

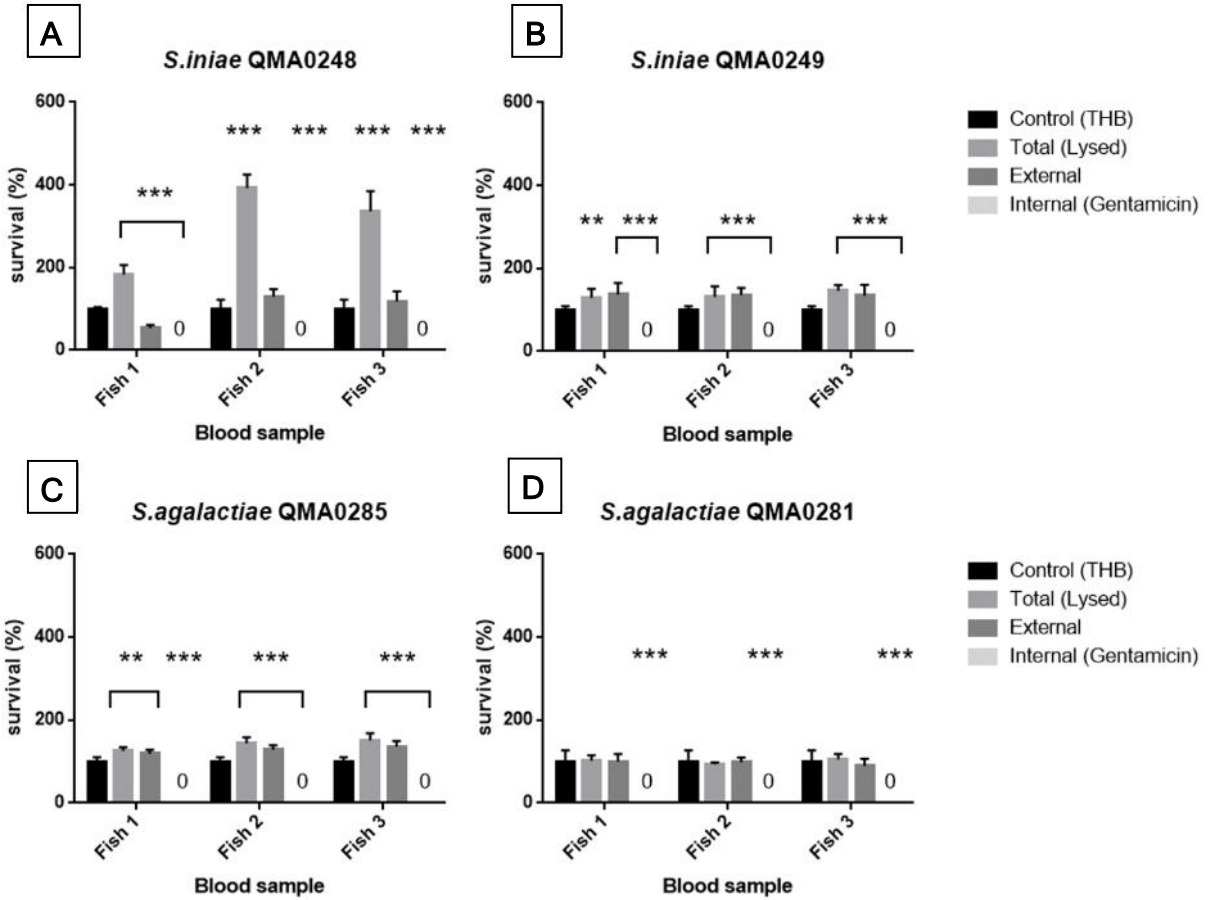

*Fig. 4 Survival or growth of S. iniae, S. agalactiae, and E. coli DH5a (not shown) in barramundi whole blood. A) S. iniae QMA0249 (acapsular); B) S. iniae QMA0248 (capsular); C) S. agalactiae QMA0281 (acapsular); D) S. agalactiae QMA0285 (capsular). In all experiments, E. coli DH5a was completely killed during 30 min incubation with barramundi blood for all fish sampled, whereas the control (incubation in broth without blood) resulted in 90-110 colonies per column. Each fish was tested in technical replicates of six for each bacterial isolate and the average use for analysis. All values normalised to broth only bacterial growth controls and statistics relative to broth control * P > 0.05, ** P = 0.01, *** P < 0.001.*

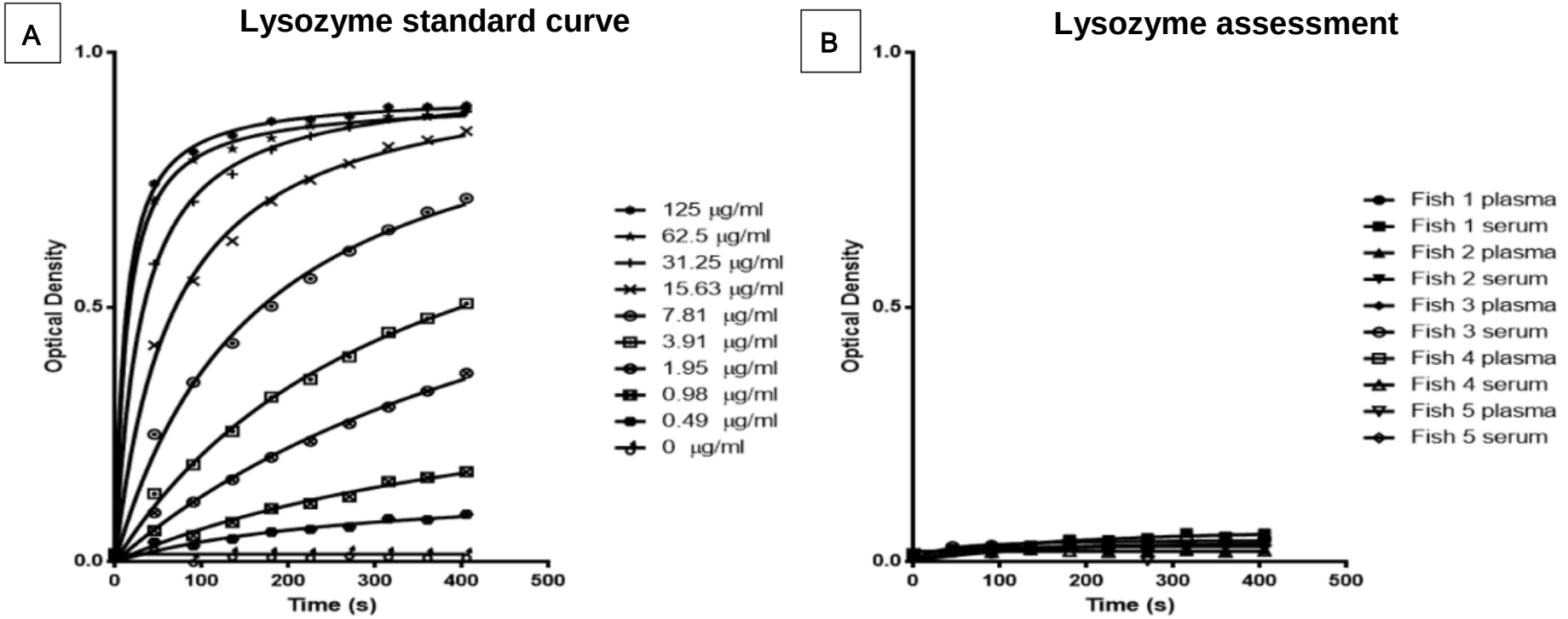

Fig.5 A) Lysozyme standard curve created using triplicate time points demonstrating sensitivity of Micrococcus lysodeikticus to lysozyme mediated lysis, kinetic reads at 450 nm recorded every 45 s for 6 min. B) Lysozyme assessment of plasma and serum harvested from five fish in parallel, minimal lysozyme activity, no significant difference from 0 mg / ml.

2.4 Discussion

S. iniae and *S. agalactiae* cause rapid onset of sepsis as a characteristic of their pathology in susceptible hosts (Eldar *et al.*, 1999, Lahav *et al.*, 2004, Delannoy *et al.*, 2013, Iregui *et al.*, 2016). However, *S. agalactiae* has not been isolated from diseased barramundi. Here we have shown that *S. iniae* can proliferate very rapidly in barramundi blood. In contrast *S. agalactiae* is inhibited but not killed. Normal bactericidal activity was functional in barramundi blood as *E. coli* DH5α was rapidly killed in barramundi blood (Fig. 4).

The comparison of capsular and acapsular strains showed that, while the virulent QMA0248 capsular strain rapidly increased in number, it did not entirely explain the survival of the bacteria since the acapsular QMA0249 was not cleared. It also appears that some form of capsule or sugar derivative is still produced by QMA0249, as seen by a dual layer in the buoyancy density assay (Fig. 2). Numbers of acapsular bacteria of both *S. iniae* and *S. agalactiae* recovered were less than that of the capsular strains suggesting while a net increase was controlled, the initial inoculum was not removed. Although there were differences in growth rate between the strains, the starting inoculum controls were used for internal normalisation of each strain. Although barramundi do not appear to be susceptible to *S. agalactiae* infection and disease, the bacteria were not cleared, unlike *E. coli* for which the initial inoculum was completely eradicated during the same incubation period. This would suggest that, while infection may occur, disease does not and it is likely that *S. iniae* has acquired mechanisms for circumventing blood-borne immunity that *S. agalactiae* has not.

Even though the route of entry of *S. iniae* into barramundi is not known, the bacteria clearly thrive in the blood, so it would be most physiologically relevant to ascertain the presence of lysozyme, unaltered, in blood. While commercial lysozyme lysed *Micrococcus lysodeikticus,* the serum and plasma collected from five barramundi contained undetectable lysozyme activity. Although this assay is commonly used, perhaps the sensitivity was too low for our samples. The lack of lysozyme

detection in the serum and plasma of barramundi may assist the pathogenesis of gram positive bacteria such as *Streptococci*, enabling rapid spread unhindered by innate soluble factors.

Lysozyme activity from fish tissues was first measured in 1972, but can be highly tissue specific (Sankaran *et al.*, 1972). Indeed lysozyme has been precipitated from rainbow trout kidney *(Oncorhynchus mykiss*), where the concentration was highest, followed by the alimentary tract, spleen, skin mucus, serum, gills, liver and muscle (Lie *et al.*, 1989).

As lysozyme levels were almost undetectable in barramundi blood a number of other factors may be responsible for killing the *E. coli* in the blood assays, including antimicrobial peptides, complement, eosinophils, the occasional blood-borne macrophage, and the neutrophils. Capsulated *S. iniae* is resistant to killing by lytic complement via either classical or alternative pathways (Barnes *et al.*, 2003), and are resistant to cationic antimicrobial peptides from fish (Buchanan *et al.*, 2005). However, their survival in the whole blood bactericidal assays described herein, imply that they must also be resistant to neutrophils as the major sentinel immune cells of the peripheral blood. The number of macrophages in whole blood was very low, however could be contributing. Peak cytokine and gene expression of human macrophage, in response to bacteria, occurs 5-10 hours post stimulation (Nau *et al.*, 2002), which is outside the scope of the barramundi blood and enriched neutrophil preparation assays.

The bacteria responsible for plague, *Yersinia pestis* has been found to preferentially invade macrophages, dendritic cells and neutrophils, subverting cellular destruction (Marketon *et al.*, 2005). Other bacteria which target and corrupt the usual function of immune cells for survival include *Mycobacterium tuberculosis* inside phagosomes (Stanley *et al.*, 2003), *Listeria monocytogenes* in the cytosol of macrophages (Birmingham *et al.*, 2008), and *Neisseria meningitidis* in brain endothelial microvascular cells (Nikulin *et al.*, 2006). Indeed, *S. iniae* has been shown to survive inside a macrophage-like cell line from rainbow trout for more than 24 h (Zlotkin *et al.*, 2003).

To investigate the relationship between the bacteria and peripheral blood immune cells, the number of external bacteria and the number of internal bacteria were investigated using a gentamicin protection assay. QMA0248 increased 2 - 4 x that of control (input bacteria), which far outnumbered the sum of internal and external CFU recovered. This suggests that the bacteria are located somewhere protected from destruction, where they can swiftly proliferate. The external bacteria recovered after 30 min incubation showed a modest increase in number (compared to input), yet the gentamicin protection assay suggested that there were no viable internalised bacteria in mature peripheral blood neutrophils. The gentamicin protection assay, while modelled on published methodology (Vaudaux *et al.*, 1979) has been primarily used with mammalian cells; there may be some leakage of the antibiotic through the fish neutrophil membrane, killing any intracellular bacteria. Alternatively, the bacteria may be sequestered from degradation within the very immune cells, which are tasked to seek and destroy them.

Post phagocytosis, *Streptococci* may escape the phagosome and exist within the cytoplasm, or live and proliferate within another vesicle inside the neutrophil. This theory would support the high numbers observed upon blood cellular lysis. Intracellular survival of *Streptococcus pyogenes* inside murine phagocytic cells has been reported to be an important mechanism for persistence and systemic invasion (Medina *et al.*, 2003). Furthermore, *S. agalactiae* has been found, also via gentamicin protection assay (in conjunction with penicillin), to infect host macrophages, and to survive and persist up to 48 h (Cornacchione *et al.*, 1998). Given that this timeframe is when the innate immune system is active, sequestering of bacteria intracellularly, especially in phagocytes, renders host immune defence ineffective and is an effective escape mechanism.

Neutrophils from channel catfish (*Ictalurus punctatus)* have been found to be intracellular hosts of *Edwardsiella ictaluri*, a gram-negative motile rod. While extracellular bactericidal activity killed *E. ictaluri*, phagocytosis was active and the intracellular bacteria were not destroyed (Waterstrat *et al.*, 1991). It is mechanisms of microbial avoidance such as this, which may allow these streptococcal

species to survive and or proliferate in the blood of barramundi, rapidly causing sepsis and further disease. It may also be simply that the rapid proliferation of *S. iniae* overwhelms the blood neutrophils with sheer number of bacteria and bacterial secretion products, masking or negating any effect of actual phagocytosis and killing.

These results, coupled with the rapid onset of septicaemia in infected fish, lead to the question of why blood borne immune factors and cells are unable clear *Streptococcus iniae* in circulation, whereas E.coli is cleared in these whole blood assays within 30 minutes. The bactericidal activity of blood cells is functional and capable, as displayed by the complete clearance of *E. coli.* Neutrophils, as the first responder cell, are the target of subsequent research to understand how *Streptococci* are able to avoid or negate the mechanisms used successfully against other bacteria; whether the cells are somehow rendered non-functional, or if the bacteria itself is able to avoid cellular destruction by stealth.

CHAPTER THREE: Neutrophils in barramundi blood: development of an enrichment procedure for mature circulating neutrophils for assessment of immune function against streptococcal species

3.1 Introduction

Blood hosts many cell types, broadly separated into red blood cells and white blood cells. Of the white blood cells, which usually comprise ~ 1% of the total blood cell population, further definition comes from lineage, based on a myeloid or lymphoid precursor origin. Of the myeloid lineage, cells may be mononuclear (monocytes and macrophages) or polymorphonuclear (granulocytes) (Akashi *et al.*, 2000). The functional role of myeloid progenitor is that of innate immunity, although monocytes may differentiate into macrophages and dendritic cells with some overlap into adaptive immunity. Granulocytes primarily function as first responder cells involved in phagocytosis, intracellular degradation, exogenous factor secretion, and cytokine production (Palmblad, 1984). Highly mobile and rapidly responsive, neutrophils are the most common granulocyte and in some species basophils and eosinophils have not yet been defined or identified (Flerova *et al.*, 2013, Ainsworth, 1992, Hine, 1992).

Granulocytes are common in human blood with neutrophils accounting for the majority of polymorphonuclear leukocytes (PMNL's), found at a density of approximately 5 million per millilitre in whole blood, or 40 - 80% of the total leukocyte count (Curry, 2016). Leukocytes, however, only represent about 1% of whole blood. Therefore to detect the response of peripheral blood neutrophils *in vitro*, enrichment or isolation techniques must be devised to reduce any interference from other cells. Moreover, some cell types such as RBCs can cause high background in fluorescence or luminescence based assays (Bochev *et al.*, 1993).

Teleost fish are highly variable when it comes to granulocyte identification and enumeration, and the species, age, treatment and cell definition create a complex data set, which is difficult to

compare or surmise (Catton, 1951). Some species are described as having heterophils, which are the most common type of granulocyte found in avian species. Some species are described as having heterophils, which are the most common type of granulocyte found in avian species though recently the presence of heterophils has been discounted as a common PMNL in teleosts (Flerova *et al.*, 2013). Differential histochemical and cytochemical stains, including myeloperoxidase, alkaline phosphatase, Periodic Acid Schiff and Diff-quick stain, may be used to identify neutrophils and other leukocytes in fish(Palic *et al.*, 2011). Moreover, the discovery of granulocyte colony stimulating factor receptor (G-CSFR) that is highly enriched on the surface of neutrophils in the cyprinids *Carassius aurata* (Katzenback *et al.*, 2012), *D. rerio* (Liongue *et al.*, 2009) and puffer fish (*Tetraodon nigroviridis*) (Santos *et al.*, 2006), has improved our ability to identify neutrophils and other granulocytes in fish. Nevertheless, the relative scarcity of mature circulating neutrophils amongst the blood cell population, coupled with their short lifespan and fragility, makes functional characterization and assays a significant challenge. Therefore, in the present study, we devise an enrichment method for peripheral blood granulocytes/neutrophils from whole blood, from barramundi, to enable analysis of immune function of this rare, yet critical, immune sentinel cell population.

All cells in the body undergo maturation processes, which eventually result in a terminally differentiated cell, fit for purpose. Neutrophils differentiate from common myeloid precursor cells and undergo a series of nuclear morphological changes, which are visible with standard staining techniques. Neutrophil maturation in mammals is mediated by interactions with bone marrow stroma, the site of granulopoiesis (Lund-Johansen *et al.*, 1993), categorised by granule composition. The granules move from azurophil through to gelatinase upon maturation, and each set of granules comprise specifically of particular enzymes and proteins (Bainton *et al.*, 1971). The earliest azurophil granules contain elastase, myeloperoxidase and lipases, involved in tissue remodelling after inflammatory damage (Elsbach, 1980, Janoff, 1985). Secondary (aka specific) and tertiary (aka

gelatinase) granules contain a number of metalloproteinases, calcium-dependent zinc-containing endopeptidases, with the purpose of extracellular matrix protein degradation (Mollinedo *et al.*, 1997). Tertiary granules also contain the preformed pool of the macrophage-1 receptor, which are rapidly mobilized during priming and may regulate neutrophil extravasation (Mollinedo *et al.*, 1997). Leucocytosis of active mature circulating neutrophils may be stimulated by compounds such as the chemotactic peptide N-formyl-Methionyl-Leucyl-Phenylalanine (f-MLP), C5a, or tumour necrosis factor alpha (Jagels *et al.*, 1995). Mature neutrophils play a vital role in antibacterial immunity, as well as clearance of dead and dying cells, tumour cells and tissue debris (Rydell-Tormanen *et al.*, 2006, Mantovani *et al.*, 2011, Caruso *et al.*, 2002).

The ontogeny of neutrophils in fish is quite different to that of mammals, hematopoietic stem cells are seeded from the kidney and head-kidney, not bone marrow as for mammals, and develop in those same organs (Kobayashi *et al.*, 2016). Many of the developmental studies in fish have been performed with zebrafish (*Danio rerio*) which allow *in vivo* observations and gene ablation (Huang *et al.*, 2008). More recently, specific transgene driven tagging of cell subsets allowed surveillance of cell maturation and function to occur, in response to inflammation or infection (Lieschke *et al.*, 2001, Ellett *et al.*, 2011). These *in vivo* models greatly enhance knowledge of cellular immunity in teleost as existing framework and accessory molecules that may be absent *in vitro* are in place (Gill *et al.*, 1991), although specific pathways may be more readily deconstructed *in vitro* (Ling *et al.*, 2000).

In mammalian neutrophils, priming is a key regulatory step to controlling unwanted cytokine production, which may result in tissue damage (Theilgaard-Monch *et al.*, 2005). Once primed by agents such as TNF-alpha, granulocyte macrophage stimulating factor and type II interferon, the cells are receptive to the secondary activating signal to unleash their chemical and biological attack (Takeshita *et al.*, 1998). The priming stage is a vital first step towards activation, and results in upregulation of macrophage-1 receptor, which is a complement receptor, comprising of CD11b (integrin alpha M) and CD18 (Integrin beta-2) (Kappelmayer *et al.*, 1993, Rainger *et al.*, 1998).

Priming also initiates assembly of the NADPH oxidase complex (DeLeo *et al.*, 1996), which is important in the production of reactive oxygen species, and prolongs neutrophil survival (Binder *et al.*, 1999). Prolonged survival of neutrophils allows enhanced cytotoxicity and sequestration of the activated neutrophils into tissues. This activation and extended lifespan of neutrophils is thought to contribute to, and exacerbate, tissue injury which may lead to multiple organ failure (Botha *et al.*, 1995, Biffl *et al.*, 1999). The resolution of neutrophils post-inflammatory response involves both apoptosis (Savill *et al.*, 1989), as well as retrograde chemotaxis back toward the vasculature (Mathias *et al.*, 2006). During the resolution phase, macrophages have been found to have neutrophilic granules in their cytoplasm indicating phagocytosis of apoptotic neutrophils (Cox *et al.*, 1995).

Enumeration of mammalian granulocytes may be done via fluorescence antibody labelling and flow cytometry, to observe a combination of receptor expression and levels. An assessment of the pattern recognition receptor CD14, adhesion molecule CD15 and the low affinity Fcγ receptor CD16 can be used for identification of granulocytes from monocytes, then further resolve neutrophils from eosinophils. Neutrophils and eosinophils are $CD14^{neg/low}$ and $CD15^{pos}$, whereas monocytes are $CD14^{high}$ and $CD15^{neg/low}$. Furthermore, mature neutrophils are $CD16^{high}$, eosinophils are $CD16^{neg}$ and monocytes are either $CD16^{neg}$ or $CD16^{intermediate}$ (Spitalnik *et al.*, 1989, Zea *et al.*, 2005). These antibodies are readily available for many mammalian species, however, reagents for fish are few, and those available are generally directed towards the developmental model of zebrafish (*D. rerio*). Simple enumeration of neutrophils may be performed with peroxidase stains and standard cell differentiation kits such as Hemacolor. However, it should be noted that the description of cellular identification, relative to the staining observed, is based on human cells, and may not always be applicable or appropriate for fish cell identification.

While Perciforms is the largest order of vertebrates, containing almost half of all bony fish, there is little similarity across the range of warm and cold water species, as well as fresh and marine environments. The order includes Cardinalfishes (Apogonidae), Cichlids (Cichlidae), Gobies

(Gobiidae) and Mackerels (Scombridae). Barramundi, of order Perciforms, are in the family Latidae, which contains 13 species. Nile perch (*Lates niloticus*) and barramundi are the two most well-known species in this family, though they are not common aquaculture species. Aquaculture, and thus research, tends to focus on high consumption or high price species, such as carp, catfish and tilapia representing the former, and salmon, trout and sturgeon the latter. Reports on blood cell populations vary, and may differ due to seasonal variation, environmental factors and whether fish are wild caught or a contained species (Gabriel *et al.*, 2004). Abiotic factors including temperature, dissolved oxygen levels and water flow quality, coupled with biotic factors such as food supply, stress and disease, may influence gene expression, protein synthesis and cellular make-up (Johnston, 2006, Bly *et al.*, 1991, Montero *et al.*, 1999). It is important, therefore, to work with as consistent and reproducible cell source as possible, for research with a non-model fish.

Regarding barramundi, description of blood cellular subsets have only been published for fresh water fish (Anderson, 1996). The mean leukocyte percentage in whole blood was ~25% and, of this, granulocytes were determined as a whole. The range for granulocytes was vast, from 0.1 to 11.7 million per ml; the average of 30 fish was 2.5 million per ml, which was equivalent to 9% of the 25% leukocyte cell count. In the present study, we aim to devise an enrichment method for peripheral blood granulocytes / neutrophils from whole blood derived from barramundi reared in brackish, recirculating water. We show that these methods permit reproducible investigation of the neutrophilic response to *Streptococci*.

3.2 Materials and Methods

3.2.1 Barramundi phlebotomy and organ harvest

Barramundi fingerlings were obtained from Australian Native Fish Enterprises (Kallangur, Australia), at a 20 - 40 mm size and maintained in recirculated, aerated, brackish water (12 - 15ppt salt), at 28°C, with water parameters checked daily and partial water exchanges performed as required. They

were fed with Ridley Native finfish diet (Ridley Aquafeeds Ltd, Narangba, Australia) twice daily, until satiation, with pellet size adjusted to suit the size of the fish. Blood was collected by caudal venipuncture with a syringe and 23G needle, immediately following euthanasia by overdose of anaesthetic (Aqui-S) when fish were 60 - 100 grams (fork length 80-100mm minimum).

Organ removal was also performed aseptically and individual organs placed in a 35 mm petri dish, containing room temperature phenol-red free RPMI (Sigma-Aldrich, Castle Hill, Australia). Blood draws were not done prior to organ harvest to avoid removal of any cells from the organs during the process (Palic, unpublished data, 2015). Neutrophils have a short lifespan, and cold temperatures damage fish neutrophils, so all work was performed at room temperature (RT) and stimulations (unless stated) at 30°C.

All individual Percoll density dilutions were made prior to use and stored at 4°C, but allowed to reach room temperature and layered to form discontinuous gradients immediately before use. Varying densities were made by diluting Percoll, with appropriate volumes of 10x Hank's Balanced Salt Solution (HBSS) (Thermofisher Scientific, Australia), and sterile water, with the ratios determined using the Percoll Calculator Web App: http://www.gelifesciences.com/webapp/wcs/stores/servlet/CategoryDisplay?categoryId=1405674&catalogId=82073&productId=&top=Y&storeId=11752&langId=-1.

3.2.2 Neutrophil isolation from kidney and head-kidney

The kidney and head-kidney were removed aseptically, washed in phenol free RPMI, and placed in a 35 mm petri dish with fresh media. A single cell suspension was made by grinding the organ through a 70 μm mesh strainer (Becton Dickinson, Australia) with the flat end of a 1 ml tuberculin syringe plunger (Becton Dickinson, Australia). The cell suspension was spun over a multistep Percoll (Sigma-Aldrich, Newcastle NSW, Australia) gradient densities assessed were from 1.06 - 1.085 g / ml. The granulocytic fraction was collected (~1.08 g / ml), washed and cytospin slides prepared. The slides

were fixed, stained for peroxidase via DAB (Sigma-Aldrich, Australia) and counterstained with Hemacolor Rapid staining kit (Millipore, Bayswater, Australia) to identify neutrophils.

3.2.3 Melanomacrophage centres in the kidney

Dense, particulate melanomacrophage centre cells heavily contaminated all kidney preparations; whole organ slides were made and stained to observe the size and prevalence. The kidney was removed, as whole as possible, washed once in Tissue-Tek O.C.T. Compound (Olympus Australia, Notting Hill, Australia) to remove excess blood, before being placed in fresh Tissue-Tek and covered by the liquid inside a small plastic mounting chamber tray. The trays were placed at -80°C overnight to freeze the block and embedded organ solid. Serial sections of 5 μm thickness were obtained using a Hyrax C60 Cryostat. The sections were warm thaw mounted onto standard glass slides, before being stained with Hemacolor as per manufacturer's instruction. Micrographs were taken using an Olympus BX41 epifluorescent microscope, images captured with an Olympus DP26/U-CMAD3 camera.

3.2.4 Development and optimisation of neutrophil enrichment

Dextran solution was assessed at various concentrations (1%, 2%, 3%, 6% w/v 0.9% NaCl) and mixed 1 : 1 with anti-coagulated blood, before 6% was selected for further optimisation of neutrophil enrichment, due to consistently cleaner separation of settlement layers. Various times were assessed before it was determined that a 30 - 60 min dextran settlement, followed by a 30 min spin at 10 x *g* gave the most reliable and consistent separations of leukocytes and complexed dextran-RBCs. During the optimisation procedure, layers were collected and cytospins were prepared for each layer, to determine where the neutrophil populations were in the gradient. The Percoll gradients were also optimised, with changes in both the densities and the number of layers, however, the 8 step gradient was found to consistently capture and separate reticulocytes and erythrocytes from white blood cells, and the RBCs had a broad range of densities, from 1.06 - 1.08 g

/ ml. Each step of the process was determined to be a requirement of the overall enrichment and loss of enrichment occurred when they were not included. All gradients were centrifuged in a swinging bucket rotor, (Eppendorf, North Ryde, NSW, Australia) at room temperature. Cell counts with trypan blue for viability assessment were performed using a standard Neubauer Chamber haemocytometer.

3.2.5 Cytospin preparations and staining

All cytospin slides were prepared with a cellspin I (Tharmac GmbH, POCD, Artarmon NSW, Australia), chambers, slide cards and funnels, centrifuged at 800 x *g* for 3 min. The slides were allowed to air dry completely before fixation for 60 s with 10% (37% formalin) / 90% (95% ethanol) which was always made prior to use. The slides was washed in reverse osmosis (RO) water and allowed to air dry. Peroxidase granules were stained with DAB (Sigma-Aldrich, Australia) as per manufacturer's instructions and allowed to air dry. Lastly, the slides were counterstained with Hemacolor, allowed to dry before coverslip mounting with Permount (ThermoFisher Scientific, Australia), allowed to dry overnight at 4°C before imaging with 40 x and 100 x magnification.

3.3 Results

3.3.1 Neutrophils of the barramundi kidney

Neutrophil isolation was initially attempted with kidney and head-kidney preparations. The kidney was loaded with black granular particles which flooded the preparation; there was no simple way to remove the silt like contamination even with multiple wash steps (Fig. 1A). While the yield of neutrophils was high, staining was inconsistent and there was difficulty identifying peroxidase positive neutrophils from peroxidase positive monocytes (Fig. 1B). The contamination was also seen at lesser levels in the head-kidney, but this varied between fish.

3.3.2 Melanomacrophage centre histology

Due to the inability to remove the melanic particles from organ preparations, sections were cut and stained with Hemacolor to assess the size and density of the melanomacrophage centres (MMC), to determine if there was a feasible way to removed them prior to single cell preparation. While there was variation between the depth of colour of the MMC black grains between cell preparation, the three fish assessed via kidney section all displayed frequent, large, dense sporadic centres throughout the length of the organ (Fig. 1C). There did not seem to be any common area or region that was not affected by the masses.

3.3.3 Dextran settlement and Percoll gradient for neutrophil enrichment

A procedure was optimised for enrichment of barramundi blood neutrophils. A blood smear demonstrated the scarcity of white cells compared to red blood cells (Fig. 2A) necessitating enrichment for immunological experimentation. Blood was drawn, mixed with 10% Citrate-Phosphate-Dextrose anticoagulant and then mixed with equal volumes of pre-filtered 6% dextran in 0.9% NaCl at room temperature. The combination was gently inverted and allowed to stand undisturbed for 30 - 60 min (Fig. 2B). Subsequently, the tube was spun for 30 min at 10 x *g* which resulted in settlement of dextran-red blood cell complexes, and consequent separation from the opaque suspension containing neutrophils at the top of the tube (Fig. 2C). This opaque fraction was collected, minimising red blood cell contamination, and further purified through an 8-step Percoll gradient, prepared fresh with room temperature gradient solutions. The gradient was spun at 400 x *g* for 30 min with no brake or acceleration, forming multiple layers of red blood cells, leaving the white buffy layer at the top of the Percoll at a density of 1.08 g / ml. This was collected via wide bore pipette, washed once with phenol free media- 0.1% FBS, counted with trypan blue for viability assessment and used for subsequent assay.

3.3.4 Differential cell counts of whole blood from barramundi and enrichment

Blood was draw from four healthy, not previously bled barramundi and haematocrit and differential cell counts performed by Brisbane Birds and Exotics Veterinary Services (BBEVS, Greenslopes, Brisbane, Australia). Cytospin slides post-enrichment were also counted to assess enrichment and range. One hundred field views of peroxidase and Hemacolour stained cytospins were counted at 40 x magnifications across a range of slides prepared on different days (Table 1).

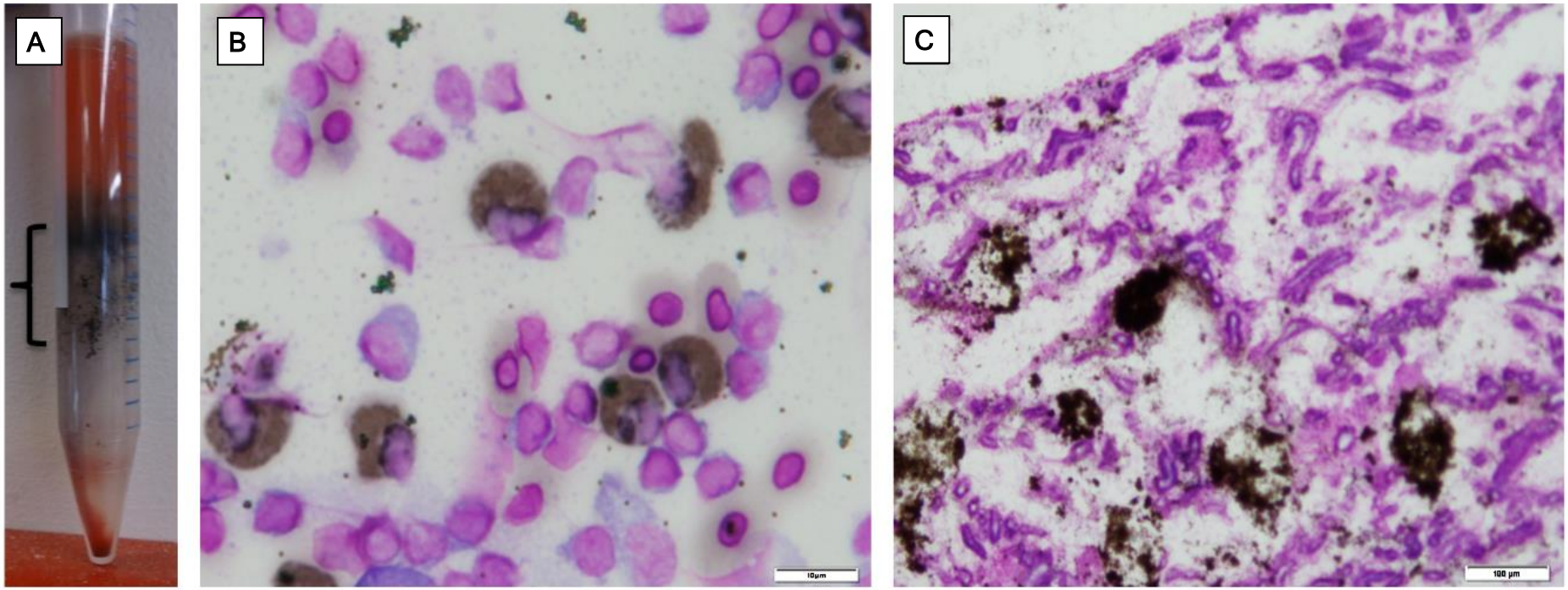

Fig. 1 Whole barramundi kidney pathology, neutrophil enrichment gradient and cytospin. A) Barramundi kidney cell suspension on Percoll density gradient, bracket indicating neutrophil rich region. B) Barramundi kidney cell suspension harvested from Percoll gradient, stained for peroxidase (brown) and cell differential via Hemacolor, 100 x magnification, 10 μm bar. C) Assessment of density and placement of MMC in 5 μm serial section of barramundi kidney, 20 x magnification, 100 μm bar.

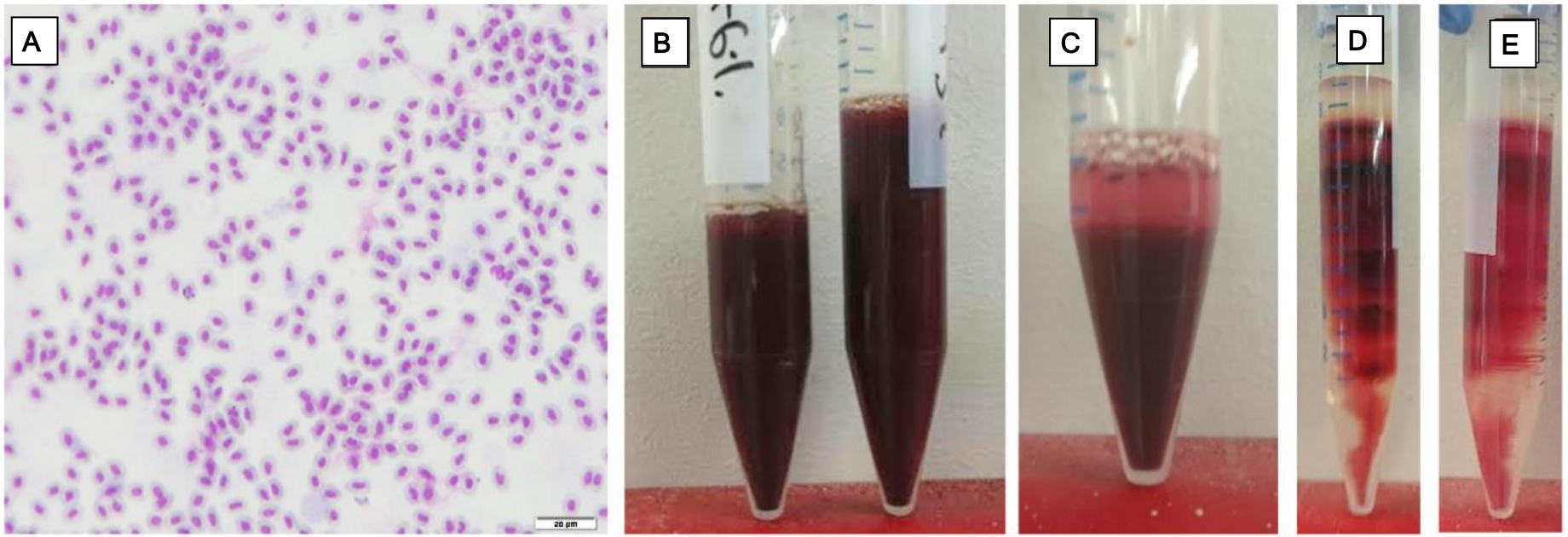

Fig. 2 Barramundi blood smear and neutrophil enrichment process. A) Barramundi whole blood smear stained with DAB and Hemacolor, 40 x magnification. B) Whole blood mixed 1:1 with 6% dextran (left) and 3% dextran (right) after 30 min gravity settlement. C) Blood treated as for B subsequently spun at 10 x g for 30 min, RT, no brake or acceleration. Percoll 8 step density gradient with 6% dextran D) - blood sedimentation and 3% dextran E) - blood sedimentation, banding at density interfaces.

Table 1. Differential cell counts on blood smears taken from healthy Lates calcarifer and reared in 15 ppt salt water. Four fish were sampled. For comparison, ranges of white blood cell % (WBC) and post dextran-Percoll neutrophil enrichment are included, 100 field views were surveyed via light microscopy at 40 x magnification.

	Whole Blood	Post Enrichment
packed RBC	30 - 43 %	n/a
reticulocytes	3.6 - 10 %	n/a
white cells	3.4 - 7.8 %	< 90%
lymphocytes (% WBC)	64 - 92 %	35 - 70%
monocytes/ macrophage (% WBC)	4 - 16 %	5 - 25%
neutrophils (% WBC)	6 - 36 %	25 - 50%

3.4 Discussion

The kidney and head-kidney are most commonly used for neutrophil studies in fish, given that the yield is substantially higher than other organs or fluid (Palic *et al.*, 2007b, Ainsworth, 1992). These are also the sites of neutrophil development and maturation, so it would be usual to have a developmental range of neutrophils in these organs, from precursors through to band cells and mature neutrophils, ready to egress in response to chemotactic signal (Sepulcre *et al.*, 2002, Hine, 1992, Flerova *et al.*, 2013). While there were many neutrophils in the head-kidney of barramundi, these appeared immature with the nucleus still developing its distinct multi-lobulated polymorphonuclear morphology. In mammals, neutrophils are classically defined as terminally differentiated with fully formed granules and segmented nuclei (Kolaczkowska *et al.*, 2013). Work in zebrafish (*D. rerio*) and other teleost, tend to identify maturation as a functional capacity for migration, phagocytosis, NET production or high levels of MPO (Havixbeck *et al.*, 2015, Bennett *et al.*, 2001). While tools for differentiation of maturation status are not available for barramundi, for these studies we consider nuclear segmentation to be an indicator of cellular maturity.
While both neutrophils and monocytes stain positive for peroxidase, neutrophils are slightly larger in size than monocytes, which have a larger, rounded nucleus, making the two cell type quite distinctly different when mature. Immature neutrophils are difficult to identify from monocytes, when they both have a “kidney bean shaped” or rounded nuclei, and both stain for peroxidase.

The method developed herein enhanced the percentage of mature neutrophils approximately three fold as a percentage of total white blood cells. The range between fish was high, even with stringent tank conditions to minimise stress and environmental differences. This was, however, also observed in the published cellular ranges of fresh water barramundi blood cells analysis where granulocytes were 2-27% of the white cell count, with a mean of 9 ± 1% (Anderson, 1996). The gradients were optimised and repeatedly assessed in an attempt to decrease the number of density layers. These changes decreased the enrichment of neutrophils, with an increased contamination of red blood

cells so the eight layer gradient was permanently adopted. The differential count indicated the reticulocyte count could be as high at 10% of the red blood cell count, and these immature red blood cells overlapped closely with neutrophil density, necessitating multiple close gradients to draw the two cell types away from each other for neutrophil harvest. While rare cell enrichment procedures often necessitate gradients and non-antigen receptor enrichment, the yield obtained from such procedures may not include all the cells in the starting population. Moreover such density based isolation may also fail to allow for, or collect, any deviations of the sub-populations that are currently ill-defined in the host.

With development of the whole blood bactericidal assay previously discussed (Chapter 2), it was determined that circulating blood neutrophils should be used as a source of neutrophil for assay comparison and consistency, especially since bacterial propagation occurs in blood during streptococcal infection. Using blood-enriched neutrophils allows comparison of bactericidal activity, to other immune functions. However red blood cells confound results, necessitating their removal.

Kidney and head-kidney cell preparations were variable in the amount of melanomacrophages, which form a centre similar to a splenic germinal centre for immune presentation (Herraez *et al.*, 1986) and may sequester toxins and free radicals (Agius *et al.*, 2003). The presence of MMC varies in size, morphology, and cellular populations in different teleost species (Wolke *et al.*, 1985, Haaparanta *et al.*, 1996, Leknes, 2007)**.** The cause of the actual variation is unknown, but may be due to stress, age or health. The fine, black, granular particles were very difficult to separate from neutrophils and gave highly variable yields. The contamination of Percoll gradients and cell suspension was readily observed numerous times. The dark melanin granules also gave high amounts of background in fluorescent and absorbance detection assays.

Previously utilised enrichment methods for mammalian blood were used for barramundi blood with little success. Ficoll, a high mass hydrophilic polysaccharide, solutions 1009 and 1119 (Sigma-Aldrich, Newcastle NSW, Australia) were used to create a two part gradient (densities 1.009 and 1.119 g /

ml). The gradients did not sufficiently removed red blood cells, or identify a distinct leukocyte layer. Cytospins of the layers displayed few granulocytes and the majority of the cells were erythrocytes.

Similar results were obtained with whole blood on a discontinuous Percoll gradient, various densities and combinations were tested but erythrocytes and reticulocytes continued to swamp the enrichment yield. Percoll, colloidal silica coated with polyvinylpyrrolidone, may be diluted with Hanks' Balance Salt Solution (HBSS) to create multi-step densities, constructed by slowly sequentially layering each solution from most dense to least dense, with blood placed on top. Similar solutions were tested including Mammalian Lympholyte (Cedarlane Labs, Bio-Scientific, Kirrawee, NSW, Australia), a combination of sodium diatrizoate and dextran and Mono-Poly Resolving Medium (MP Biomedicals, Seven Hills, NSW, Australia) a Ficoll-Hypaque solution, but none separated leukocytes from RBCs sufficiently. As well as various density solutions, simple adherence was also assessed; the panning method may be used for mammalian cells to separate lymphocytes and erythrocytes from myeloid derived cells (plated with or without antibody). This method did not separate any cell populations in fish blood; RBCs adhered to glass slides and plastic trays as readily as target leukocytes within 10 - 20 min, as assessed by Hemacolor staining.

Removal of RBCs was also attempted via osmotic lysis which did not succeed, which may be due the osmoregulatory ability of barramundi, as they can quickly acclimatise from fresh water to marine environments and *vice versa*. The lysis solution of ammonium citrate is commonly used for red blood cell lysis in mammals; however this buffer is most effective on non-nucleated RBCs. Since fish have nucleated RBCs, this solution failed to remove RBCs. It was vital to several assays (respiratory burst and myeloperoxidase release) that RBC contamination be minimised, to be able to ascertain if the innate cells were functional against whole bacteria and pathogenic associated molecular patterns.

Mammalian neutrophils require a two-step priming and activation process, and the complex of macrophage -1 (CD11b/ CD18) is only present in tertiary granules, found in band and segmented neutrophils. This suggests that only mature neutrophils can become fully activated, and partake in

inflammatory processes of microbial and cellular clearance (Hertzog, 1938, Martinelli *et al.*, 2004). Healthy humans do not have immature neutrophils in their peripheral blood, and immature neutrophils may be an early indicator of systemic inflammation from sepsis (Nierhaus *et al.*, 2013). Whether this stands true for fish neutrophils is unknown. However, it supports the notion that using a source of mature cells would be more appropriate for measuring immune response, rather than a mixed population, which may contain a range of developmentally immature cells. Examining the peritoneal cavity immune response of rainbow trout (*Oncorhynchus mykiss*) and *Yersinia ruckeri* infection, it was postulated that in a resting state, resident macrophages are the primary phagocyte, however under inflammatory conditions, mature neutrophil influx make neutrophils the most common phagocyte (Alfonso *et al.*, 1998). Such an influx was also observed in the swim bladder of carp (*Cyprinus carpio*), red sea bream (*Pagrus major*) or tilapia (*Oreochromis niloticus),* upon injection of *E. coli*. The rapid influx of cells peaked at 48 h, indicating that fish, like mammals, have reserves of mature, highly mobile neutrophils, which can be called to regions of inflammation when needed (Matsuyama *et al.*, 1999). Comparison of resting kidney neutrophils and inflammatory neutrophils, derived from heat killed bacteria injected into the peritoneal cavity, revealed carp (*C. carpio*) showed a marked increase in inflammatory neutrophil activity over the resting population. In the same set of experiments, ayu (*Plecoglossus altivelis*) showed little difference, with both populations highly active for the ability to produce reactive oxygen species upon stimulation (Serada *et al.*, 2005). This suggests that pre-priming of neutrophils may be required for some fish species, but not others, especially the resting population in kidney, compared to those neutrophils found in the periphery. Therefore, studies involving neutrophilic response are best done with mature circulating neutrophils.

CHAPTER FOUR: What are the *in vitro* interactions between blood neutrophils with *S. iniae* / *S. agalactiae* and how do the bacteria circumvent these immune strategies?

4.1 Introduction

Streptococcus iniae rapidly proliferates in barramundi blood, demonstrating that there are escape mechanisms operating that protect the bacteria from the innate cellular and humoral immune system, and for long enough for numbers to amplify to an overwhelming level. How *S. iniae* is able to do this seems to be enhanced by, but not dependent on, the polysaccharide capsule, since acapsular QMA0249 survived, but did not appear to proliferate. Low lysozyme levels in the blood of healthy barramundi, as demonstrated in this thesis, coupled with capsule-mediated complement resistance reported previously (Barnes *et al.*, 2003) contribute towards an explanation of lack of clearance by humoral factors. However, there is currently no explanation regarding how *S. iniae* avoids elimination by the major sentinel immune cells of the blood system, the neutrophils. The breakdown in neutrophil bacterial clearance may occur as a result of failure of one or more key processes. These may include the ability to sense or detect invading microbes, migration towards them, engagement and subsequent phagocytosis, and destruction of the bacteria by exogenous and endogenous antimicrobial factors.

In this study, we employ circulating, functionally mature enriched neutrophil populations from barramundi blood, to elucidate the methods of neutrophil evasion by *S. iniae*. The source of neutrophils for studies over the years has compared and contrasted with circulating blood neutrophils, organ sourced neutrophils, to those collected via inflammatory chemotaxis. Obviously, sourcing neutrophils is related to the ease and availability of collection from the research model used, most frequently blood from humans (Maqbool *et al.*, 2011), bone marrow from mice (Swamydas *et al.*, 2013) and haematopoeitic tissue (kidney and head-kidney) in fish (Palic *et al.*, 2007a). While blood contains mature neutrophils, in small animal models this may not

provide a yield sufficient for studies without pooling samples. Bone marrow, much like the kidney and head kidney in fish, are sources of granulopoiesis, therefore there will be arrange of maturity levels of cells which may alter or underestimate responses (Berkow *et al.*, 1986). The blister based model of inflammation causes rapid influx of neutrophils, likely in response to c5a, (Kuhns *et al.*, 1992), but is by and large, a tissue injury model without a defined antigen (Jenner *et al.*, 2014). Comparison of neutrophils source from humans assessed *ex vivo* stimulated blood, and *in vivo* cantharidin blister, skin windows and intra-dermal injection of UV-killed *E.coli*. They found no significant differences of expression of activation markers or phagocytic capacity (Maini *et al.*, 2016).

We determine whether host sentinel cells are functional *in vitro*, and compare *S. iniae,* non-pathogenic *S. agalactiae* and *E. coli,* to determine how *S. iniae* may block or subvert neutrophils, thereby allowing rapid proliferation, sepsis and death.

4.2 Materials and Methods

4.2.1 Bacterial strains and culturing

S. iniae QMA0248 and *S. iniae* QMA0249 were isolated from infected barramundi at a recirculating aquaculture farm during an acute and chronic outbreak of streptococcosis respectively. Both were isolated from previously vaccinated fish; QMA0248 is capsular whereas QMA0249 is acapsular with a frameshift mutation in *cpsE* resulting in truncation of the protein and a deletion of the genes *cpsF* through *cpsM* (Millard *et al.*, 2012). *S. agalactiae* QMA0285 and QMA0281 were both isolated from dead wild giant grouper *Epinephelus lanceolatus* (Cornacchione *et al.*, 1998). QMA0285 is virulent and capsular (Cornacchione *et al.*, 1998) while QMA0281 is acapsular with a deletion from mid-cpsB through to mid cpsE. Strains were stored without sub-culture at -80°C in Todd-Hewitt Broth (THB) containing 20% glycerol. Strains were grown routinely on Columbia agar base containing 2% defibrinated sheep blood at 28°C. For liquid culture, strains were grown in THB under microaerophilic conditions with minimal agitation to prevent settling.

4.2.2 Experimental animals and husbandry

Barramundi fingerlings were obtained from Australian Native Fish Enterprises (Kallangur, Australia) at a 20 - 40 mm size and maintained in recirculated, aerated, brackish water (12-15 ppt salt), at 28°C, with water parameters checked daily and partial water exchanges performed as required. They were fed with Ridley Native finfish diet (Ridley Aquafeeds Ltd, Narangba, Australia) twice daily, until satiation, with pellet size adjusted to suit the size of the fish. Blood was collected by caudal venipuncture with a syringe and 23G needle following overdose of anaesthetic (Aqui-S) when fish were 60 - 100 grams (fork length 80 - 100mm minimum).

Acute inflammatory plasma was prepared from an intraperitoneal injection with 100 μl of QMA0248 autogenous formalin killed vaccine as previously described (Aviles et al., 2012). The fish were sacrificed at 24 h post-vaccination, bled and the plasma collected from five fish was pooled and stored at -20°C.

4.2.3 Neutrophil isolation from peripheral blood

Freshly drawn anti-coagulated blood was mixed 1 : 1 v/v with sterile filtered 6% dextran (Mr 450,000-650,000 from *Leuconostoc* spp. Sigma-Aldrich, Castle Hill, Australia) in 0.9% NaCl, all solutions at room temperature. Tubes were gently inverted 3 - 5 times to ensure mixing, and allowed to stand undisturbed for 30 - 60 min, followed by a 30 min centrifugation at 10 x *g*. The uppermost opaque layer was removed via wide bore transfer pipette and placed on an 8 step Percoll (GE Healthcare, Parramatta, Australia) gradient, of densities, 1.092, 1.089, 1.086, 1.083, 1.08, 1.75, 1.07, 1.06 g / ml. All gradients were centrifuged in a swinging bucket rotor, (Eppendorf, North Ryde, NSW, Australia) at room temperature with no brake and the lowest acceleration setting. Cell counts with trypan blue for viability assessment were performed using a standard Neubauer Chamber haemocytometer.

4.2.4 Neutrophil migration

Neutrophil chemotaxis was assessed in a 3 μm pore 96 well microchamber plate (Neuroprobe, Gaithersburg, Maryland, USA). In a similar procedure to standard Transwells (Cooper *et al.*, 1995) media is placed in the bottom well, and cells on top of the filter membrane. Lower chamber contained 300 μl phenol red free RPMI- 0.1% FBS with or without stimuli and cells were added at 5 x 10^6 cells / ml, 30 μL per well. Conditions included f-MLP (Sigma-Aldrich, Castle Hill, Australia) (200 μg / ml), acute inflammatory plasma (diluted 1:3 with media), zymosan (Sigma-Aldrich, Castle Hill, Australia) (100 μg / ml), bacteria (MOI of 1), or tissue culture medium alone. The plate was incubated for up to 90 min at 30°C, no significant loss of viability was observed via cytospin and histochemical stain or via flow cytometry during this timeframe. The top filter membrane was removed, plate centrifuged at 400 x *g* and 200 μl removed. Cells were mixed 1 : 1 v/v with trypan blue and counted on a haemocytometer via light microscope to assess migration and viability. Biological replicates of six fish were used for this experiment.

4.2.5 Neutrophil ROS production

Respiratory burst was measure via chemiluminescence, whereby luminol substrate is oxidised and detected in a microplate luminometer (Hyslop *et al.*, 1984, Wymann *et al.*, 1987). A Fluostar Optima (BMG Labtech, Germany) was used to capture luminescence. Blood was drawn and neutrophils enriched as described above, counted and resuspended to a final concentration of 1 x 10^7 cells / ml in phenol red free RPMI- 0.1% FBS using 100 μl of cell suspension per reaction. A 1 M stock solution of luminol was made in DMSO and frozen at -20°C. From this, a working stock of 10 mM was made with 0.2 M borate buffer, pH 9.0. Both borate buffer and luminol were thawed to room temperature just prior to use, mixed and used immediately. The solutions were not reused or refrozen once thawed.

Cell stimulations were set up in a 96 well flat bottom black plate (Greiner, Germany). Cells were added into the wells, then in a separate plate, stimuli and luminol were combined 1 : 1 and 100 μl combined solution transferred to the cells via multichannel and read immediately, every 30 s for 30 to 180 min. Positive controls included calcium ionophore (CaI) (Sigma-aldrich, Australia) (4 μg / ml) and Phorbol 12-myristate 13-acetate (PMA) (Sigma-aldrich, Australia) (2 μg / ml). For bacteria stimulations, single bacterial colonies were picked from an agar plate and grown overnight to mid to late log-phase in Todd-Hewitt broth. *E. coli* and *S. iniae* were diluted to an OD_{600} of 0.45 and *S. agalactiae* 0.3. All bacterial stimulations were performed at a multiplicity of infection (MOI) of 1. Where cell numbers allowed it, technical replicates of three were assessed, with biological replicates of 3 - 8 fish per experiment.

4.2.6 Neutrophil peroxidase release

Detection of neutrophil degranulation may be observed as MPO exocytosis, utilising 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-Aldrich, Australia) as a substrate which is altered to a soluble pale blue end product, prepared as per manufacturer's specifications. The chromogenic substrate acts as a hydrogen donor to peroxide and the colour change can be assessed spectrophotometrically at 370 nm or 620-650 nm or stopped with acid and the yellow end product read at 450 nm. A sensitive and specific reagent for the detection of blood haemoglobin and peroxidases, it has been adopted for assessing pooled cells from whole zebrafish (*D. rerio*) kidneys (Palic *et al.*, 2007a) or fathead minnows (*Pimephales promelas*), from which neutrophils were separated and assessed for degranulation (Palic *et al.*, 2005). This assay was adapted and optimised for barramundi blood-enriched neutrophils.

In brief, barramundi blood was collected, neutrophils enriched, counted as per standard procedure above, and resuspended to $5x10^6$ cells / ml in phenol red free RPMI- 0.1% FBS and 50 μl per well employed for each assay. Using a 96 well flat bottom plate, cells were stimulated with bacteria or

positive controls including. Controls were as for the ROS assay (CaI 4 μg / ml, PMA 2 μg / ml). For bacterial and control stimulations, all culturing, dilutions and conditions were performed as described for ROS assay. Two sets of wells were set up for all stimulations, and after an incubation period of 30 min at 30°C, Triton-X (Sigma-Aldrich, Australia) was added (0.02%), to lyse fish cells, liberating contents to assess total peroxide content. This measure of total peroxidase, via cellular lysis, reflects all MPO within the neutrophil, which may be released into a phagosome for microbial degradation, or exogenously released for degrading larger organisms. While immature cells may contain MPO, mature cells are the primary instigators of MPO mediated release via azurophilic granule degranulation. Where cell numbers allowed it, technical replicates of three were assessed, with biological replicates of cells from 3 - 5 fish per experiment. Once stimulation was complete, and detergent added to positive wells for 2 min for lysis, 50 μl of TMB solution was added to the wells.

To calibrate the assay, a standard curve titration of peroxidase was added to each plate and read using a Fluostar Optima luminometer (BMG Labtech, Germany) with appropriate filters for colourmetric detection. The percent release was calculated as previously published as follows: % release = $[(OD_{stimulated} - OD_{background})/ (OD_{lysed} - OD_{background})] \times 100$ (Palic *et al.*, 2007a).

4.2.7 Neutrophil DNA release

DNA release was measured in microplate format by adapting a previously published method, used to assess pooled fathead minnow (*P. promelas*) kidney neutrophil preparations (Palic *et al.*, 2007b). This assay was adapted for barramundi blood enriched neutrophils in a similar way to the MPO release, with CaI and PMA as positive controls. In brief, neutrophils were enriched and resuspended in phenol red free RPMI/ 0.1% FBS at 5 x 10^6 cells / ml, using 50 μl per well for assay in a black 96 well flat bottom plates (Greiner, Germany). Stimuli were added at 50 μl per well, bacteria added as previously described at an MOI of 1. The plates were incubated for 60 min at 30°C, with unstimulated cells used as the baseline for background DNA release. After the incubation period, a set of control wells were treated with Triton-X, as for MPO release, to gain maximal DNA-SYTOX

signal. Sytox Green Nucleic Acid Stain (Life Technologies, Fisher Scientific, Australia) was added at a final concentration of 0.1 μM and incubated in the dark for 5 min before the fluorescence was read with output designated as Arbitrary Fluorescence Units (AFU, excitation 504 nm, emission 523 nm) using a Fluostar Optima microplate reader (BMG Labtech, Germany) with appropriate filters for fluorescence detection. Bacteria alone were also assessed, and there was minimal signal change from 0 to 2 h indicating the SYTOX signal was not affected by the bacteria.

4.2.8 Neutrophil phagocytosis and degranulation

The ability of innate immune cells to phagocytose may be assessed via engulfment of fluorescently labelled bacteria, detected via flow cytometry. The ability of cells to morphologically arrange around bacteria, and engulf them into a phagosome, is dependent on actin microtubule rearrangement, which may be blocked with cytochalasin D (cytoD) (Casella *et al.*, 1981). The difference therefore between non-treated and cytoD treated neutrophils, reflects active phagocytosis, and not non-specific binding of bacteria to the outside of cells.

The basis of this assay was an amalgamation of several published methods, whereby labelled bacteria were incubated with enriched cell populations (Staali *et al.*, 2006, Luft *et al.*, 2002). This method had been used previously in fish, to examine phagocytosis of fluorescently labelled yeast cells (*Saccharomyces cerevisiae*) by gilthead seabream (*S. aurata* L.) head-kidney enriched leukocytes (Rodriguez *et al.*, 2003).

Bacteria were labelled with BacLight™ Red Bacterial Stain (QMA0248, QMA0285) or BacLight™ Green Bacterial Stain (QMA0249, QMA0281, *E. coli* DH5α) (Thermofisher Scientific, Australia) at 1 μM / ml for 15 min then washed extensively with PBS to remove excess dye and avoid dye leakage or transfer. Two strains of bacteria (virulent strain dyed red, acapsular green) were added to cells at an MOI of 1 (each) and incubated in flow cytometry tubes at 30°C for 60 min. The tubes were washed once with 2 ml 1 x PBS and spun at 400 x *g* before final resuspension in 300 μl.

To determine active phagocytosis, one set of duplicate tubes was pre-treated with 10 mM cytochalasin D to depolymerise actin (which inhibits phagocytosis), for 30 min at 30°C. The amount of bacteria binding with cytochalasin D treatment is both bacteria that would have been phagocytosed, plus any attached on the outside of the host cells. This is compared to cells which were not treated and would be able to engulf the bacteria. The cytoD also remained in the subsequent bacterial incubation. Cell mixtures were analysed with a BD LSR II Analyser with filters 660-Red, 655-Violet and resulting data processed with FlowJo, version 6. Live gates were placed around viable cells based on forward and side scatter, and the entire gate analysed for 660-Red and 655-Violet signal comparing cytoD treated conditions to the matching untreated.

4.2.9 Neutrophil viability

To assess the effect bacteria may have on the viability of blood enriched neutrophils, Alexa Fluor® 488 Annexin V/ Dead Cell Apoptosis Kit (ThermoFisher, Australia) was employed according to the manufacturer's instructions and the results assessed by flow cytometry. Camptothecin, a potent inhibitor of topoisomerase I, was used at 150 μg / ml as titrated and optimised, as a positive control to induce apoptotic cell death (Wall *et al.*, 1966). The negative control was unstimulated cells. Annexin buffer, Alexa Fluor® 488 annexin V and propidium iodide (PI) working stocks were all prepared as instructed on kit specifications sheet, however, two changes were made; PI stock solution was used at a further 1:50 dilution, and 2 μl (rather than 5 μL) Alexa Fluor® 488 annexin V was used per tube. Annexin V labels intracellular phosphatidylserine, identifying apoptotic cells and PI labels DNA in cells that have lost membrane integrity.

Bacteria were grown overnight to mid-log phase, and prepared as for previous assay, added at an MOI of 1. Enriched blood neutrophils were prepared as for previous assays. Post washing and counting, the cells were resuspended in annexin-binding buffer to 1×10^6 cells / ml, 100 μl per flow cytometry tube. A volume of 2 μl Alexa Fluor® 488 annexin V and 5 μl of 100 μg / ml PI were added to each cell-bacteria mixture, and incubated for 60 min at room temperature. After the incubation

period, suspensions were further diluted up to 400 μL with 1X annexin-binding buffer, mixed gently, and analysed on the BD LSRII analyser using filters appropriate for fluorescein (FITC) and tetramethylrhodamine (TRITC) or Texas Red® dye.

On a 2-dimensional scatter plot, three groups were identified, live (double negative), apoptotic (single FITC/ Alexa 488 positive), and dead (PI positive). Analysis of data was performed with FlowJo, version 6.

4.2.10 Bacterially produced nucleases

Nuclease activity was assessed via measuring the degradation of calf thymus deoxyribonucleic acid (Sigma-Aldrich, Australia) based on published methods for similar assessment of *Aeromonas hydrophila* in carp (*C. carpio*) studies (Brogden *et al.*, 2012). The DNA was reconstituted as per product recommendations, at 1 mg / ml in molecular biology grade water. To create a standard curve of nuclease activity, micrococcal nuclease from *Staphylococcus aureus* was used in DNase buffer (3 mM MgCl2, 3 mM CaCl2, 300 mM Tris; pH 7.4). Using 200 μl per well, a standard curve of nuclease (200 units top dose, serially diluted) was constructed with DNA at 1 mg / ml and DNase buffer. Samples of nuclease or bacterial supernatant or homogenate were assessed in parallel. For assessment of bacterial production, overnight late log phase bacteria were centrifuged (~15,000 x *g*), the supernatant collected, and pellet washed thoroughly in PBS then homogenised with glass beads (115 μm, Sigma-Aldrich, Australia), at 9000 rpm for 45 s in a Roche Magnalyser (Roche Diagnostics). Unbroken cells and debris were removed by centrifugation, in a microfuge at full speed for 5 min, and 50 μl of the resulting supernatant assayed for nuclease activity.

The plate was incubated at 37°C for 4 h, HydraGreen (ACTGene, Inc., Piscataway, USA) was added at a dilution of 1:8000, and the plate read, with excitation at 480 nm and emission at 520 nm. Calculations were performed using the untreated standard curve value as 100%, and utilisation of

substrate calculated as a percentage (Cannavo *et al.*, 2013). The bacterial solutions were assessed in triplicate.

4.3 Results

4.3.1 Neutrophil migration

Transwell chambers, Dunn slide chamber and under agarose diffusion migration assessments were not able to detect migration under any of the conditions assessed, including the positive control f-MLP.

Using the NeuroProbe microchamber plate, acute vaccine plasma resulted in migration of enriched neutrophils, with a mean and standard deviation of 56 ± 18% with six fish. No other conditions resulted in any migration (Fig. 1).

4.3.2 Neutrophil ROS production is functional but not stimulated by bacteria

Blood enriched neutrophils were able to produce reactive oxygen species as determined by luminol signal, measured as a kinetic assay. However, there was very high variability in ROS production between individual fish (biological replicates), when cells were stimulated with positive controls CaI and PMA (Fig. 2A). Therefore, in order to examine the effects between treatments, individual fish were considered independently and patterns within and between individuals observed (Fig. 2B). Stimulations with bacteria, (*S. iniae*, *S. agalactiae* and *E. coli* DH5α) did not cause enriched neutrophils to produce reactive oxygen species, whilst the positive controls did, albeit to a varying degree between individuals (Fig. 2C, 2D).

4.3.3 Neutrophil peroxidase release is functional but not stimulated by bacteria

Peroxidase release was detected for positive controls (CaI and PMA) via TMB substrate reduction, but was not detected in blood-enriched neutrophils incubated with bacteria over 30 min (or even during up to 180 min of incubation) (Fig. 3).

4.3.4 Neutrophil DNA release is not Streptococcus specific or capsule dependent

The ability of neutrophils to release DNA as a sticky web and capture microbes is a difficult event to capture. Plate bound DNA release prevents washing and inadvertent destruction of extracellular DNA. Whole cell lysis was used to determine 100% available DNA and tests were all normalised to these values (Fig. 4A). The blood enriched neutrophil preparations produced 8 - 15% DNA release for bacterial stimulations, however there was no significant difference between *E. coli* DH5α and streptococcal species and, amongst the *Streptococci*, no significant difference between capsular and acapsular strains (Fig. 4B). In contrast to the luminol and MPO assays, the DNA release assay was robustly replicated between individual fish, therefore data from five fish were analysed together.

4.3.5 Neutrophil phagocytosis hilighted unique behaviour from S. iniae

Competitive phagocytosis of capsular and acapsular bacteria was performed due to insufficient numbers of neutrophils from individual fish to allow discrete assessment of all parameters, and it was considered risky to mix neutrophils from different individuals, where non self-reactions between immune cells may confound the data. No increase in fluorescent signal, indicating bacterial presence in, or on, blood cells was observed for *S. agalactiae* or *E. coli* DH5α. *S. iniae*, however, both capsular QMA0248 and acapsular QMA0249, displayed positive peak fluorescent signal. This was also detected when cytoD was included to block active phagocytosis. Histogram overlays showed that both red labelled capsular (QMA0248) and green labelled acapsular (QMA0249) bacteria were present in high numbers regardless of the pre-incubation blockage of actin activity (Fig. 5).

4.3.6 Neutrophil viability was not affected by bacterial interaction

Cell death cause by bacteria would diminish the effectiveness of the immune response against it, so the effect of *Streptococcus* strains on blood neutrophils was tested to determine if this was an avenue of immune evasion. Viability and cell-death was measured with annexin V and propidium iodide; there was no significant cell death or induction of apoptosis detected in cells from five fish

incubated with the four strains of *Streptococcus* and *E. coli* DH5α, (Fig. 6). The positive control demonstrated the functional viability of the assay, inducing significant apoptosis within the 1 h incubation period.

4.3.7 Bacterially produced nucleases were minimal in supernatant and lysate

Detection of nucleases may be observed by breakdown of DNA, either in agar or in a plate based assay. Plate agar plaque clearance zone assays were performed but results were not clear (data not shown). The assessment was repeated using calf DNA and a micrococcal DNase standard curve. The variation within conditions was very high, only QMA0249 showing consistent levels.

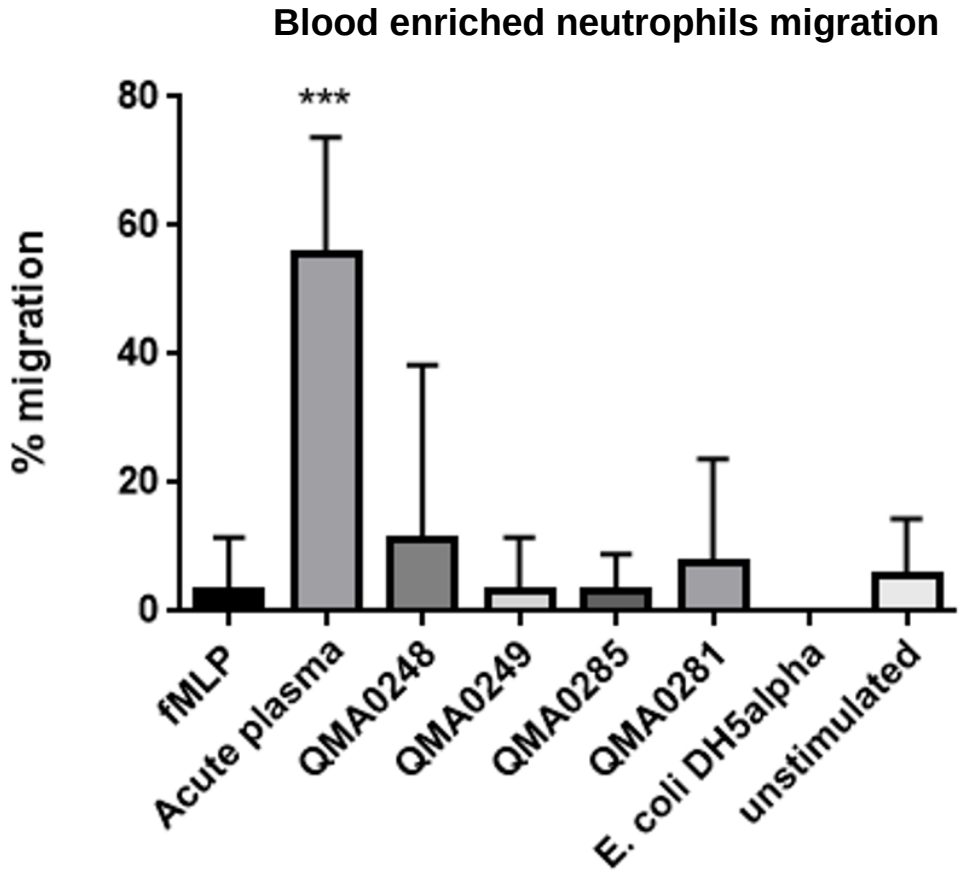

Fig.1 Migration of blood enriched neutrophils through Neuro Probe ChemoTx®Disposable Chemotaxis System plate, 3 μm pore size, towards chemoattractants or whole bacteria at an MOI of 1 . Percent migration determined as proportion of total cells. Results calculated using average and standard deviation of six fish. Analysis via one way Anova, relative to unstimulated, $p \leq 0.001$ indicated by asterisks.

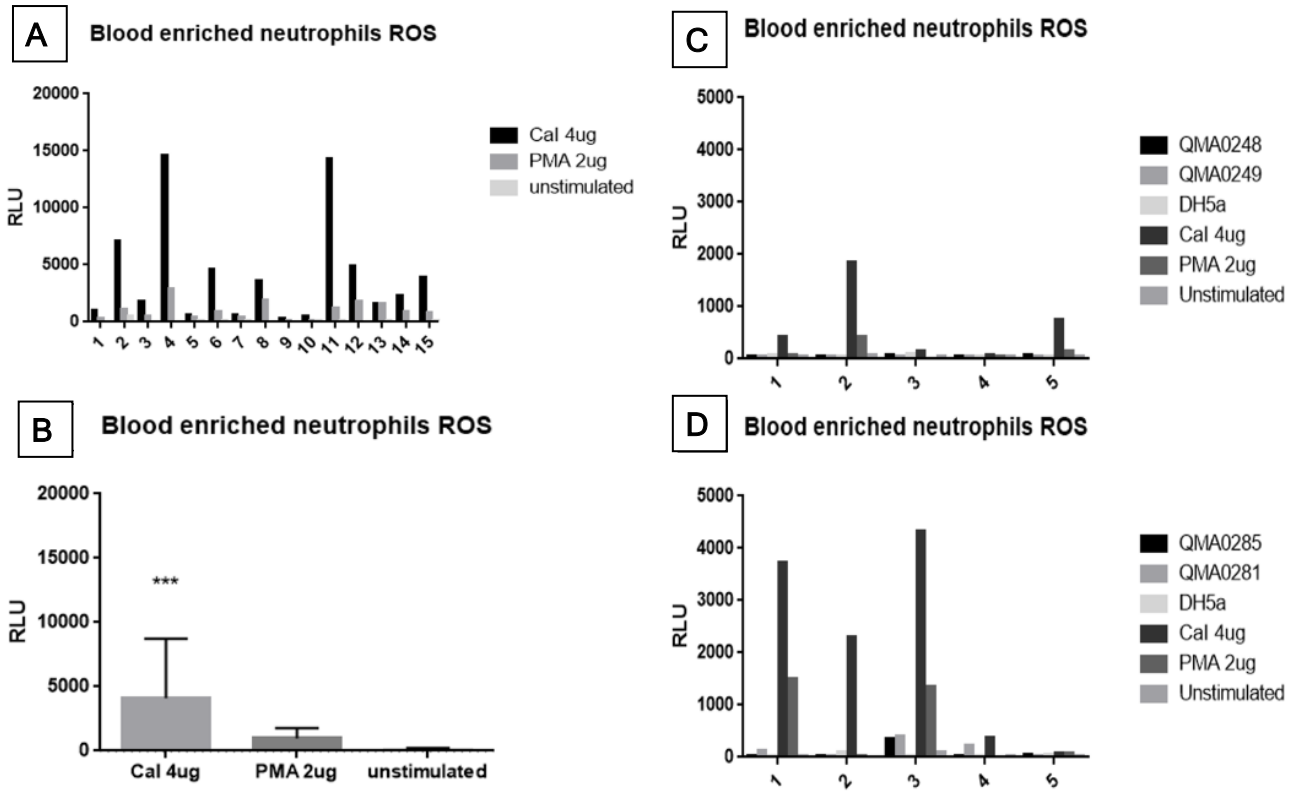

Fig. 2. Blood enriched neutrophils are able to produce reactive oxygen species. A) Individual variation is vast for blood enriched neutrophils stimulated with calcium ionophore (CaI) or phorbol 12-myristate 13-acetate (PMA) as detected via luminol assay, area under curve calculations over 20 min. B) The variation of response is such that combining individual fish response diminishes statistical significance. CaI was significant $p \leq 0.001$ compared to unstimulated but PMA was not. C) There was little to no respiratory burst detected when neutrophil preparations were incubated with S. iniae QMA0248 (capsular), QMA0249 (acapsular) or E. coli DH5α, while the cells were able to respond to CaI / PMA. D) There was little to no respiratory burst detected when neutrophil preparations were incubated with S. agalactiae QMA0285 (capsular), QMA0281 (acapsular) or E. coli DH5α, while the cells were able to respond to CaI / PMA. Control assessment (A & B) combines two experiments of a total of 15 individual fish, assessment of bacterial stimulation with five individual fish.

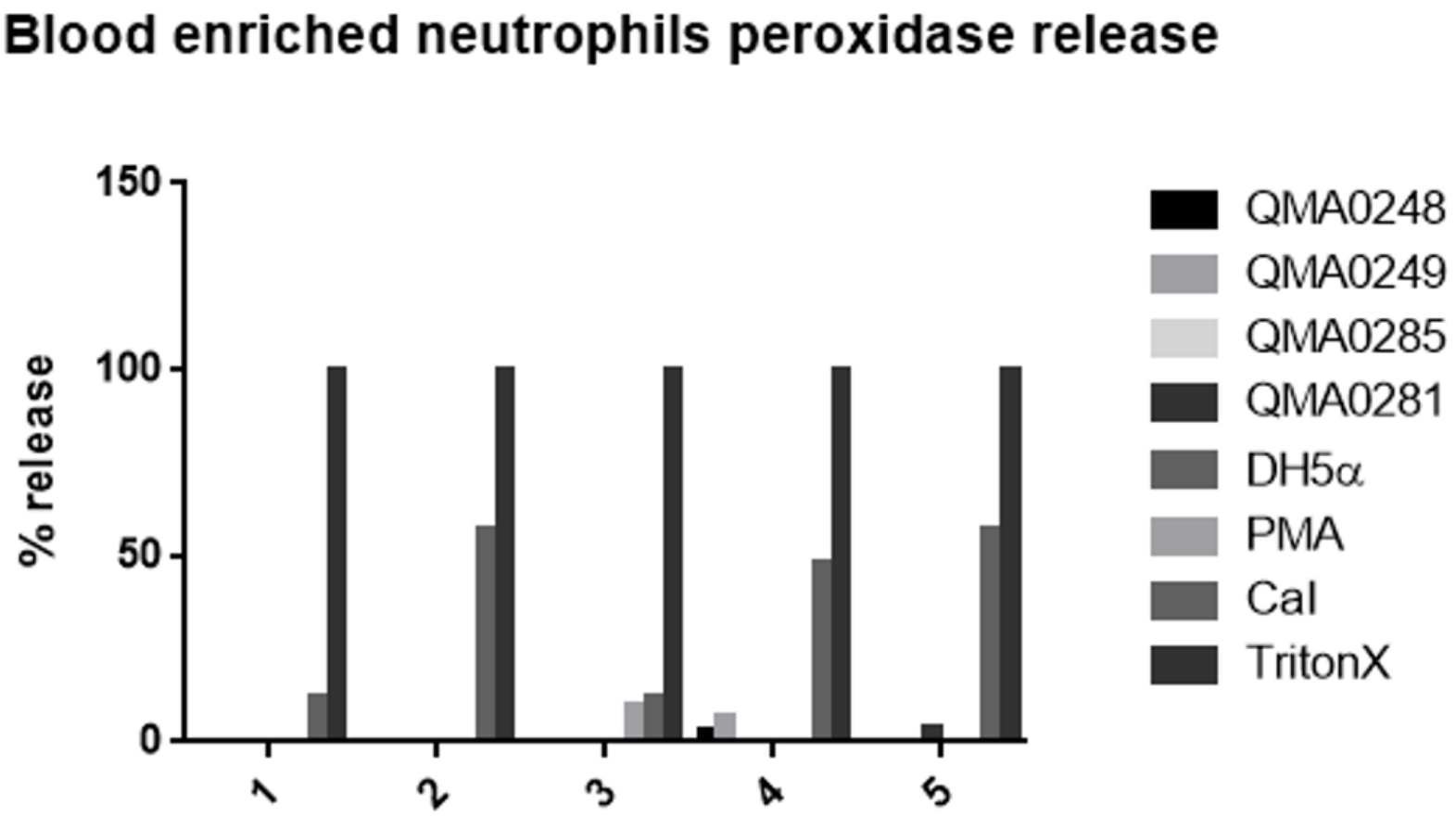

Fig.3. Peroxidase release from blood enriched neutrophils. Plate based peroxidase release of enriched neutrophils detected with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate whereby percent release calculated via the formula: % release = [(ODstimulated -ODbackground)/ (ODlysed - ODbackground)] x 100. Maximal signal obtained from triton lysed cells which was normalised to 100%. Results represent five individual fish.

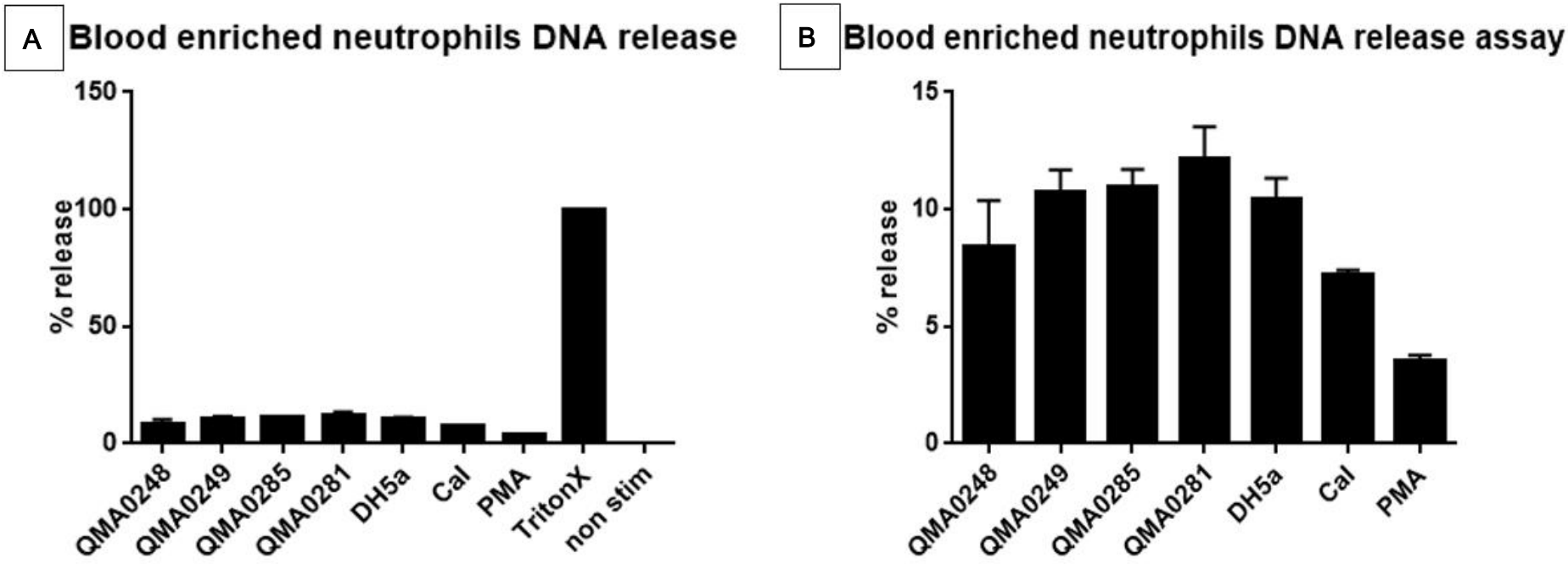

Fig.4. DNA release from blood enriched neutrophils. Plate based DNA release of enriched neutrophils, stimulation detected via Sytox green fluorescence, whereby percent release calculated via the formula: = [(ODstimulated -ODbackground)/ (ODlysed - ODbackground)] x 100. A) Maximal signal obtained with triton-X lysed cells which was normalised to 100%. B) Stimulation with bacteria produced a range of DNA release however there was no significant difference between capsular and acapsular or either S. iniae or S. agalactiae and no difference between Streptococcus and E. coli DH5a. Results calculated using average and standard deviation of five individual fish.

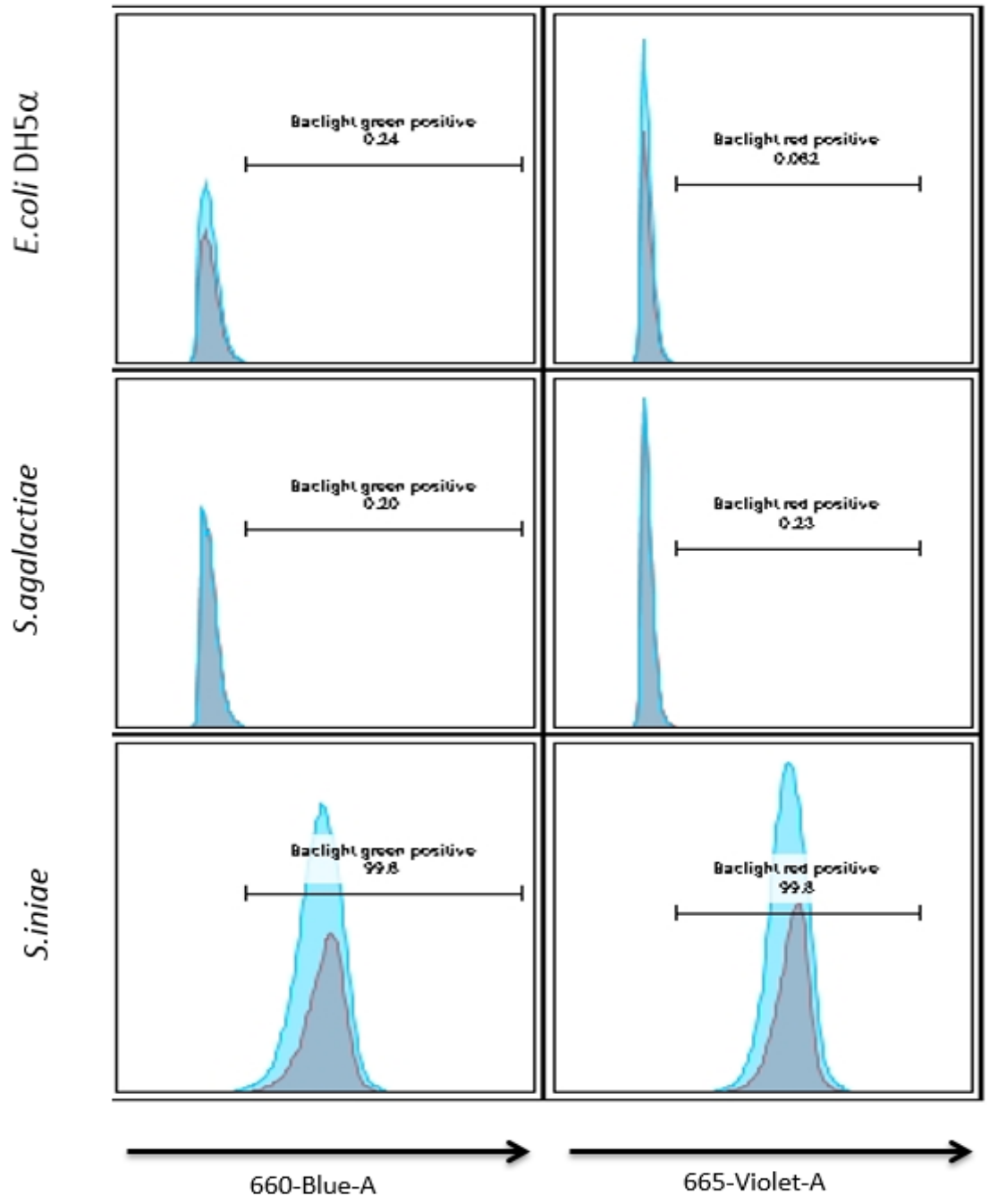

Fig. 5. Phagocytosis of Baclight red (capsular) and Baclight green (acapsular) bacteria by blood enriched neutrophils. Neutrophil enriched from blood were incubated with both capsular and acapsular S. iniae or S. agalactiae simultaneously, both at a multiplicity of infection of 1. E. coli DH5α was labelled with baclight green and incubated alone. Conditions were done in duplicate, one tube received a pre-incubation with cytoD to block actin rearrangement to indicate non-specific binding of bacteria to the cells. Overlayed histograms are cytoD (blue) over untreated (grey). Controls included FITC-labelled 2 μm beads and unstimulated cells, all of which were negative both with and without cytoD.

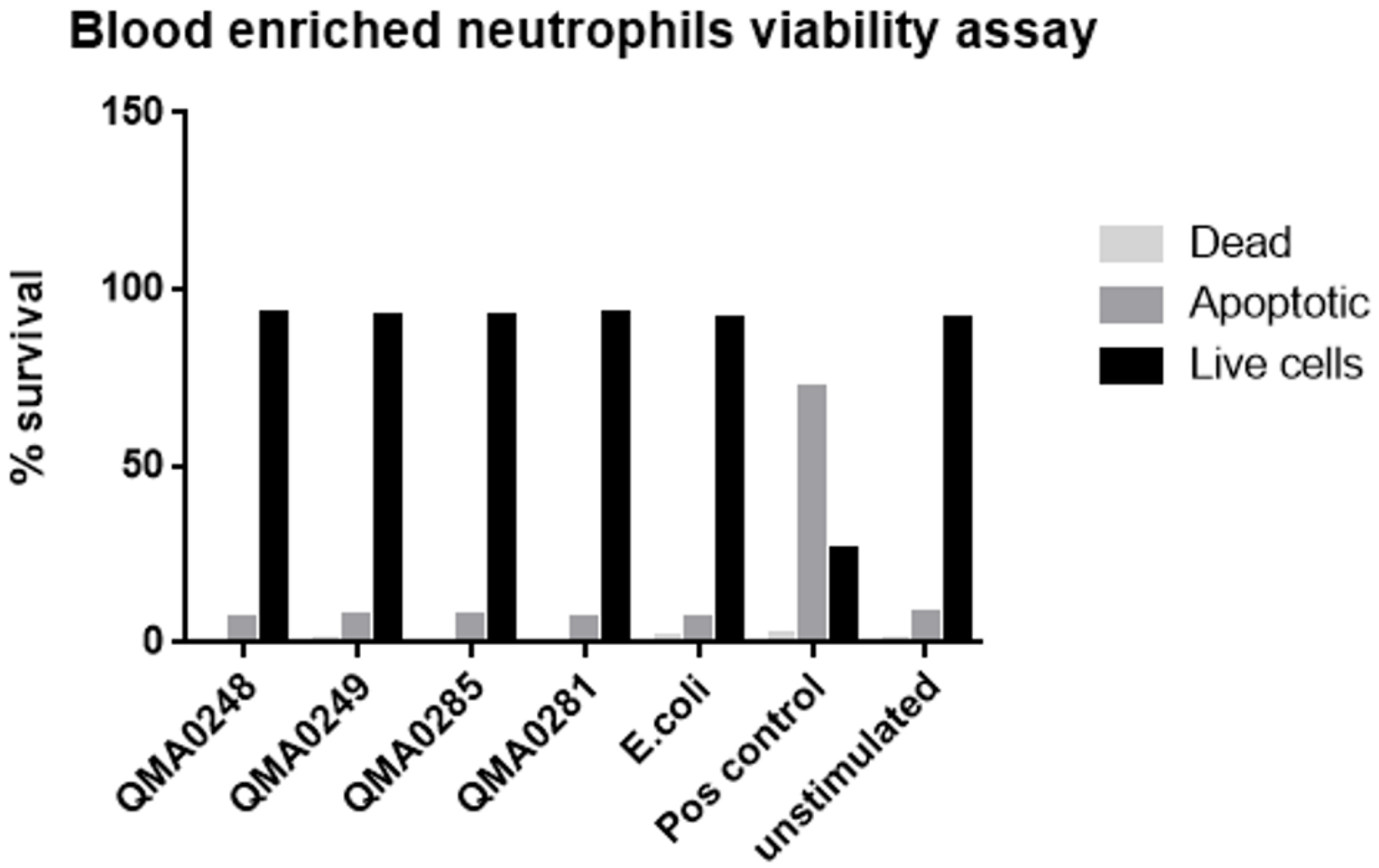

Fig.6. Assessment of viability and apoptosis of enriched neutrophils incubated with bacteria at an MOI of 1. Incubation of enriched neutrophils with S. iniae, S. agalactiae (capsular or acapsular) and E. coli DH5α showed no increase in apoptotic or dead cells. Apoptosis was achieved in the same incubation time frame with camptothecin. Flow cytometric analysis of five individual fish, graph representative of one fish.

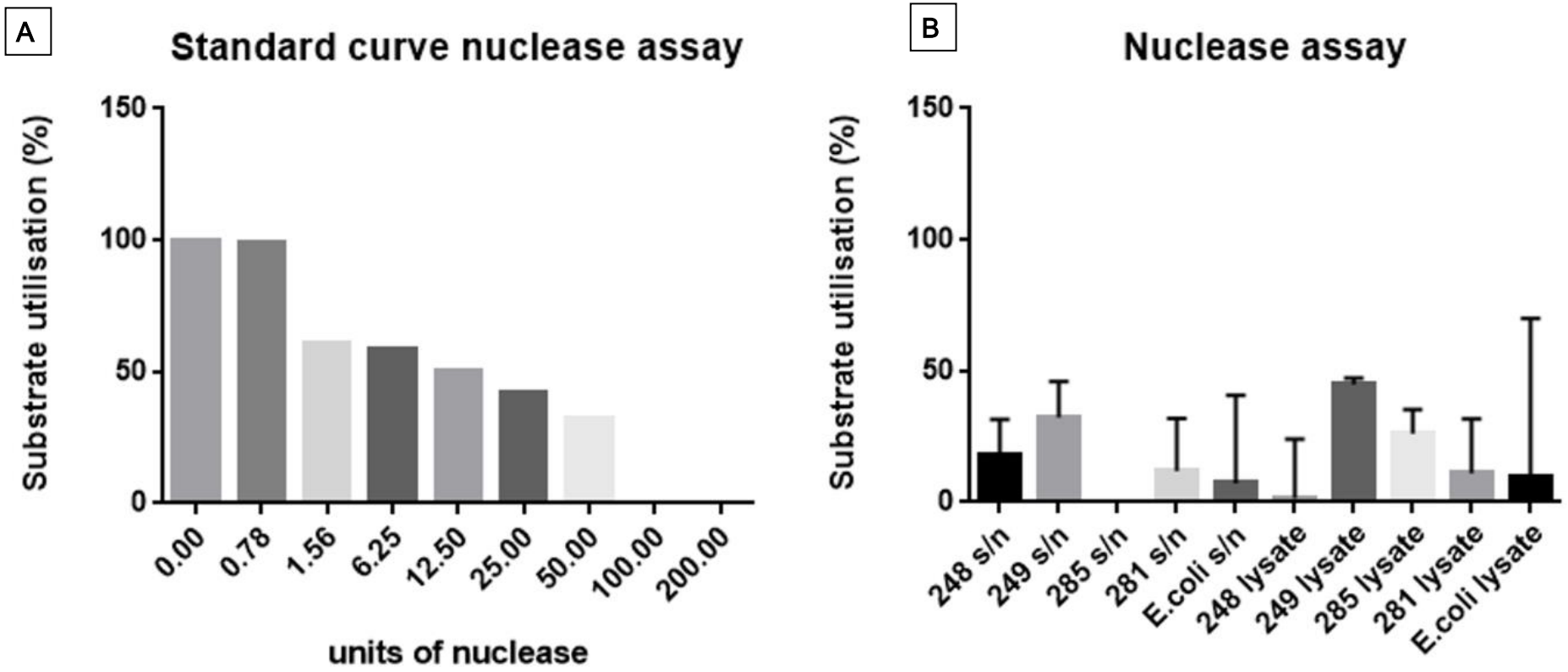

Fig.7 A) Standard curve of micrococcal degradation of calf DNA, observed as loss of sytox green DNA labelling signal. B) Mid- late- log bacterial supernatants and cell lysates were assessed for strains QMA0248, QMA0249, QMA0285, QMA0281 and E. coli DH5α. Bacterial solutions asessed in triplicate.

4.4 Discussion

The migration of neutrophils along a chemoattractant gradient is a vital part of targeting microbial invasion. Upon microbial invasion, neutrophils rapidly deploy from circulation, to the microbial entry site to combat spread (Abadie *et al.*, 2005). The chemotactic recruitment of neutrophils is coupled with pro-inflammatory mediators released by the host cells, prompting endothelial cells to express adhesion molecules, and immune cell to produce neutrophil activating factors (McCormick, 2016, Peveri *et al.*, 1988).

While *in vitro*, *in vivo* and genomics studies continued to expand the field of chemokine sensitivity and receptor expression in fish, zebrafish (*D. rerio*) are a convenient model for transgenic studies of inflammatory response. Whole fish inflammatory studies were rapidly advanced by the construction of a transgenic zebrafish, which constitutively expressed GFP under the neutrophil myeloperoxidase promoter (Renshaw *et al.*, 2006). A tail clip model of wound inflammation showed, in the transgenic zebrafish, that the GPF neutrophils were rapidly recruited to the site of damage. Infection challenges also verified, (via gene expression in the Tg (mpx:gfp) zebrafish) that both CXCL8-l1 and CXCL8-l2 were upregulated upon exposure to *Salmonella typhimurium* (de Oliveira *et al.*, 2015). Comparative whole genome analysis found chemokine, putative zebrafish CXCL8 (aka interleukin-8), transcript similarity to be 48%- 57% of the human CXCL8 (DeVries *et al.*, 2006). When IL-8 activity was blocked, there was a reduced neutrophil recruitment and less bacterial clearance, demonstrating the importance of the chemoattractant.

The migration of enriched neutrophil preparations from barramundi was only observed for cells exposed to acute inflammatory plasma, which contains a cocktail of cytokines and activating factors. With a distinct lack of information on chemokine and receptor expression in *Lates calcarifer*, it is not known if IL-8 is secreted during an acute inflammatory reaction, and if this is what the neutrophils are responding to. Additionally assessed as a positive control, the tripeptide f-MLP (N-Formylmethionine-leucyl-phenylalanine), has been identified it as a potent polymorphonuclear

leukocyte chemotactic agent (Harlan *et al.*, 1985). The mechanism of action for f-MLP is as a mimic of oligopeptides released by tissue to recruit granulocytes and direct the innate immune response to the area of invasion or damage. In humans, f-MLP mediated neutrophil migration is thought to be CD11/CD18 integrin (Mac-1) dependent (Carulli *et al.*, 1997, Carrigan *et al.*, 2007, Furie *et al.*, 1991).

Information regarding f-MLP response in teleosts reports an increased in bone development in larval zebrafish (*D. rerio*) (Shin *et al.*, 2011). It has also been used as a positive control for migration assays of goldfish (*Carassius auratus* L.) kidney cells (Katzenback *et al.*, 2009). The same was not observed for plaice (*Pleuronectes platessa* L.) kidney cells (Nash *et al.*, 1986) or carp (*C. carpio*) head-kidney cells, where no migration was observed when cells were stimulated with recombinant human CXCL8 or f-MLP (Stakauskas *et al.*, 2006). The present results demonstrated that enriched neutrophils from barramundi retain the ability to migrate post-enrichment in response to acute plasma, but it is not known what specifically they are responding to, within the plasma. The tripeptide f-MLP did not promote migration in four assay systems, suggesting that this pathway is non-functional in barramundi.

The inability to clearly define barramundi enriched neutrophil migration may be a reflection of lack of *in vivo* structural factors assisting neutrophils. In mammals, these receptors are primarily β2 integrins and selectins, which mediated rolling and passing through cellular matrix layers. Additionally, some infections, as with *Streptococcus pneumoniae*, seem to facilitate migration via alternative receptors, such as the binding lectin galectin-3 which was not observed with *E. coli* (Sato *et al.*, 2002, Alves *et al.*, 2010). This highlights the complexity of leukocyte extravasation, which may also be pathogen specific. While chemoattractants obviously function in the fish, and were present in acute inflammatory plasma, these are only one part of the migration signalling package used *in vivo,* to facilitate diapedesis. The lack of *in vitro* response to bacteria indicates soluble factors or structural accompaniments were lacking.

Once neutrophils have migrated and reached the source of inflammatory stimuli, they produce anti-microbial factors to degrade, capture or neutralise the cause. The ability to produce ROS has been detected in echinoderms (Coteur *et al.*, 2002), marine bivalves (Abele *et al.*, 2002) and plants (Bailey-Serres *et al.*, 2006, Mittler *et al.*, 2004), indicating the evolutionary conservation of this method of microbial clearance. In teleosts, ROS have been detected in gilthead seabream (*S. aurata)* (Olavarria *et al.*, 2010), mullet (*Mugil cephalus*), flounder (*Platichthys flesus*) (Ferreira *et al.*, 2005) and rainbow trout (*O. mykiss*) (Sharp *et al.*, 1993). This conservation, therefore, infers importance of this immune mechanism, which could be assessed in relation to pathogenic, non-pathogenic, capsular and acapsular bacterial strains, to determine if *S. iniae* subverts or neutralises the effect. Detection of ROS may be performed with luminol, ($C_8H_7N_3O_2$), which becomes chemiluminescent when oxidized. The production of free radical ROS when cells become activated, causes the oxidation of luminol, and detection can be performed in a time-dependent manner (Kobayashi *et al.*, 2001, Bochev *et al.*, 1993). The area under the curve is a standard measure of activity and the response occurs rapidly and signal diminishes within 20 - 30 min. The ability of cells to produce ROS indicates immune responsiveness, and has been used as a measure of cell activity (Kobayashi *et al.*, 2001). While the depth of responsiveness was variable between fish, inactivity toward whole bacteria was unexpected. The neutrophil preparations were fairly stable regarding numbers, viability and purity; however, the lack ROS output suggested the assay was lacking something, which the small immune stimulant positive controls were able to bypass. While neutrophil viability is always of concern during *in vitro* assays, viability assessments found neutrophil enrichment preparations were consistently greater than 90% several hours post enrichment. The ROS assay was read out for 180 min; however, the burst stimulated by CaI and PA was initiated rapidly and was spent within 20 – 30 min. There was no further signal after the initial respiratory burst with controls or bacterial stimuli. The assay does not discriminate between types of ROS produced, so further break down of the pathway could ascertain functional and responsiveness to bacteria, and if *Streptococci* block or neutralize any of these degradative products.

Utilising a similar experimental approach, detection of MPO, which converts hydrogen peroxide to corrosive acid during respiratory burst, was assessed. Using TMB, which is a substrate for horseradish peroxidase, activity of MPO was assessed normalised to lysed cells (Suzuki *et al.*, 1983, Menegazzi *et al.*, 1992). Similarly to the ROS assay, the control chemicals (CaI and PMA) demonstrated enriched neutrophil cell suspensions were able to release peroxidase, but again, there was little activity stimulated by whole bacteria. This may similarly tie in with the cells being devoid of a crucial component needed to process and respond to whole bacteria *in vitro*. Time of stimulation used was 30 min, as adapted and re-optimised from a time course published using fathead minnow neutrophils stimulated with CaI and PMA (Palic *et al.*, 2005), suggesting that the incubation period was sufficient for MPO release.

The ability to produce NETs has been identified in numerous animals including human, cows, fish and cats (Urban *et al.*, 2006, Grinberg *et al.*, 2008, Palic *et al.*, 2005, Wardini *et al.*, 2010). Identifying NETs may be done via microscopy using DNA stains, including Hemacolour kits through to fluorescent dyes including SYTOX and SYBR green. The DNA component of the web is most often targeted for labelling; given it is the most abundant component of the NET. Visualisation is difficult, given the NET is a three dimensional web, and that two dimensional visualisation only allows the observation of a slice the whole structure. Neutrophil elastase may also be fluorescently labelled for NET identification. NE is a cytotoxic serine protease and is stored in granules. These are released after neutrophil stimulation and can cause degradation of outer membrane proteins including laminin (basement membrane protein), fibronectin (extracellular matrix protein), collagen (connective tissue protein), proteoglycans (glycosylated connective tissue protein) and elastin (connective tissue protein) (Drujan *et al.*, 2002, Janoff *et al.*, 1968). NE is often labelled with an antibody, which can be an issue for cross-species reactivity, although most research has been performed on murine models, where a lack of NE increases susceptibility to gram negative bacteria (Belaaouaj *et al.*, 1998). Histones, however, are highly conserved and an antibody is available which

labels a diverse range of host targets. The Millepore antibody targets histone H2B, which is one of the five main histone proteins involved in the structure of chromatin in eukaryotes (Brinkmann *et al.*, 2004). Many AMPs are associated with histone H2B, due to the rich basic amino acids at the N-terminus, and frequently carry positively charged and hydrophobic residues. Histone derived antimicrobial peptides have been identified in several fish, including catfish (*Parasilurus asotus*) (Park *et al.*, 1998b), rainbow trout (*O. mykiss*) (Fernandes *et al.*, 2002), halibut (*Hippoglossus hippoglossus*) (Birkemo *et al.*, 2003) and Atlantic salmon (*S. salar*) (Richards *et al.*, 2001). Low molecular weight AMPs can penetrate cell membranes, and once inside, interfere with DNA, protein synthesis, protein folding, and cell wall synthesis (Wu *et al.*, 1999).

Many attempts were made to stimulate NET production with barramundi cells, but none proved convincing by fluorescent or light microscopy. Given the delicate nature of the web like structure, a plate-based DNA release assay was performed in preference to slides. This was based on a previously published method with zebrafish (*D. rerio*) neutrophils (Palic *et al.*, 2007a). While plate-bound cells (neutrophils need to adhere prior to NETosis) showed a moderate level of DNA release, this did not seem to be specific to any bacterial strain or species. A time course was initially assessed, with seemingly no differences from 30 min to 3 hr, so subsequent work was incubated for 1 h. Interestingly, the signal obtained for bacteria was higher than that of the usual immunological stimuli used for neutrophil assessment, CaI and PMA, and controls indicated the fluorescent dye was not labelling the bacteria themselves. For all bacterial stimulations, an MOI of 1 was used, as this was deemed physiologically relevant to the initial stages of infection, and given neutrophils are the first responders, it could be argued that they would be sensitive to pathogens. The moderate signal showed no species specificity, or significant difference between bacteria, with or without, a capsule indicating what was being measured was fairly non-specific.

Phagocytic engulfment is one of the three duties of neutrophils, along with release of exogenous factors and production of NETs, all in response to pathogenic stimuli and initiating an immune

response. Neutrophils can internalize non-opsonised and opsonised particles. Opsonised particles bind via cell surface Fc receptors and β2 integrins, and the bacterial polysaccharide capsule has been reported to subvert engulfment (Hyams *et al.*, 2010). The bacterium is enveloped in neutrophil plasma membrane, which then buds off into the cytoplasm. The microbe containing phagosome matures via fusion with secretory vesicles and granules, and eventually fuses with a lysosome resulting in a phagolysosome (Nordenfelt *et al.*, 2011). While early and late vesicles bind during the maturation process, the lysosome is the ultimate degradative package, with a pH ≤5.0, and high concentrations of proteases to degrade engulfed endocytic cargo (Reeves *et al.*, 2002). The early azurophil granules containing peroxidase and MPO have been confirmed in fish (Palic *et al.*, 2007a), secondary granule constituents, lactoferrin and lysozyme, and tertiary vesicle albumin and ALP remain ill-defined.

The processes of neutrophil engulfment and digestion have been observed in whole body imaging in zebrafish studies, where engulfment of *Shigella flexnerii* by zebrafish neutrophils was observed as early as 20 min post infection, and once inside the cell they were subsequently rapidly degraded (Mostowy *et al.*, 2013). Since *E. coli* is not known to be a pathogen of barramundi, it was anticipated that the laboratory strain of the bacterium, devoid of its virulence factors, would be an easy target for engulfment and destruction. Therefore the lack of phagocytosis was unexpected, but previously observed with *S. iniae* and human blood or cell lines (Fuller *et al.*, 2001). These results suggest the barramundi immune cells use an alternative process to clear microbes, but having assessed MPO and ROS already without result, none seem candidate pathways of bacterial clearance. It is possible other cells or humoral factors are involved, which would have been removed or decreased during the separation process, or assay conditions are simply not favourable for whole bacterial response.

The phagocytosis of bacteria is hampered by presence of a capsule, so the competitive phagocytosis assay presented two strains of the same bacterial species, one capsular and the other acapsular (Lin *et al.*, 2004, Smith *et al.*, 1999, Houde *et al.*, 2012). It was expected that the acapsular strain would

present an easier target for neutrophilic engulfment than the capsular strain, identifiable by flow cytometry via differential fluorescent labelling. However, *S. iniae* was bound by blood neutrophils regardless of presence of capsule, as the neutrophil population gave high fluorescent signals from both red label capsular QMA0248, and green label acapsular QMA0249. The binding was actin independent, as duplicate control samples that were treated with, and subsequently incubated with, cytoD, showed the same level of fluorescence as the untreated. CytoD inhibits actin polymerisation, necessary for structural rearrangement and phagosome formation during phagocytosis (Casella *et al.*, 1981) and is routinely used to assess non-specific adherence of bacteria (Hyams *et al.*, 2010). Since there was no binding of either *S. agalactiae* or *E. coli* DH5α, this seems to be an *S. iniae*-specific phenomenon, perhaps alluding to a mechanism of immune escape, allowing it to cause disease in barramundi. Alternative dyes were investigated, as standard two dimensional microscopy would still not indicate if the bacteria were stuck on the outside, attached and covered in host proteins, or somehow inside the cell. Unfortunately, bacterial dyes that only fluoresce once the microbe is inside an acidified phagosome required fixation of the microbes. This fixation process would likely ablate neutrophil response, which is best seen with live, proliferating bacteria. Streptococcal challenges in zebrafish (*D. rerio*) with heat-killed *S. iniae* proved to be non-fatal, compared to live microbes (Neely *et al.*, 2002).

Phagocytosis by neutrophils has been reported to occur by different mechanisms to that employed by macrophages. By studying phagocytosis in zebrafish, it has been reported that while macrophage are effective at phagocytosing microbes in, or on, any substrate (solution or substrate), neutrophils were not effective at suspension phagocytosis, and needed microbes to be surface-associated (Colucci-Guyon *et al.*, 2011). While flow cytometry offers the ability to detect both intra and extracellular bacteria, the interaction of cells and bacteria is primarily fluid-borne, and settlement of cells and bacteria in the hour time frame may create inconsistencies. Ideally, bacteria should be centrifuged onto a surface, or grown as a biofilm, but flow cytometry would not be applicable as a

method of detection. Neutrophils can stick quite rapidly and firmly to both glass and plastic, which creates recovery issues. Detection problems may also arise if a dye is used for visualisation, as division would subsequently dilute signal to progeny bacteria. The same report (Colucci-Guyon *et al.*, 2011) also indicated motility was related to the phagocytic ability of neutrophils, again suggesting neutrophil movement is surface receptor mediated. The more bacteria the neutrophil engulfed, the slower it became, and once a number of bacteria were collected, the neutrophils degranulated into the phagosome, killing all microbial occupants. The fluorescence signal indicating *S. iniae* was stuck on the neutrophils, may have needed more time for engulfment and digestion of the microbes, although phagocytosis has been reported to occur much more rapidly (Renshaw *et al.*, 2006). It is possible the bacteria have found a way to utilise the host's immune cells to delay destruction, allowing rapid proliferation to occur.

Bacteria that instigate or block apoptosis, necrosis and inflammatory mediated tissue damage, often hide within the cells of the immune system. The pathogen *Listeria monocytogenes* has adapted to exist in the cytosol via production of a cytolysin, listeriolysin O, which facilitates phagosomal escape, by targeting the membrane (Hara *et al.*, 2007). *Salmonella typhimurium* induces pyroptosis, an inflammatory caspase 1-dependent cell death (Franchi *et al.*, 2006), and *S. flexneri* kills macrophages by inducing apoptosis (Chen *et al.*, 1996) or necrosis (Carneiro *et al.*, 2009). It was not apparent that *Streptococcus* used any means of induced cell death on fish neutrophils, to avoid degradation or capture, though only apoptosis and death, not pyroptosis, were assessed.

Nucleases may be used by microbes to facilitate escape from extracellular traps and these DNA nucleases may be membrane bound or secreted (de Buhr *et al.*, 2015, Juneau *et al.*, 2015, Morita *et al.*, 2014, Derre-Bobillot *et al.*, 2013). Recent research in swine identified membrane vesicles (MV) produced by *Streptococcus suis* that contain proteases and nucleases, and are used to escape immune strategies such as NETs (Haas *et al.*, 2015). These vesicles were also able to promote activation of nuclear factor-kappa B in some immune cells, which caused a cytokine cascade

resulting in increased permeability of the blood brain barrier (BBB). The BBB is a tightly fortified barrier, primarily comprising of brain capillary endothelial cells (Gloor *et al.*, 2001). Though such pathways of CNS invasion are yet to be confirmed in teleost, the pathogenesis of *S. iniae* causing meningitis in barramundi suggests similar mechanisms may occur to allow the bacteria to cross. Assessment of late phase supernatant and bacterial lysate indicated a variable level of nuclease activity, the most consistent being lysate from the capsular deficient *S. iniae*, QMA0249. While this was not supported by such activity in the wild type strain (QMA0248), perhaps lacking the anti-phagocytic capsule encourages redundant escape mechanisms to become more apparent. In assessing the activity of nucleases, supernatant may be too dilute, and bacterial homogenate may not release nucleases bound to cellular membranes. Further classification would require specialised purification, which were not sought, due the high variation obtained with the DNA degradation assay.

Collectively, these results showed *in vitro* migration of enriched neutrophil was possible, although the acute plasma which instigated the attraction contains many products that could be the key chemotactic agent. The attraction towards whole bacteria obviously occurs *in vivo*, but was not observed *in vitro*. Likewise, chemical stimulants were able to induce a range of immune responses (ROS, MPO), which were not mirrored with whole bacteria. So, while responsive anti-microbial pathways demonstrated the cells were able to react (DNA release), assessing their response towards whole bacteria *in vitro* was challenging. Given the propensity for fatal disease in barramundi caused *by S. iniae*, but not observed with *S. agalactiae* or *E. coli*, only the actin-independent attachment to neutrophils suggested a species-specific mechanism of pathogenesis (Buchanan *et al.*, 2008). This capsule-independent interaction with neutrophils may be advantageous to *S. iniae*, exploiting the remodelling of host tissue tight junctions. Intercellular junctions are considered the main thoroughfare where adherent neutrophils penetrate the endothelium, for trans-endothelial migration (Burns *et al.*, 2000). This migration through tissues facilitates colonisation of the central

nervous system, which is characteristic of *S. iniae* pathology in barramundi. This is seen equally for both capsule positive and capsule negative isolates (Millard *et al.*, 2012). This suggests the capsule is not the only, or most important, virulence factor or that there are redundant systems in place (Lowe *et al.*, 2007). This allows capsule-deficient mutants to still be able to cause infection, albeit with an altered pathology (Millard *et al.*, 2012).

CHAPTER FIVE: General Discussion

Food security continues to be an important issue as the global population grows and populous countries, such as China, have a rapidly growing middle-upper class (Worldbank). This increase in socio-economic standing drives consumptive markets to meet the increasing demand. Seafood production in China has doubled in the last 50 years, while daily intake has increased fourfold, compared to that of the rest of the world (Villasante *et al.*, 2013). The seafood industry in Australia tends to export high-end products like tuna, abalone and rock lobster, while importing lower end canned and processed fillets. The vast majority of seafood Australians consume is imported from Asia (Government, 2016). With approximately 85% of Australians living within 50 kilometres of coastline, fishing both recreationally and commercially exerts pressure on local ecosystems, and aquaculture is an increasingly attractive way to balance supply, without depleting wild stocks.

Aquaculture allows fish farming to be controlled and optimised for high productivity, with fast growing species, like barramundi, growing from fingerling to market size in 12 - 18 months. With fish density in farming pens being much higher than that found in nature, stress and disease susceptibility are important balances, against yield and growth rate (Andrews *et al.*, 1971, Riche *et al.*, 2013, Duan *et al.*, 2011). Stress can lead to increased disease, which is the largest limitation of aquaculture expansion (Meyer, 1991). Vaccination of finfish has been used for many years (Gudding *et al.*, 2013), and is used increasingly in place of antibiotics (Bravo *et al.*, 2007, Sommerset *et al.*, 2005, Defoirdt *et al.*, 2011). Vaccines for fish farming include bacterins (formalin killed bacterial cell suspensions), live attenuated and DNA vaccines, and are ideally polyvalent to offer protection against multiple strains (Toranzo *et al.*, 2009). Some reports indicate vaccinations may in fact drive bacterial evolution, and escape mutants are subsequently resistant to humoral immunity generated from previous vaccination (Gandon *et al.*, 2001, Millard *et al.*, 2012). Truncation of host life cycle has also been reported to drive pathogen evolution (Nidelet *et al.*, 2009), as well as use of brood-stock with limited genetic diversity (Poisot *et al.*, 2011) which may occur during the domestication process

(Hedrick *et al.*, 2000). Endemic infections may also adapt to become more virulent, as observed and reported for virus infections in the salmonid industry (Mochizuki *et al.*, 2009, Garver *et al.*, 2006). Understanding the multifaceted interplay between host health and pathogen transformation, is vital for vaccine development, from early innate responses, right through to long term humoral immunity.

Neutrophils are the first responders to pathogen-mediated inflammation, but are rare in barramundi blood. Mobilisation may occur from the developmental reservoirs, the kidney and head-kidney, upon infection. An increase of mature cells in the circulation is advantageous, as blood cells are in immediate, direct contact with invading *Streptococci*, which utilise blood as a favoured medium for growth and dissemination. Migration of neutrophils is rapid and the movement in mammals is mediated by endogenous IL-8 produced by endothelial cells during acute inflammation (Huber *et al.*, 1991). The factors, receptors and involvement of various epithelial cells are undefined in fish. The chemoattractant f-MLP did not induce migration of neutrophils from barramundi; however acute inflammatory plasma was a potent inducer of migration. Acute inflammatory plasma is likely to contain a cocktail of cytokines and chemokines, so further work is required to fractionate acute plasma to identify the key components involved in neutrophil migration. The attraction of barramundi neutrophils seems more complicated than dendritic cells, which easily move through *in vitro* Transwells via extension of dendrites (Zoccola *et al.*, 2015). Neutrophils that are GFP tagged, via the MPO promotor, are visibly trackable and able to rapidly respond to tissue damage in transgenic zebrafish (*Danio rerio*) (Renshaw *et al.*, 2006).

Mature blood neutrophils in fish originate and develop in the kidney and head-kidney. Like their mammalian equivalents, when fully mature they possess multi-lobed nuclei and they function to recognise and respond to pathogen associated molecular patterns. Neutrophils phagocytose microbes; release exogenous antibacterial products or cast DNA based webs to opsonise pathogens and create a greater target and thus immune response. While whole blood from barramundi was

able to kill *E. coli* DH5α, *Streptococcus* was not contained, indeed the virulent capsular disease-causing *Streptococcus iniae*, QMA0248, proliferated rapidly in fresh barramundi blood. This rapid inflation of bacterial population only occurred with the capsular strain, indicating the capsule likely plays a role in protecting the bacteria from host defences, allowing replication. Conversely, the acapsular stain (QMA0249) was not killed; while proliferation was suppressed in blood, the bacteria were not cleared. Likewise, the two *S. agalactiae* strains, which are not known to cause disease in barramundi, were contained proliferation wise but not killed. This suggests that there are other mechanisms in place for clearing non-disease causing bacteria, or that rapid proliferation in blood is a requirement for overt, acute disease outbreak. Potential neutrophil factors that enabled QMA0248 to proliferate so rapidly were further examined, but neither ROS nor MPO release was induced by any of the bacterial strains used. This suggests a priming prompt may have been missing, although the control stimuli induced degranulation and release of ROS and MPO without any additional initiation signal (Condliffe *et al.*, 1998). One of the pitfalls of working with non-model organisms is that comparisons to mammalian systems may be inappropriate. Indeed, the complexity of barramundi neutrophil assessment was confounded by using methods optimised for common research species (primarily humans and murine), and in attempting to characterise NETs and DNA release, we assumed that function would be similar to mammalian, murine or cyprinid systems previously investigated (Rocha *et al.*, 2015, Palic *et al.*, 2007b, Kessenbrock *et al.*, 2009, Ermert *et al.*, 2009, Brogden *et al.*, 2014). The release of DNA was not specific to exposure to *Streptococcus,* so whether this was true NETosis or not remained clouded by technical challenges of capturing a fine 3D web on 2D slides. The discovery of NETs in 2004 (Brinkmann *et al.*, 2004) opened new pathways of understanding of neutrophil activity, and methodologies continue to evolve to follow and assess the phenomenon (de Buhr *et al.*, 2016).

The method utilised by infective *S. iniae* to escape death via blood cells may also involve avoidance of phagocytic capture and degradation; indeed adherence of virulent *S. iniae* to enriched barramundi

neutrophils seemed to inhibit cellular engulfment. The engulfment of microbes by neutrophils occurs in a plasma membrane-derived vacuole, so blockage of host cell detection, phagosome budding or early escape from an enveloping vacuole, would allow *Streptococcus* to exist on, or in, host cells. Since pre-treatment with cytochalasin D made no difference to the signal of *S. iniae* bound to host cells, this indicates the adherence was actin-independent. Phagocytosis in mammals is mediated by receptors, primarily Fc mediated (Yokota *et al.*, 1992) or complement receptor (Brown, 1991). These are both actin-dependent processes, though complement envelopment may occur with a 'sinking' action with little cellular disturbance, and minimal oxidative burst or inflammatory response (May *et al.*, 2001). Lesser known integrin and lectins may also drive phagocytosis, with little to no inflammatory signal (Blystone *et al.*, 1994). This may well be a pathway used by *Streptococcus*, as the resultant lack of inflammatory signal would be supported by the absence of ROS or MPO release. There are also pathways mediating phagocytosis of apoptotic cells that suppress inflammation (Vollmers *et al.*, 1997, Fadok *et al.*, 2000), which would assist streptococcal pathogenesis, although there was no apoptosis or cell death caused by bacteria in these studies. Most phagocytosis pathways are actin dependent (Dupuy *et al.*, 2008), although clathrin/dynamin pit engulfment may be actin-independent (Tse *et al.*, 2003). A clathrin lined pit mechanism of particulate endocytosis is receptor-mediated and frequently associated with digestion and absorption of metabolites, rather than immune clearance (Sorkin, 2004).

The surface proteins and polysaccharide capsule of many *Streptococci* have studied extensively, and it is considered a primary virulence factor (Lindahl *et al.*, 2005, Yamamoto *et al.*, 1999, Wang *et al.*, 2015, Thurlow *et al.*, 2009, Sellin *et al.*, 2000, Bellais *et al.*, 2012). The capsular polysaccharide (*cps*) operon of *S. iniae* consists of 21 genes, for some of which a function has not been determined, but many show strong homology to other *Streptococcus* species (Lowe *et al.*, 2007). Several capsular replacement gene mutants have been produced, but no single gene has been found to be responsible for capsule production and virulence (Lowe *et al.*, 2007, Locke *et al.*, 2007a). Indeed

acapsular *S. iniae* QMA0249, with a frameshift mutation in *cpsE* resulting in truncation of the protein and a deletion of the genes *cpsF* through *cpsM* (Millard *et al.*, 2012), was still able to cause disease in fish, albeit a chronic pathology rather than the acute form caused by *S. iniae* QMA0248. This suggests a redundancy in survival mechanism(s), which are not reliant on the capsule, though virulence was altered. While proliferation of the acapsular strain was restricted in the bactericidal assay, indicating capsular dependence, all other immunological assays demonstrated that the presence of a capsule was not the pivotal factor to bacterial survival.

Additional *S. iniae* virulence factors, such as M-like protein (SiM), are not critical in resistance to host cell phagocytosis, or invasion or adherence to epithelial layers (Locke *et al.*, 2007b). Vaccination with recombinant SiM, elicited an antibody response in barramundi, but did not result in challenge protection (Aviles *et al.*, 2013). Similarly, other virulence factors such as C5a peptidase (Locke *et al.*, 2008) and streptolysin S (Fuller *et al.*, 2002) did not contribute to *S. iniae* pathogenesis in fish. Also considered important to Group A *Streptococcus* virulence, is an IL-8 protease SpyCEP, and the homologue in *S. iniae* has been named CepI (cell envelope proteinase of *S. iniae*) (Zinkernagel *et al.*, 2008). While there is very little information on this protease, the ability to degrade IL-8 may assist early bacterial establishment, negating the rapid influx of neutrophils by blocking the IL-8 chemoattractant. The acute inflammatory plasma used for migration studies with enriched blood neutrophils, demonstrated rapid migration, but it unknown if this is IL-8 mediated. In mammals, neutrophil chemokines secreted early in acute inflammation include IL-8 (Harada *et al.*, 1994) and C-X-C motif chemokine 10 (CXCL10) (aka IP-10) (Wong *et al.*, 2004). Homologues of CXCL10 have been identified in carp and zebrafish (*D. rerio*) (Chen *et al.*, 2008, Bird *et al.*, 2015).

These results underline the need for further characterisation of the surface of *S. iniae*, as identification of a mechanism to block the binding or invasion of neutrophils (and/or other host cells) could halt proliferation and subsequent disease. While the capsule is vital to bacterial proliferation, it was not a primary factor involved in escape from immune degradation and capture.

There must be other molecules involved in colonisation, evasion, suppression and invasion of *S. iniae* in host blood and tissues (Baiano *et al.*, 2009).

While vaccines are currently strain specific, the need for polyvalent coverage is exemplified by fish stock losses in North America, the Middle East and the Asia-Pacific (Agnew *et al.*, 2007), and vaccine failures here in Australia, and elsewhere (Bachrach *et al.*, 2001, Millard *et al.*, 2012). While this work focuses on innate immunity and not adaptive immunity, more directly related to vaccine and antibody production, understanding how the bacteria enter the fish and proliferate, despite active neutrophil migration may identify candidate targets for better vaccines.

Neutrophil processes and pathways in fish continue to be defined, and with the rapid expansion in the field of bioinformatics, coupling *in vitro* cellular studies such as those presented in this thesis, together hold the key to understanding complex interactions *in vivo*. The binding of *S. iniae* to neutrophils, without activation, may well be the method they employ to cross the blood brain barrier into the central nervous system. In human neonatal BBB invasion, Group B *streptococcus* utilize lipoteichoic acid binding to endothelium to mediate membrane transgression (Doran *et al.*, 2003). Furthermore, *E.coli* K1 specifically targets immune cells to sequester it from the immune system. Specific binding via FcγRIa (CD64) allows invasion of macrophages and dendritic cells, and receptor gp96 targets neutrophils, allowing the bacteria protective passage through the host (van Sorge *et al.*, 2012, Mittal *et al.*, 2011). The *S. iniae* factors involved in this process may be excellent targets for cross-serotype vaccination, and should be a priority for further research.

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Appendix 1.

A reliable method for enrichment of neutrophils from peripheral blood in barramundi (*Lates calcarifer*)

Kelly-Anne Masterman and Andrew C Barnes

Abstract

Neutrophils are a short-lived, terminally differentiated, innate immune cell, that are critical first responders during infection. Research into neutrophil-pathogen interactions in fish has primarily employed cells derived from the pro-nephros and nephros. Since these sites are also the location of neutrophil and other immune cell development, there may be some ambiguity in maturation and functional ability of these cells, and difficulty in differentiating the effects of neutrophils from those of macrophages and monocytes. In contrast, peripheral blood circulating neutrophils are mature and ready to respond, thus it may be more physiologically relevant to use these cells for immune studies when evaluating interactions with blood-borne pathogens. The enrichment of tropical, euryhaline fish blood cells cannot follow classic mammalian enrichment methods for several reasons: Fish have nucleated red blood cells (RBC's), a high number of reticulocytes, a very low number of granulocytic leukocytes and an osmotic tolerance, rendering techniques such as water lysis ineffective. Enrichment of neutrophils while minimizing RBC contamination is imperative for studies where luminescence or fluorescence signals may be confounded by background from an overabundance of RBC's. We have optimized a method for enriching neutrophils from peripheral blood with an initial settlement step employing 6% dextran (Mr 450,000-650,000) for 30-60 min at room temperature, followed by density separation on an 8-step Percoll density gradient. This method provides a cell suspension comprising 20 - 50% neutrophils, free of contamination from reticulocytes. These are then suitable for luminometric or fluorometric downstream analyses.

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Neutrophils are a key early responder innate immune cell involved in detection and clearance of microbes. Isolation and/or enrichment of neutrophils can be problematic due to manipulation sensitivity and a short lifespan (Sepulcre *et al.*, 2011). In teleosts, the head-kidney functions as the mammalian bone marrow equivalent, seeding granulocytic precursors which develop into neutrophils. These cells mature in the trunk kidney before moving into peripheral blood circulation for immune surveillance (Fijan, 2002). Upon microbial encounter, neutrophils phagocytose the foreign entity, secrete exogenous antimicrobial agents or cast neutrophil extracellular traps (NETs) (Brinkmann *et al.*, 2004, Finco-Kent *et al.*, 1987, Palic *et al.*, 2005). Assessment of blood neutrophils has proven difficult due to the low number of circulating cells (less than 5% in teleost) (Havixbeck *et al.*, 2016) and interference by red blood cells (RBC). Immature RBC, known as reticulocytes, may occur in a range of percentages in different teleost species (Catton, 1951), and have a range of densities that overlap with neutrophil density. This reduces the effectiveness of a routine Percoll density gradient separation, so alternative measures were sought to remove RBC, and particularly reticulocytes, from blood neutrophil preparations. Lysis of red blood cells using ammonium citrate buffer was ineffective since fish blood cells are nucleated. Fresh water osmotic lysis was also unsuccessful as barramundi are euryhaline and can tolerate a broad range of salinities. We have developed a method to enrich neutrophils from barramundi (*Lates calcarifer*) blood, utilising dextran settlement prior to Percoll density gradient separation to enrich neutrophils.

Barramundi juveniles (40 – 100 mm fork length) were obtained from a local commercial hatchery and kept in aquaria with recirculated brackish water (15 ppt) at 28 +/- 2°C. Water quality was checked daily for ammonia, nitrite, nitrate and pH, and water exchanges (~5 - 10% v / v) were done regularly as required. Fish were fed at a maintenance rate (approx. 5% body weight daily) twice per day, with a commercial diet for barramundi (Ridley Aqua Feed). Prior to bleeding, fish were anaesthetised with a lethal overdose of Aqui-S (Lower Hutt, New Zealand). Blood was collected via

caudal venipuncture, with a heparinised 23G needle and immediately mixed with 10% final volume citrate-phosphate-dextrose anticoagulant. This mixture was added to an equivalent volume of 6% dextran (M_r 450,000-650,000 from *Leuconostoc spp*. Sigma-Aldrich, Castle Hill, Australia) in 0.9% NaCl, 0.22μm sterile filtered. The tube was inverted 3 - 5 times before being allowed to settle undisturbed at room temperature (RT) for 30 - 60 min. Once settled, the tube was centrifuged at 10 x *g* (at RT) for a further 30 min with no brake and acceleration set to the minimum in an Eppendorf 5810R centrifuge. The upper opaque layer (Fig. 1A) was removed via wide bore pipette and placed on top of a freshly prepared 8 step Percoll (GE Healthcare, Parramatta, Australia) gradient, of densities 1.092, 1.089, 1.086, 1.083, 1.08, 1.75, 1.07, 1.06 g / ml. The Percoll gradient was centrifuged at RT, 400 x *g* for 30 min, with no brake and minimum acceleration (Fig. 1B). The uppermost buffy layer was collected and washed once with phenol-red-free RPMI-1640 (Sigma-Aldrich, Castle Hill, Australia). Cell counts and cytospins (Tharmac Cellspin, POCD, Artarmon, Australia) were prepared to assess yield and enrichment of neutrophils. Standard differential cell counts from blood smears from 4 fish were also performed by Brisbane Bird and Exotics Veterinary Service to enable comparison with cell counts post-neutrophil enrichment (Table 1).

Table 1. Differential cell counts derived from blood smears taken from healthy *Lates calcarifer* reared in brackish (15 ppt salt) recirculating water. Four fish were sampled. For comparison, ranges of white blood cell % (WBC) and post dextran-Percoll neutrophil enrichment are included, 100 field views were surveyed via light microscopy at 40 x magnification.

	Whole Blood	Post-Enrichment
packed RBC	30 - 43 %	n/a
reticulocytes	3.6 - 10 %	n/a
white cells	3.4 - 7.8 %	> 90%
lymphocytes (% WBC)	64 - 92 %	35 - 70%
monocytes/ macrophage (% WBC)	4 - 16 %	5 - 25%
neutrophils (% WBC)	6 - 36 %	25 - 50%

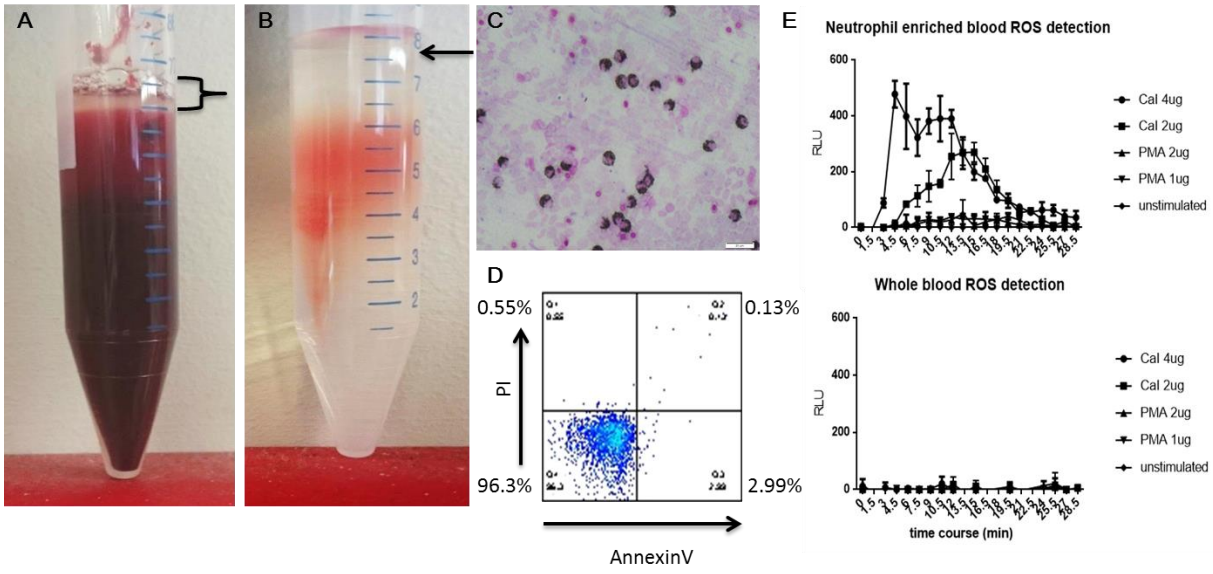

Fig.1. Peripheral blood neutrophil enrichment from barramundi. A) Barramundi blood mixed 1 : 1 with 6% dextran, 30 min gravity settlement followed by 30 min centrifuge at 10 x *g*, bracket shows opaque layer collected for layering onto Percoll density gradient. B) Subsequent Percoll gradient,

arrow indicates location of buffy layer where neutrophils reside. C) Enriched neutrophils from blood stained with peroxidase (brown) and hemacolor at 40 x. Micrographs were taken using an Olympus BX41 epifluorescent microscope, images captured with an Olympus DP26/U-CMAD3 camera. D) Flow cytometric assessment of neutrophil viability and apoptosis post-enrichment. E) Reactive oxygen species (ROS) luminol assay demonstrating respiratory burst activity of enriched neutrophil preparation (E top) and whole unprocessed blood (E bottom).

Large polymer solutions, such as high molecular weight dextran (≥ 40 kDa), can be used to bind RBC in a reversible process to make large linear or branched aggregates (Neu *et al.*, 2008). These complexes can be allowed to settle via gravity or gentle centrifugation leaving the leucocytes at the top. The leucocytes were further enriched via Percoll, a colloidal silica solution, using gradients of differing concentrations of Percoll prepared in Hank's Balanced Salt Solution (HBSS with calcium and magnesium, no phenol red, Sigma-Aldrich, Castle Hill, Australia) and ultrapure water (Sigma-Aldrich, Castle Hill, Australia). The resultant cellular enrichment gave a yield of 2 - 3 x 10^6 cell per ml of whole blood and the neutrophil percentage of this cell preparation ranged from 25 - 50% as assessed via 3,3'-diaminobenzidine peroxidase (DAB, Sigma-Aldrich, Castle Hill, Australia) and Hemacolor® (HC) staining (Merck Millipore, Bayswater, Australia) of cytospin slides. Neutrophils were identified via brown peroxidase staining, only expressed in neutrophils and monocytes, and via HC staining in which neutrophils were identified with a multi-lobed horseshoe-shaped nucleus (Fig. 1C and 1D). Optimisation experiments to reduce the number of layers in the multilayer Percoll gradient determined that enrichment was reduced, or contamination with other cell types increased, if any of the eight density layers were omitted

Since *in vitro* manipulation of neutrophils can cause activation and apoptosis, neutrophil respiratory burst activity was assessed via luminol assay to confirm cell functionality, as adapted from a human blood luminol assay (Carulli *et al.*, 1995). In brief, enriched blood neutrophil cell preparations were resuspended to 5x10^6 cells per ml in RPMI, and seeded into a flat bottom black 96-well plate

(Greiner, Germany) at 100 μl per well. Stimulatory agents were diluted and mixed with 10 mM luminol (Sigma-Aldrich, Castle Hill, Australia) in 20 M borate buffer, at pH9, and 100μl added to cell wells including negative and unstimulated control wells. The luminescent signal was immediately read every 30 s for 30 min using a BMG FLUOstar (BMG Labtech, Ortenberg, Germany). Units are expressed as relative luminescent unit and wells were set up in triplicate. Whole blood diluted 1:4 in RPMI assessed for luminol based ROS activity consistently gave little to no signal (Fig. 1E), luminol possibly being absorbed by RBCs rather than oxidised via ROS to produce chemiluminescence. Additionally, in order to determine whether the multi-step enrichment procedure was killing neutrophils, viability was assessed during each cell count via trypan blue exclusion and confirmed on several occasions via live/ dead cell kit based on uptake of annexin V and propidium iodide, which was detected using flow cytometry. In every case, viability was always greater than 90% (Fig. 1D).

In conclusion, these combined methods allow a reproducible enrichment of mature, circulating neutrophils from teleost blood. These may be used for further immunological assessment as evidenced by respiratory burst in response to positive controls. This enrichment process will substantially assist with assay design and development specifically for mature neutrophils, and progress understanding of the vital first responder role neutrophils play in pathogenic clearance. This should prove advantageous compared to evaluation of function of potentially immature cells derived from haematopoietic tissues such as head-kidney. Indeed, peripheral neutrophils in goldfish (*Carassius auratus*) seem to be deployed from the head-kidney reserve during inflammatory challenge (Havixbeck *et al.*, 2016). The rapid extravasation of neutrophils supports the theory that neutrophils play a vital role in the early regulation of the innate response. In murine systems, myeloperoxidase secreted by stimulated neutrophils has been shown to enhance both phagocytosis and killing of microorganisms (Lincoln *et al.*, 1995) and trigger cytokine release, which in turn activate macrophages (Wei *et al.*, 1986). Studies of these regulatory functions by mature circulating

neutrophils necessitate enrichment procedures to enhance numbers of quiescent cells for *in vitro* activation.