THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

On the origin of Group B *Streptococcus* from disease outbreaks in wild marine fish in Australia

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BSc Honours

MSc

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2014

School of Biological Sciences

Abstract

Since 2007, 96 wild Queensland groupers, *Epinephelus lanceolatus*, were found dead from Karumba (Gulf of Carpentaria) in Northern Territory to Brisbane in southern Queensland, Australia. In twelve cases, *Streptoccocus agalactiae* (Group B *Streptococcus*, GBS) was isolated in pure culture from eyes and internal organs. GBS has been also isolated from other marine species (Javelin grunter, Giant catfish and Squaretail mullet) and caused an outbreak in three species of wild stingrays (Eastern shovelnose ray, Mangrove whipray and Estuary ray) in a public aquarium also in Queensland. These findings have raised public and industry concern over threats to human health from potential transmission between fish and humans, over environmental impacts on other aquatic animals, and over the potential threat posed to the growing Australian aquaculture industry in which, to date, there have been no reported cases of this disease in spite of the devastation caused to the industry overseas. Therefore, my thesis set out to determine whether GBS isolated from dead wild fish in Australia is indeed virulent in Queensland grouper and causative of mortality, to try to identify from where these infections in wild fish originated, to determine potential risk to human health and to other aquatic animals, and to begin to identify mechanisms by which these Australian isolates may cause fatal disease in Queensland grouper. This information may be employed in future risk assessment of potential human transfer, in mitigating further outbreaks in wild fish through understanding origin of infection, and in future development of preventative measures such as vaccination for farmed fish.

In order to confirm that *S. agalactiae* was the cause of the mortality in wild fish, experimental challenges were conducted by injection, immersion and through the oral route. To better understand why these strains of GBS are pathogenic in fish, we conducted a series of cellular immune assays using juvenile Queensland grouper head-kidney leucocytes in the presence of different piscine and terrestrial isolates. Whole-genome sequencing analysis of the piscine and terrestrial isolates provided information on potential origin and potential for transfer between species, and also informed us on critical virulence factors that could be used during pathogenesis of GBS. Moreover, genome analysis gave intriguing insight into the evolution and adaption of GBS to fish as a host and to life in the aquatic environment.

Based on results from infectivity trials, a local piscine isolate of *Streptococcus agalactiae* demonstrated high virulence in juvenile Queensland grouper. By injection it caused 100% morbidity and mortality regardless of challenge dose, and mortalities were significantly higher in fish challenged by immersion, suggesting that skin mucus plays an important role in the initial steps of the infection. Oral challenge by admixture with feed resulted in development of clinical symptoms in ~ 10 % of fish challenged with the highest dose, and a high level of asymptomatic carriage was detected by histology and PCR but no mortalities were recorded, suggesting a possible natural oral route of infection, but that other factors are required for overt disease. Clinical signs and pathologies typical of streptococcal infections

were successfully reproduced and corresponded to what has been described in the wild cases in Australia and elsewhere.

To determine putative origin of GBS in wild fish in Australia, multilocus sequence typing (MLST) and capsular polysaccharide (CPS) were derived from whole-genome sequences and revealed that all piscine isolates from Australia were serotype Ib and fell into ST-261 lineage first identified in 1986 from Tilapia in Israel. Overseas, ST-261 has only been found in fish and poikilotherm animals, and has never been associated with humans. High similarity and identical point mutations in the capsular operon of the piscine isolates suggests a common source of infection and subsequent passage amongst these animals.

Mature macrophages and neutrophils from the head-kidney leucocytes are the major phagocytic cells of *E. lanceolatus* compared to the smaller and less complex monocytes. Quenching of reactive oxygen species (ROS) production coupled with high phagocytic indexes and elevated immune cell mortality rate caused by some terrestrial GBS isolates suggests potential for transmission from terrestrial animals to fish, in line with previous research in Brazil and the USA.

Whole-genome sequence analysis revealed a significant genome reduction in the piscine GBS isolates compared to the human and terrestrial strains, characterised by the loss of metabolic functions. Phenotypically, while piscine and terrestrial isolates shared the same enzymatic hydrolysis profile, their ability to ferment carbohydrates differed greatly, corroborative of substantial reduction in carbohydrate metabolic genes. Genomic mutations observed in the capsular polysaccharide were phenotypically relevant, with complete loss of the capsule in two of the piscine isolates. Marine GBS isolates grew more slowly than terrestrial isolates in rich medium, but this difference, though still significant, was less pronounced at lower temperature (at 28°C compared to 37°C). Most virulence factors found in mammalian isolates were missing from fish strains, but a few were conserved. As meningitis is common to GBS infection in both humans and fish, this thesis provides a basis to identify factors conserved between terrestrial and marine isolates of GBS that are critical to development of meningitis.

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.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer reviewed publications

Delamare Deboutteville, J., Bowater, R., Condon, K., Reynolds, A., Fisk, A., Aviles, F., Barnes, A.C., 2014. Infection and pathology in Queensland grouper, *Epinephelus lanceolatus*, (Bloch), caused by exposure to *Streptococcus agalactiae* via different routes. Journal of Fish Diseases, doi: 10.1111/jfd.12273.

Delamare Deboutteville, J., Bowater, R., Ben Zakour, N.L., Barnes, A.C. Multilocus sequence typing and capsular operon analysis of Group B *Streptococcus* infecting wild marine fish in Australia indicate that they have not arisen as a result of recent transfer from a terrestrial source; *in preparation*, 2014

Delamare Deboutteville, J., Bowater, R., Stanford, J., Barnes, A.C. Development and validation of a specific immunohistochemistry method for the detection and localization of *Streptococcus agalactiae* infections in fish tissues; *in preparation*, 2014.

Delamare Deboutteville, J., Kawasaki, M., Zoccola, E., Millard, C., Nefedov, M., Bowater, R., Barnes, A.C. Cellular immune functions of Queensland grouper (*Epinephelus lanceolatus*) pronephros leucocytes in response to *Streptococcus agalactiae* from marine and terrestrials origins analysed by flow cytometry; *in preparation*, 2014.

Ben Zakour, N.L., **Delamare Deboutteville, J**., Stanton-Cook, M.J., Senabouth, A., Bowater, R., Barnes, A.C., Beatson, S.A. Tracking the origin of bacterial outbreaks in marine environments using next-generation sequencing; *in preparation*, 2014.

Conference abstracts

Delamare Deboutteville, J., Bowater, R., Fisk, A., Reynolds, A., Aviles, F., Barnes, A.C. Experimental study of the susceptibility of Queensland grouper, *Epinephelus lanceolatus*, to infection with *Streptococcus agalactiae* by different routes of exposure. *Conference abstract: 1st Australasian Scientific Conference on Aquatic Animal Health*, Cairns, Australia, 2011.

Delamare Deboutteville, J., Bowater, R., Condon, K., Ben Zakour, N.L, Barnes, A.C. *Streptococcus agalactiae* mortalities in wild Queensland grouper: possible routes of transmission and origin of marine strains. *Conference abstract: 53rd Australian Society for Microbiology*, Brisbane, Australia, 2012.

Delamare Deboutteville, J., Bowater, R., Stanford, J., Barnes, A.C. Development and validation of a specific immunohistochemistry method for the detection and localization of *Streptococcus agalactiae* infections in fish tissues. *Conference abstract: 2nd Australasian Scientific Conference on Aquatic Animal Health*, Cairns, Australia, 2013.

Delamare Deboutteville, J., Bowater, R., Condon, K., Ben Zakour, N.L, Barnes, A.C. Emerging Group B *Streptococcus* in wild marine fish in Australia belong to ST-261 and are highly pathogenic in Queensland grouper by injection and immersion. *Conference abstract: 16th International Conference on Diseases of Fish and Shellfish,* Tampere, Finland, September 2013.

Publications included in this thesis

No publications included

Contributions by others to the thesis

Jenny Stanford was the histologist on the project, and performed sectioning, staining and mounting of fish tissue sections on slides relevant to Chapter 2. Rachel O Bowater provided valuable contribution on histopathology interpretations (including pictures of Gram-Glynn-positive bacteria from infected tissues) relevant to Chapter 2. Kelly Condon conducted PCR validation on tissue samples for all Queensland grouper tested in experimental challenge trials relevant to Chapter 2. Nouri L Ben Zakour and Mitchell J Stanton-Cook for writing the bioinformatic pipelines used for the assemblage, annotation and analysis of genomic information relevant to Chapter 3 (extraction of MLST and CPS operon data) and Chapter 5 for extraction of GBS isolates virulence factors. Andrew C Barnes provided invaluable scientific-contribution to the conception and design of the project, including significant critical editorial guidance relevant to the thesis as a whole including all Chapters.

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

None

Acknowledgements

I particularly want to thank my main supervisor Andy Barnes, who brought during these three years of research, invaluable scientific-input and support. His encouragement and guidance have greatly contributed to improve my scientific writing skills, which is for me probably one of the greatest achievements of this experience. I would like to thank Rachel Bowater who brought this exciting scientific project to UQ for Andy and I. Rachel has been of a great support with all aspects of histology and pathology interpretations, I am grateful for all these skills that I have learned. I would like to thank Adam Reynolds for his depth of expertise, providing fish from multiple Queensland grouper spawnings, for all of the experimental challenge trials; this was not an easy task and without fish many aspects of this project could not have been achieved. My thanks also go to Andrew Fisk who spent time to assist me with the upgrading and set-up of aquaria for the suitable running of experimental challenge trials. I am very grateful to Luck Hammond and Michael Nefedov who provided technical assistance with epifluorescence microscopy and flow cytometry respectively; both gave me the passion for these two extraordinary scientific tools. Special thanks to all my fellow research members from the Aquatic Animal Health Laboratory including Cecile Dang, Candice Millard, Kelly-Anne Masterman, Chan Nguyen Dieu Hoai, Timothy Green and Fabian Aviles for their friendship, and my sincerely gratitude goes to Minami Kawasaki and Emmanuelle Zoccola for their countless support in the lab. I would like to thanks the Australian Government for their support with an International Postgraduate Research Scholarship that enabled me to complete this PhD and the University of Queensland for their financial support with the UQ Centennial Scholarship. Finally I wish to thank the Fisheries Research and Development Corporation Aquatic Animal Health Subprogram for financial support of this research.

Keywords

Streptococcus agalactiae, *Epinephelus lanceolatus,* microbiology, next-generation sequencing, immunohistochemistry, multilocus sequence typing, capsular polysaccharide, leucocyte, respiratory burst, phagocytosis.

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 060503, Microbial Genetics, 20%

ANZSRC code: 070707, Veterinary Microbiology, 40%

ANZSRC code: 060804, Animal Immunology, 40%

Fields of Research (FoR) Classification

FoR code: 0605, Microbiology, 80%

FoR code: 0608, Zoology, 20%

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List of Abbreviations used in thesis

16S rRNA	small subunit rRNA of bacteria and archea
ANOVA	analysis of variance
ATCC	American type culture collection
BBB	blood-brain barrier
BLASTn	basic local alignment search tool nucleotide
BMECs	brain microvascular endothelial cells
bp	base pair
BRIG	blast ring image generator
BSA	bovine serum albumin
CC	clonal complex
CCD	charge-coupled device
CFU	colony forming unit
CPS	capsular polysaccharide
DABCO	1,4-Diazabicyclo[2.2.2]octane
DAPI	4',6-Diamidino-2-phenylindole
DCF	2',7'-dichlorodihydrofluorescein
DCFH	2',7'-dichlorodihydrofluorescin
DCFH-DA	2',7'-dichlorodihydrofluorescin diacetate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
FFPE	formalin-fixed paraffin-embedded
FISH	fluorescence *in situ* hybridisation
FITC	fluorescein isothiocyanate
GBS	Group B *Streptococcus*
GG	Gram-Glynn
HKL	head-kidney leucocyte
ICC	immunocytochemistry
IgG	immunoglobulin G
IgM	immunoglobulin M

IHC	immunohistochemistry
i.p	intraperitoneal injection
LED	light-emitting diode
Log	logarithm
LPS	lipopolysaccharide
MAMPs	microbe-associated molecular patterns
MLST	multilocus sequence typing
MOI	multiplicity of infection
NGS	next generation sequencing
OD	optical density
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PBST	phosphate buffer saline triton X-100
PFA	paraformaldehyde
PI	propidium iodide
PMA	phorbol 12-myristate 13-acetate
ppt	parts per thousand
PRRs	pattern recognition receptors
PTG	peptidoglycan
RNA	ribonucleic acid
RNAse	ribonuclease
rRNA	ribosomal ribonucleic acid
ROS	reactive oxygen species
rpm	rotation per minute
SBA	sheep blood agar
SLV	single locus variant
SNP	single nucleotide polymorphism
ST	sequence type
THB	Todd-Hewitt broth

Chapter 1

General Introduction

BACKGROUND TO THE PROBLEM

Since 2007, 96 wild Queensland groupers, *Epinephelus lanceolatus*, a protected species under the Fisheries Regulation 2008, were found dead between Brisbane and Karumba (Gulf of Carpentaria) (Bowater *et al.*, 2012). Most deaths occurred in highly urbanised areas including Cairns and Port Douglas, with peak mortalities occurring in winter. In many of these cases, *Streptococcus agalactiae*, also referred to as Group B *Streptococcus* (GBS), was isolated in pure culture from eyes and internal organs by the Queensland Primary Industries and Fisheries (QPIF) Tropical & Aquatic Animal Health Laboratory (TAAHL) in Townsville. This is the first reported case of streptococcosis due to *S. agalactiae* in wild fish in Queensland, and to our knowledge, of any fish in Australia. This appears to be a newly emerging bacterial disease of Queensland groupers and other marine fish species. Emerging infectious diseases pose a threat to ecosystem biodiversity, and there is growing evidence to support the link between human environmental disturbance and emerging infectious diseases of wildlife populations (Daszak *et al.*, 2001).

S. agalactiae has caused large-scale wild fish kills in several countries including the USA, Brazil, Kuwait and Israel (Baya *et al.*, 1990; Evans *et al.*, 2006a; Plumb *et al.*, 1974). Natural epizootics of streptococcosis from *S. agalactiae* occurred in Klunzinger's mullet (*Liza klunzingeri*), Bluefish (*Pomatonus saltatrix*), Striped bass, (*Morone saxatilis*) and Squeteague (*Cynoscion regalis*) (Baya *et al.*, 1990; Evans *et al.*, 2006a). GBS has also caused disease epizootics with significant on-farm losses on aquaculture farms, in red tilapia (*Oreochromis* sp.), red hybrid and Nile tilapia (*Oreochromis niloticus*) grown in Thailand, Malaysia and Columbia (Abuseliana *et al.*, 2010; Chen *et al.*, 2007; Hernández *et al.*, 2009; Musa *et al.*, 2009; Suanyuk *et al.*, 2008; Zamri-Saad *et al.*, 2010), and Gilthead seabream (*Sparus aurata*) in Kuwait (Glibert *et al.*, 2002). The bacterium has recently been isolated in China affecting Barcoo grunter (*Scortum barcoo*) and Ya-fish (*Schizothorax prenanti*) (Geng *et al.*, 2012; Liu *et al.*, 2013c).

S. agalactiae is also a significant bacterial pathogen of humans, causing septicaemia and meningitis in newborn babies and disease in immuno-compromised humans (Dermer *et al.*, 2004). It also causes mastitis in cows, and disease in wild and captive saltwater crocodiles, dolphins, frogs and lizards (Amborski *et al.*, 1983; Bishop *et al.*, 2007; Evans *et al.*, 2006b; Hetzel *et al.*, 2003; Keefe, 1997; Zappulli *et al.*, 2005).

In 2009 sick Javelin grunter (*Pomadasys kaakan*) were caught by a recreational fisherman in Cairns and confirmed to be infected with *S. agalactiae*. Javelin grunter are popular recreational fish, caught also by commercial fishermen and sold in seafood shops and the finding of *S. agalactiae* resulted in public and industry concern over food safety. Further research is needed to determine the genetic relatedness of the Australian fish *S. agalactiae* strains to human *S. agalactiae* strains in north Queensland, to ascertain any public health risk in consuming and handling infected fish.

Following the discovery of infected Javelin grunter, a small survey of fish was conducted in Cairns inlet by QPIF Fisheries & TAAHL targeting six different fish species. Of these, Squaretail mullet

(*Ellochelon vaigiensis*) tested positive for *S. agalactiae*. Mullet are caught locally by recreational bait fisherman and are sold fresh or frozen as bait. It is currently not known how many different fish species are infected with *S. agalactiae*, how widespread the disease is, or how the bacteria are spread.

There is evidence that *S. agalactiae* is also present in wild stingrays, and that the disease has already spread to south east Queensland through the translocation of an infected, wild-caught stingray from Cairns. In May 2009, over 40 stingrays died in a disease epizootic at a large commercial display aquarium in south east Queensland. Pathology and bacteriology confirmed eight stingrays (comprising three different species) died from meningitis and bacterial septicaemia from *S. agalactiae*. The first mortality was the wild stingray translocated from Cairns. Subsequent mortalities were local-caught species from Moreton Bay (south east Queensland), and other previously healthy stingrays translocated from north Queensland.

Streptococcal infections in animals may be acquired through ingestion of food contaminated with the bacteria. Mortality in a dolphin was reported following ingestion of mullet infected with *S. agalactiae* during a large wild fish kill in Kuwait Bay (Evans *et al.*, 2002). Similarly, an Amazon river dolphin in a display aquarium in San Francisco suffered abscesses after being exposed to food fish containing *Streptococcus iniae* (Pier and Madin, 1976). It is possible that Queensland groupers may have acquired infection by eating bacteria-containing food (e.g. mullet, juvenile stingrays), or infected water (e.g. ballast water). Currently, nothing is known about how *S. agalactiae* is transmitted in these fish. Further research is also required to establish infection models in Queensland grouper to determine infectious doses and routes of transmission. This will enable risk analysis of transmissibility and also provide invaluable samples for development and optimisation of diagnostic methodologies.

To date, there is evidence that *S. agalactiae* infects Queensland grouper (*E. lanceolatus*), Squaretail mullet (*E. vaigiensis*), Giant catfish (*Netuma thalassina*), Javelin grunter (*P. kaaakan*) and three species of wild stingrays (northern and south-eastern species). Queensland groupers are a target candidate species for aquaculture and currently broodstock Queensland groupers are held at the QPIF Northern Fisheries Centre (NFC). They have been successful at inducing spawning and producing juveniles for aquaculture farm-grow-out trials, thus *S. agalactiae* infection poses a serious threat to this infant industry. Although barramundi (*Lates calcarifer*) aquaculture is substantial in north Queensland, and in regions proximal to Cairns, *S. agalactiae* has not been reported in barramundi in Queensland and it is unknown whether barramundi are susceptible or resistant to *S. agalactiae* infection, or whether they carry the organism asymptomatically.

The recent emergence of *S. agalactiae* as a serious pathogen of protected wild fish is of serious concern yet the origin of the bacterium in Australian waters is unknown. Pathogens can be endemic or exotic, and can arrive in Australia in imported live or fresh fish, frozen bait, ballast water or marine organisms. Furthermore, pathogens can be transmitted through the translocation of live, infected or disease-carrying fish within Australia. Recently molecular methods determined the phylogenetic

relatedness of piscine, dolphin and human *S. agalactiae* strains, and indicated that different strains came from different geographical regions (Evans *et al.*, 2008). Similar methods will be used to elucidate the origin of *S. agalactiae* in wild fish in Australia, and determine whether it is exotic or endemic. Genetic comparisons of the different piscine strains of *S. agalactiae* with human and veterinary isolates will enable epidemiological tracing of infection and identification of sources of infection allowing accurate risk analysis and reassure the seafood industry and consumers in the safety of Australian seafood products.

Our knowledge on the interactions between GBS and Queensland grouper immune system, or fish immune systems in general is limited. In order to cause disease, bacteria may utilise a range of virulence factors to divert or subvert defence mechanisms mounted by the host immune system; these strategies may be confrontational or evasive. Understanding how these pathogens circumvent cellular and humoral immune pathways is critical to understanding why these bacteria are causing disease in this species.

Continuing our research on the origin of GBS in wild fish is necessary to further our understanding of disease transmission, phylogenetic relatedness of isolates, immune response in Queensland grouper, and ultimately on disease prevention through biosecurity protocol and development of protective vaccines. Using infection models, standard molecular and immunological-based techniques, and next generation sequencing technologies, I intend to build and refine upon preliminary data our knowledge for a clearer understanding of this newly emerging disease in aquatic animals from a pathology, genetic, and immunological perspective. To achieve this I aim to address three fundamental questions:

AIMS

1. Can GBS cause mortality experimentally in Queensland grouper and what are the modes of transmission of the disease?

- Evaluate the effect of injection doses in an injection challenge model.

- Determine if the disease could be transmitted through the water in an immersion challenge model.

- Perform an oral challenge model to test if the disease can be transmitted through infected feeds.

2. How genetically related are the piscine isolates to overseas and local strains of *S. agalactiae* isolated from humans and others animals?

Genome sequencing of multiple pathogenic isolates of GBS using the next generation Illumina HiSeq 2000 platform.

- Assemble, annotate and analyse genomic information *in silico*.

- Conduct global population studies of isolates based on genetic variations amongst seven housekeeping genes using a multilocus sequence typing approach (MLST).

- Determine molecular serotype of isolates based on serologically relevant variation in the capsular operon for local population studies.

3. How does the innate immune system of *E. lanceolatus* respond to GBS?

Perform *in vitro* immunological studies by challenging primary head-kidney leucocyte cultures from Queensland groupers with different GBS isolates for which we have complete genomes.

- Determine relative phagocytosis rates of Queensland grouper leucocytes with different strains of GBS by flow cytometry.

- Determine the respiratory burst activity of Queensland grouper leucocytes following phagocytosis of differing strains by flow cytometry.

- Determine the capacity of the various GBS isolates to kill and cause apoptosis in Queensland grouper leucocytes by flow cytometry.

RATIONAL & SCIENTIFIC BACKGROUND

Are GBS primary pathogens of Queensland grouper?

From the 96 cases of dead Queensland groupers (as of 2011), GBS was successfully recovered from eight individuals, thus it is necessary to investigate if GBS is the primary cause of morbidity or a secondary opportunistic coloniser. To determine the most natural transmission scenario, and the virulence potential of GBS in Queensland grouper, three *in vivo* experimental infection challenges will be evaluated respectively by intraperitoneal injection, immersion, and oral challenges in order to fulfil the Koch's postulates, which states that (1) the microorganism must be isolated in pure culture from a moribund animal; (2) the microorganism when introduced into a healthy individual should induce the clinical signs of the disease; (3) the microorganism must be re isolated from the inocula and the challenged experimental fish and proved to be identical to the original microorganism. To test the Koch's postulates in Queensland grouper, we will use a GBS strain previously isolated from a diseased grouper.

GBS genetic diversity: introduction to the pan-genome

Interpretation of bacterial diversity has been based for many years on the clonal population concept, where population structure is not influenced by horizontal genetic exchange (Gupta and Maiden, 2001). However, with the increasing number of new genomes being sequenced, the traditional definition of a bacterial species has been challenged. Indeed, increasing numbers of new genes are discovered even after sequencing many genomes of the same species (Medini *et al.*, 2005). Thus, a good way to describe a bacterial species is to consider its **pan-genome** (all the genes of a bacterial species). The pan-genome is composed of the **core genome** that includes all the genes present in all the strains of the same bacterial species, these genes are responsible for the basic aspects of the biology of the species and its **dispensable or accessory genome** containing the pool of genes present in two or more strains, or unique genes specific to single strains; these genes contribute to the species diversity (Medini *et al.*, 2005; Tettelin *et al.*, 2008). Mathematical models based on multi genome analyses have shown that GBS has an "open" pan-genome of 2713 genes (core genome: 1806 and accessory genome: 907) that is estimated to grow up to 33 new genes for every new strain sequenced (Tettelin *et al.*, 2005). An open pan-genome characterises bacterial species that have the capacity to colonise various ecological niches (Medini *et al.*, 2005).

There are three mains mechanisms by which genetic material can be exchange horizontally within or between bacterial species: (1) by **transformation**, DNA is acquired from the environment; (2) by **transduction**, when the DNA is delivered by a bacteriophage; and (3) by **conjugation,** when DNA is exchanged by contact between organisms (Medini *et al.*, 2005).

Where did the Australian piscine strains come from?

There are over 1762 strains of GBS that have been referenced around the world (Kong *et al.*, 2008).

A large majority of these have been isolated from human and bovine clinical cases. However, *S. agalactiae* is known to be a multi-host pathogen also presents in marine mammals, amphibians, reptiles, and teleostean fish, which raise questions regarding the origin of the piscine strains.

In Australia, GBS appears to be an emerging pathogen in marine fish. Our ability to accurately identify the origin of the strains that cause disease in fish is crucial to future epidemiological studies. There are two quite different ways to approach epidemiological studies. The first one is to determine at the global scale how genetically related are the strains causing disease in Australia compared to other countries around the world (long term or global surveillance) — this approach examines variations that accumulate very slowly within the core genome. The second works at the local scale to establish if the strains isolated from different diseased marine fish in Queensland are the same or different (local surveillance for short term studies) and to determine how are they related to Australian human and bovine clinical strains — this local approach examines variations that accumulate rapidly within the accessory genome which is under strong selective pressure of the environment and the host immune system.

To determine the genetic diversity of Australian isolates and their relatedness to one another and to others isolates around the world we aim to use two molecular typing methods of increasing resolution: MLST and capsular genotyping that will enable positioning of the Australian GBS isolates respectively at the global and local scales.

Genomic diversity

Genomic diversity of GBS at the local scale has been analysed using a wide range of traditional and DNA based methods, including ribotyping, pulsed-field gel electrophoresis (PFGE), random amplified polymorphism DNA (RAPD) and multiplex PCR; restriction enzymes and specific primers are used to characterise individual loci, or unknown regions of the genome (Brochet *et al.*, 2006; Evans *et al.*, 2008; Maiden *et al.*, 1998; Pereira *et al.*, 2010). These techniques give maximal variation within the population but often suffer from poor reproducibility of the result between laboratories, lack of discriminating power to differentiate between isolates, and they are unable to quantitatively measure the genetic variability among isolates.

Pulsed-field gel electrophoresis (PFGE)

PFGE is useful for local outbreak analysis because it can detect variations in the genetic profile between isolates within a defined geographical region. A recent research conducted in Brazil has studied the genetic profile of GBS strains isolated from Nile tilapia, human and cattle using PFGE (Pereira *et al.*, 2010). While, it has been shown that human and bovine strains belong to genetically distinct lineages (Dogan *et al.*, 2005; Evans *et al.*, 2008; Sukhnanand *et al.*, 2005), other reports showed that isolates from human and bovines shared similar genetic profile by PFGE suggesting a common origin (Bisharat *et al.*, 2004; Martinez *et al.*, 2000; Oliveira *et al.*, 2006). The 27 strains of GBS isolated from six different Brazilian states where disease outbreaks occurred were distributed

among five distinct PFGE types with a predominant pattern being present in seven out of the nine farms studied, suggesting a possible transmission of this dominant clone by Brazilian tilapia hatcheries to other farms throughout Brazil. Comparison of the genetic profile (PFGE) of the fish, human and bovine strains indicated that these isolates were genetically unrelated.

Multilocus enzyme electrophoresis (MLEE)

At the global level, population structures (or clonal complexes) of many important infectious agents have been revealed by multilocus enzyme electrophoresis (MLEE), a method assessing the electrophoretic mobilities of conserved enzymes involved in important biochemical processes (Brochet *et al.*, 2006). However, results from MLEE are often difficult to compare between laboratories, and the method as well as other current typing techniques can only detect a small number of alleles within the population, leading to poor discrimination between isolates.

Multilocus sequence typing (MLST)

To overcome this issue and improve our capacity to identify clones within complex bacterial populations, a fully portable technique named multilocus sequence typing (MLST) has been developed to identify alleles from several loci directly from the nucleotide sequences of internal fragments of housekeeping genes that encodes for the enzymes that were assessed by MLEE (Maiden *et al.*, 1998). In MLST, GBS strains are assigned a sequence types (STs) based on the composition of their allele numbers. The data generated are easily transferable between labs with results being uploaded onto a MLST database comparable over the world. The use of the following seven housekeeping genes: *adhP* (alcohol dehydrogenase), *pheS* (phenylalanyl tRNA synthetase), *atr* (glutamine transporter protein), *glnA* (glutamine synthetase), *sdhA* (serine dehydratase), *glcK* (glucose kinase) and *tkt* (transketolase), which are spread across the core genome, will give a much higher level of resolution. MLST compared to MLEE and other DNA based methods is the only method enabling to infer on the phylogenic relationships between isolates (Brochet *et al.*, 2006).

Recent research has investigated the origin of the GBS strains that have caused disease outbreak in wild mullet in Kuwait using a MLST approach to study the genetic relatedness among GBS isolated from fish, dolphin, bovine and human (Evans *et al.*, 2008). All fish isolates from Kuwait, Brazil, Israel and the USA were capsular serotype Ia, and were assigned six different sequence types. At least two divergent pathogenic populations of GBS were identified in fish. Results showed that the fish isolates from the USA, Honduras, Israel and Brazil formed a distinct genetic population of strains that were not related to human and bovines strains, whereas the Kuwait fish and dolphin lineage (ST-7) clustered with human strains associated with neonate sepsis in Japan to form a single clonal complex. Interestingly, each ST was confined to fish strains isolated from a specific geographical location. The authors suggested a human or dolphin origin to the piscine isolates in Kuwait and the possibility for the ST-7 strains to be zoonotic.

MLST will enable us to conduct epidemiological study of population structures at the global level,

allowing us to draw inference on the origin of the Australian strains, and to determine how they are related phylogenetically to other strains from around the world. However, MLST will not detect similarities or genetic differences within the accessory genome of piscine strains isolated from different locations along the coast of Queensland over the last three years. Thus we need a molecular typing tool with higher discriminatory power to assess small genetic variation for local epidemiological studies.

Capsular Polysaccharide (CPS) genotyping

The capsular polysaccharides (CPSs) are the major virulence factors in GBS (Kong *et al.*, 2008; Martins *et al.*, 2010; Millard *et al.*, 2012) and are present in most encapsulated Gram-positive bacteria. To date, GBS strains are classified into 10 different serotypes (Ia, Ib, II–IX) based on variations in the capsular polysaccharide (Kong *et al.*, 2008; Slotved *et al.*, 2007). While microbiology still relies on conventional serology tests to catalogue related bacteria strains according to their composition of capsular polysaccharide using specific antisera, the technique has some limitations as results diverge between laboratories and some strains are still nonserotypeable. This can be explained as many of the new strains are isolated from animals and that most of the available antibodies have been raised against human antigenic proteins. Molecular serotyping of isolates based on serologically relevant variations in the capsular operon have permitted identification of untypeable isolates (Kong *et al.*, 2008), and thus will be used at the local scale to study clinical diagnostic samples from Australia.

Capsular specificity genes are present in the accessory genomes, and can be exchanged within the same species or between species by lateral genetic transfer in order to quickly adapt to new environment (Martins *et al.*, 2010; Medini *et al.*, 2005). The polysaccharide capsule is made up by the arrangements of four sugar components into a unique repeated unit (Brochet *et al.*, 2006). The capsule inhibits host immune detection of the pathogen by interfering with the complement pathway (Miller and Neely, 2005), and reducing phagocytosis (Locke *et al.*, 2007).

Mutations in the *cps* genes of the capsular operon change rapidly in response to intense selective pressure of the environment, vaccination and the host immune system (Millard *et al.*, 2012). Mutations change the structure of the capsule resulting in new serotypes. Gene clusters that encode the biosynthesis (enzymes) that makes the major polysaccharide antigens are represented in the Figure 1.1.

By analysing highly polymorphic genes amongst the capsular operon (‘molecular serotyping’), streptococcus strains can be differentiated meaningfully at the local scale.

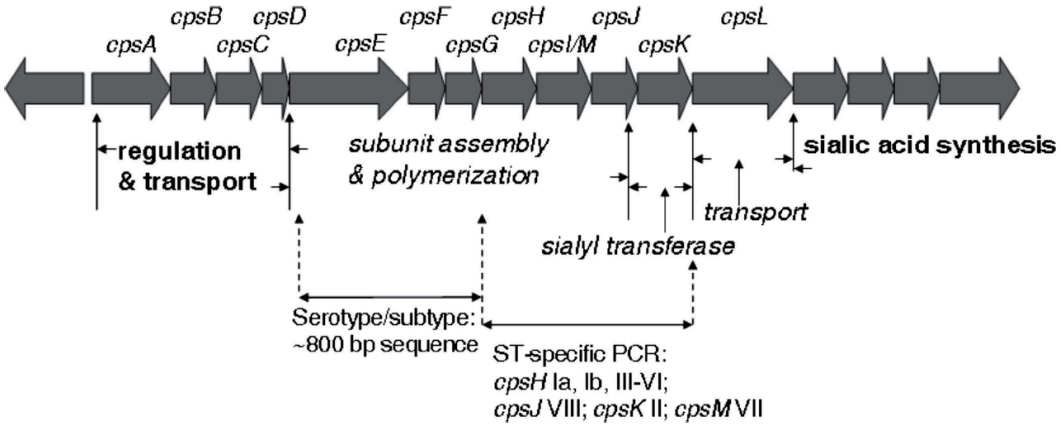

Figure 1.1. Schematic diagram of GBS *cps* gene cluster showing (labels at the bottom) conserved (bold) and variable (italic) regions and sites of sequencing and serotype-specific PCR (and mPCR/RLB) for serotype identification (From (Kong *et al.*, 2008)).

Bacterial infectious diseases in the era of genomics

In less than a year, with the software and hardware available two decades ago, Fleischmann *et al.*, (1995) were the first to sequence a complete genome of a free-living organism: *Haemophilus influenzae*. Advent of the latest next generation sequencing (NGS) technologies coupled with rapid development of bioinformatics tools have significantly accelerated our understanding of the mechanism of bacterial pathogenesis (Croucher *et al.*, 2013; Forde and O'Toole, 2013; Walker and Beatson, 2012). These techniques have been increasingly used in human epidemiology, and have revealed their power to pinpoint the origin of outbreak in hospitals, local communities or trace the evolutionary history of a pathogen on a global scale (Cheung and Kwan, 2012; Maamary *et al.*, 2012; Sherry *et al.*, 2013).

Research into *S. agalactiae* has been one of the major drivers of this genomic revolution, with several complete genome sequences of human and bovine GBS isolates available in the public domain (Glaser *et al.*, 2002; Richards *et al.*, 2011; Singh *et al.*, 2012; Tettelin *et al.*, 2005; Tettelin *et al.*, 2002). Comparative genomics of multiple strains of GBS originated from different host species can give great insights into the diversity of the species, their pathogenic potential and how they evolve under selection in the different environments, host and their immune systems (Brochet *et al.*, 2006; Lin *et al.*, 2011; Richards *et al.*, 2011; Tettelin *et al.*, 2005).

These technologies are now being used to study microbial ecology outside the human clinical field in highly complex system such as the marine environments (Kisand *et al.*, 2012; N'Guessan *et al.*, 2012; Wood *et al.*, 2013).

Delannoy *et al.*, (2012) were the first to report a draft genome sequence of a nonhemolytic GBS (serotype Ib, ST-260, from clonal complex CC 552) isolated in 2008 from diseased-farmed tilapia (*Oreochromis* sp) in Honduras. A recent comparative genomic study, including seven strains of GBS

from fish and poikilotherm, indicated that they form part to at least two distinct lineages that vary by their strategy of host adaptation (Rosinski-Chupin *et al.*, 2013). While the first group of strains is part of a clonal complex (CC 7) known to infect multiple host such as fish, human and cattle, the other strains from the ST260–261 complexes diverged long ago from the human and cattle lineages to adapt specifically to fish. This adaptation was correlated with a massive gene inactivation and changes in gene expression with a number of metabolic functions and virulence factors being affected (Rosinski-Chupin *et al.*, 2013). In the last two years, a number of new genome sequences from tilapia GBS isolates from China and Brazil have been sequenced and deposited on GenBank (Liu *et al.*, 2012; Liu *et al.*, 2013a; Pereira *et al.*, 2013a; Wang *et al.*, 2012).

Comparison of the bacterial pan-genome of different strains isolated from different animals can revealed conserved antigen targets than can be used to better inform during bacterial vaccine design (Maione *et al.*, 2005; Pereira *et al.*, 2013b; Schubert-Unkmeir and Christodoulides, 2013; Tettelin, 2009).

Subsequent to our initial plan, NGS technologies became more attractive as the availability and cost fell dramatically. More importantly, there has been a substantial advance in the software pipelines required for analysis of the data generated by these methods. Consequently we sequenced the entire genomes of 23 Australian *S. agalactiae* isolates from fish, crocodile, cattle and human by Illumina HiSeq 2000, from which all the information required for MLST and *cps* serotyping were derived, but would also offer the potential to discover novel markers associated with Australian pathogenic isolates further down the track.

Why are these strains of GBS pathogenic in Queensland grouper?

Although the relevance of GBS to cause disease in human and cattle is quite clear, information on the potential of *S. agalactiae* isolates from human or bovine to infect others host such as fish is limited. Pereira *et al.*, (2010) have shown in their infectivity challenges that human and bovine strains were able to infect and cause meningoencephalitis in Nile tilapia. Interestingly, the genetic patterns from the piscine, human and bovine assessed by PFGE presented few similarities suggesting that genetic lineage was not a prerequiste for GBS to cause disease interspecies.

Fish cellular immune system

The major interface between host and pathogen is the cellular immune system, in particular the phagocytic cells including macrophages and neutrophils that act collaboratively with other cells amongst the leucocyte populations to play a central role in both innate and adaptive immunity (Ellis, 1977, 1999). The innate immune system is an early non-specific immune response in teleosts that interacts with the adaptive immune response (specific) to maintain to healthy state (Magnadóttir, 2006). Macrophages and neutrophils are specialised in the killing of non-self micro-organisms. In fish they are found in all tissues and organs and are predominant phagocytic cells (Secombes and Fletcher, 1992). They play a key role in non-specific immunity because these cells have evolved in

such a way that they are able to utilize pattern recognition receptors (PRRs) to recognize pathogen/microbe associated molecular patterns (PAMPs/MAMPs) such as lipopolysaccharide (LPS), peptidoglycan (PTG), mannose and bind to these surface molecules to ingest and destroy invading bacteria (phagocytose). Following phagocytosis, macrophages enter a state of heightened respiratory activity termed respiratory burst during which they produce a variety of reactive oxygen and reactive nitrogen species (ROS and RNS respectively), which are considered to be one of the most important microbicidal defences of phagocytes cells, including macrophages and neutrophils (Secombes and Fletcher, 1992; Stafford *et al.*, 2002).

Ultimately most invading bacteria are killed by phagocytic cells — so how do virulent GBS circumvent these cells? We propose to conduct *in vitro* immunological assays (phagocytosis, respiratory burst activity and killing performance of the bacteria) to evaluate key functions of these cells when challenged with various isolates and to correlate *in vitro* observations with potential putative genetic determinant of virulence that will be mined from the genomic data generated above.

Chapter 2

Susceptibility of Queensland grouper, *Epinephelus lanceolatus*, to infection by *Streptococcus agalactiae* via different routes of exposure

ABSTRACT

Since 2007, 96 wild Queensland groupers, *Epinephelus lanceolatus*, have been found dead in north east Australia. In some cases, *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) was isolated, although whether it was causative of mortality is unknown. An isolate from the wild grouper case was employed in experimental challenge trials in hatchery-reared juvenile Queensland grouper by different routes of exposure. Intraperitoneal injection challenge resulted in rapid development of clinical symptoms including bilateral exophthalmia, hyperaemic skin or fins and abnormal swimming. Death occurred in, and GBS was re-isolated from, 98% fish injected and was detected by PCR in brain, head-kidney and spleen from all mortalities, regardless of challenge dose. Challenge by immersion resulted in lower morbidity with a clear dose response. Whilst infection was established via oral challenge by admixture with feed, no mortality occurred. Histology showed pathology consistent with GBS infection in wild mortalities, in organs examined from all injected fish, from fish challenged with medium and high doses by immersion, and from high dose oral challenge. These experimental challenges suggest that GBS is likely causative of the historic mortalities in wild Queensland grouper, with consistent pathology between experimental and wild fish. Natural infection may occur through the water and possibly through the oral route, though fish challenged by the latter route recovered.

2.1 INTRODUCTION

S. agalactiae is an important pathogenic Gram-positive bacterium, emerging globally in a diverse range of fish species (Bowater *et al.*, 2012; Duremdez *et al.*, 2004; Evans *et al.*, 2002; Evans *et al.*, 2009; Hernández *et al.*, 2009; Jafar *et al.*, 2009; Olivares-Fuster *et al.*, 2008). Also known as Group B *Streptococcus* (GBS), it is capable of causing bacterial disease in wild and cultured fish species from temperate and tropical latitudes (Evans *et al.*, 2006a) and infections have been reported in fish from various habitats including estuarine, freshwater and saltwater environments (Bowater *et al.*, 2012; Eldar *et al.*, 1994; Evans *et al.*, 2006a). GBS has caused significant wild fish kills in several countries including USA, Israel, Kuwait, China and Brazil (Eldar *et al.*, 1994; Evans *et al.*, 2002; Godoy *et al.*, 2013; Plumb *et al.*, 1974; Zhang *et al.*, 2013). Natural epizootics of streptococcosis from *S. agalactiae* have occurred in Klunzinger's mullet (*Liza klunzingeri*), Bluefish (*Pomatonus saltatrix*), Striped bass (*Morone saxatilis*) and Squeteague (*Cynoscion regalis*) and, most recently, Queensland grouper (*Epinephelus lanceolatus*) (Baya *et al.*, 1990; Bowater *et al.*, 2012; Glibert *et al.*, 2002; Evans *et al.*, 2006a). GBS has also caused disease epizootics with significant on-farm losses in aquaculture, in red hybrid tilapia *Oreochromis niloticus* x *Oreochromis mossambicus*, Nile tilapia (*Oreochromis niloticus*) and Snubnose pompano (*Trachinotus blochii*) cultured in Thailand, Malaysia, Indonesia and Columbia (Amal *et al.*, 2012; Amal *et al.*, 2013b; Chen *et al.*, 2007; Hernández *et al.*, 2009; Lusiastuti *et al.*, 2012; Musa *et al.*, 2009; Suanyuk *et al.*, 2008) and Gilthead seabream (*Sparus aurata*) in Kuwait (Glibert *et al.*, 2002).

The giant Queensland grouper, *Epinephelus lanceolatus*, is one of the two largest species of grouper (Chiu *et al.*, 2012) and one of the largest bony fish inhabiting the Great Barrier Reef (Bowater *et al.*, 2012). Since 2007, 96 dead wild Queensland groupers were recovered along the north east coast of Australia, from south of Brisbane to the far north of Karumba in the Gulf of Carpentaria (Bowater *et al.*, 2012). Most deaths occurred in highly urbanised areas including Cairns and Port Douglas, with peak mortalities occurring in winter (Bowater *et al.*, 2012). Wild Queensland grouper deaths were postulated to result from bacterial septicaemia and meningitis, possibly caused by *S. agalactiae*, which was isolated from several dead fish. Surveys in these areas have revealed the presence of GBS in other marine finfish including two infected Javelin grunter, *Pomadasys kaakan*, a marine Giant catfish, *Netuma thalassina*, and a Squaretail mullet, *Ellochelon vaigiensis* (Bowater *et al.*, 2012). *S. agalactiae* has also been isolated in the Estuary stingray, *Dasyatis fluviorum*, Mangrove whipray, *Himantura granulata*, and Eastern shovelnose ray, *Aptychotrema rostrata* during two separate outbreaks in a marine display aquarium in Queensland (Bowater *et al.*, 2012). Some of these animals were initially caught in northern Queensland, where many of the grouper had died, and were then translocated to the aquarium.

The source of infection and the route of transmission in Queensland grouper are currently unknown. It is possible that *S. agalactiae* is an endemic pathogen in fish in Australia or that it has been introduced into the country (e.g. via ballast water, imported live fish or baitfish). Queensland grouper may have acquired the disease by ingesting native prey already infected with GBS or by feeding on infected

baits. Animals can acquire streptococcosis by ingesting food containing streptococcal bacteria, resulting in bacterial septicaemia and death. For example, emerald monitors have died from severe septicaemia following ingestion of infected mice with *S. agalactiae* (Hetzel *et al.*, 2003). In Kuwait Bay, following a massive fish kill caused by *S. agalactiae*, a bottlenose dolphin was found dead after feeding on infected mullet (Evans *et al.*, 2006b). The disease could also be transmitted through water (sewage or contaminated water) and be a potential source for infection in Queensland grouper and other finfish in Australia. Genotyping showed that isolates from dead fish samples during a massive fish kill in Kuwait Bay and sewage water samples were identical (Jafar *et al.*, 2009).

Possible routes of infection include transmission through oral exposure, inoculum via trauma or via water-born exposure. Queensland grouper are known to feed on juvenile stingrays, turtles and saltwater crocodiles. Indeed, one of the dead grouper contained a stingray barb in its stomach (Bowater *et al.*, 2012). It is possible that perforation of the stomach or other internal organs following ingestion of prey may result in a possible site of entry for *S. agalactiae* in Queensland grouper. This study determines for the first time whether *S. agalactiae* causes infection, pathology and mortality in Queensland grouper and investigates the possible route of transmission of the disease through experimental challenges.

2.2 MATERIALS AND METHODS

Experimental fish and husbandry

Juvenile Queensland grouper (*E. lanceolatus*), weighing approximately 30 g, measuring between 60–70 mm total length were obtained from the Northern Fisheries Centre in Cairns and held in full strength seawater at 35 ppt salinity in 100 L circular plastic tanks. Quarantine aquaria were organized into two replicate sets of eight tanks each in a biosecure quarantine room. Each set of eight tanks was equipped with its own recirculation system consisting of 1 x 200 L sump, 50 L pre-seeded bio-filter, and a protein skimmer. For infectivity challenges, fish were randomly assigned to each tank such that there were eight individuals per tank (injection and immersion challenges), and 10 individuals per tank for the oral trials. Uninfected control fish were maintained in a similar but separate holding system located in a different room. The temperature of all systems was maintained at 28°C by three heating and cooling systems with a recirculation rate of approximately 120 L per hour. Fish were fed a maintenance diet comprising a commercial 4 mm floating diet (Ridley Aqua Feed, Narangba, QLD) once a day, equivalent to 5% body weight. Water exchange (15%) was performed every week and water quality was checked for ammonia, nitrite and pH twice daily after first introduction and then on a weekly basis once fish were established.

Bacterial strains and growth conditions

In early 2011, two GBS strains previously isolated from dead wild Queensland grouper: QMA0280 (from 2010) and QMA0285 (from 2008) were both confirmed to be virulent in a pre-injection challenge; and as the GBS strain isolated from grouper late 2011 was not yet available, it was decided

to use strain QMA0285 for subsequent replicated experimental challenges. QMA0285 was retrieved from stock (-80°C) and routinely cultured on sheep blood agar (SBA) (Oxoid) for 48 hr at 28°C. From pure culture, a single colony was inoculated into 5 mL Todd Hewitt Broth (THB) and incubated overnight at 28°C with 100 rpm agitation. Bacterial identity was confirmed from a 100 μL aliquot by PCR using the AgaF/AdyR primer set as previously described (Kawata *et al.*, 2004). Cells from the remaining 4.9 mL of the culture were collected by centrifugation 15 min at 10 000 x g and washed twice in phosphate buffered saline (PBS). Pellet was resuspended in PBS to a final optical density of 1.0 at 600 nm. An OD_{600} = 1 for GBS was previously estimated to be between 1 and 3 x 10^8 cfu mL^{-1} by viable count on blood agar.

Experimental infection trials

Prior to exposure, fish were allowed to acclimatize for a period of four weeks.

Injection challenge. For the injection challenge, pathogen dose titration using serial 10-fold dilutions of QMA0285 was prepared such that final cell suspensions were approximately 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 cfu mL^{-1}. Fish were anaesthetised with Aqui-S in accordance with the manufacturer directions for marine finfish (20 mg L^{-1}). The two replicate cohorts of eight fish per treatment were challenged by intraperitoneal injection (i.p.) with 100 μL of each 10-fold dilution of GBS (QMA0285) giving a final inoculum per fish ranging from 10^3 to 10^8 cfu $fish^{-1}$. Replicates of eight sibling fish housed in the separate holding system were injected with 100 μL of a sterile PBS as negative controls.

Bath exposure. For the immersion challenge, three treatment doses: 10^4, 10^5, 10^6 cfu mL^{-1}, along with replicate handling control sentinel groups (unchallenged placed in a separate tank, but within the same flow-through recirculating system) were employed. Replicate cohorts of eight further fish were injected intraperitoneally (10^6 cfu $fish^{-1}$) using a sub-sample of the same challenge inoculum used for immersion as a positive control to confirm bacterial viability and virulence had not been lost between injection and immersion experiments. Each treatment had two replicates of eight fish each, one replicate tank in each of the quarantine aquarium systems. Cohorts of eight grouper per treatment were maintained outside of their original tank in aerated 10 L bucket and exposed separately for 60 min to one of three serial 10-fold dilutions of GBS strain QMA0285 ranging from 10^4 to 10^6 cfu mL^{-1}. All buckets were covered with a lid to prevent aerosol dispersion of the challenge inoculum. After 1 hr, fish were returned to their respective tanks and water from immersion buckets was disinfected (8 mL of a 12.5% sodium hypochlorite w/v solution added per 100 L of contaminated water for 1 hr) before being drained. The handling sentinel groups were held in clean 10 L buckets for 60 min prior to returning to their tanks. The eight positive control fishes were injected as described above.

Oral exposure. Pelleted feed containing GBS (QMA0285) was prepared daily as follows: commercial feed was autoclaved to ensure no other viable bacteria were present. Everyday a fresh overnight THB culture of QMA0285 was obtained in the stationary phase and standardised to an optical density at 600 nm of 1.0 (OD_{600}). Then, using a high quality fish oil (Ridley Aqua Feed), emulsions of the bacterial suspension were prepared at two different concentrations, 10^8 cfu mL^{-1} and 10^5 cfu mL^{-1}, and coated

onto the feed (approximately 25 pellets/fish/day) by vortexing the mixture until all the emulsion was absorbed. The resulting coated feed was determined by viable count to contain approximately 10^6 cfu $fish^{-1}$ and 10^3 cfu $fish^{-1}$ for the two respective treatments. Two replicate cohorts of 10 fish per treatment were used. Juvenile Queensland groupers were fed once a day with freshly prepared food as described above for a period of five weeks.

In all cases fish were given 24 hr care during the challenge period. Any fish showing gross clinical signs of disease (exophthalmia, erratic swimming and darkening) were immediately euthanized by anaesthesia (Aqui-S) and counted as a mortality in accordance with animal welfare and ethics requirements (ie. scheduled euthanasia as opposed to death as an endpoint). For injection and immersion challenges, mortalities were recorded every 4 hr. For the oral challenge, as the mortality rate was very low or nil, the trial was maintained for five weeks. All remaining fish at the end of the challenges were euthanized by immersing fish in Aqui-S at a dose rate of 40 mg L^{-1} until unresponsive and then the cervical spine was severed.

Confirmation of infection by bacteriology and PCR

Brain, head-kidney, liver and spleen were aseptically sampled from all treatment, control and sentinel fishes, streaked onto SBA plates, and incubated at 28°C for up to three days. For detection of GBS by PCR directly from the internal organs, a small piece of the brain, head kidney, liver and spleen was sampled and placed into absolute ethanol and kept at 4°C until processed. Several colonies from each organ of all sampled fish were collected using 1 μL sterile loop and resuspended in PCR tubes (200 μL) containing 50 μL of ultra pure DNAse, RNAse free water (Gibco). When no growth occurred, regions of the SBA plate were swabbed for PCR. Samples were analysed by PCR using a direct lysis method and the AgaF/AdyR primer pair as described previously.

Histopathology

Gills, eye, heart, head-kidney, caudal-kidney, spleen, liver, muscle, skin, stomach, brain, intestine, pyloric caecae and swim bladder were sampled from all dead or euthanized fish for histology. Organs were fixed in 4% formaldehyde for 48 hr, tissues rinsed to remove excess fixative and transferred to 70% ethanol. All samples were stored at 4°C until being processed for histology. Sections were cut at 5 μm and stained with Gram-Glynn stain prepared according to (Bancroft and Gamble, 2008). Slides were analysed by light microscopy with an Olympus BX41 compound microscope. A fish was considered positive for GBS by histopathology if Gram-positive cocci were visualised by microscopy with Gram-Glynn stain inside any organs or tissues, and if the fish also showed pathology indicative of infection. Pathology indicative of infection ranged from mild, to moderate to severe changes, including inflammation of the organs or tissues.

Fluorescence *in situ* hybridization (FISH)

For FISH, all oligonucleotide probes used in this study have been previously described and evaluated (Artz *et al.*, 2003; Kempf *et al.*, 2000; Trebesius *et al.*, 1998; Trebesius *et al.*, 2000). Probes GBS 5'-GTAAACACCAAACMTCAGCG (Table 2.1) have been designed to target specifically the 16S rRNA region of *S. agalactiae*. The probes were synthetised and 5'-labeled with the cyanine dyes Cy5 and Cy3 (Sigma Aldrich, NSW, Australia). *In situ* hybridization was performed according to previous methods (Amann *et al.*, 1990). In brief, pure GBS bacterial cell suspensions and closely related streptococci (*Streptococcus iniae*, *Streptococcus pyogenes*) were immobilised by spotting an aliquot (20 μL) of fixed cell (4% paraformaldehyde PFA) suspension on Teflon coated microscope slides (Biofusion Pty. Ltd.) and left to air dry at room temperature. Thereafter, cell membranes were permeabilised by an ascending ethanol series (50%, 80% and 96% 3 min each). The thick bacterial peptidoglycan layers were digested using lysozyme (5 mg mL^{-1}), mutanolysin (50 U mL^{-1}), pepsin (ready to use solution, Sigma), or proteinase K (100 μg mL^{-1}) separately or in combination for 15, 30, 45 and 60 min. All enzymes were diluted in 10 mM Tris-HCl, pH 8.0. Enzymatic digestion was stopped with PBS and samples re dehydrated in an ethanol series then air-dried. 20 μL of hybridization buffer (0.9 M NaCl; 0.02 M TRIS-HCl pH 8.0; 0.01% SDS) containing 20% formamide and 25 ng of each oligonucleotide was added and samples incubated overnight at 46°C in a humid chamber. After hybridization slides were transferred to a washing buffer (0.225 M NaCl, 0.02 M TRIS-HCl, 0.01% SDS) and incubated for 15 min at 48°C. Subsequently, DNA of both bacteria and host cells was stained with 1 μg mL^{-1} 4′,6-diamidino-2-phenylindole (DAPI) for 5 min. GBS specific probes labelled with the Cy3 or Cy5 dyes were applied simultaneously with probe Eub338-fluorescein isothiocyanate (FITC), complementary to a portion of 16S rRNA unique for the domain *Bacteria* (formerly known as *Eubacteria*). Aliquots of the same samples were tested in parallel with probe Non-Eub338, complementary to Eub338, to control non-specific binding probes.

Following tests on pure bacterial suspensions, the probes were used to examine infected tissue sections from Queensland grouper's organs on histology slides. Both whole bacterial cell (WBC) suspensions and formalin-fixed and paraffin-embedded (FFPE) organs tissue sections were analysed on the BX61 confocal laser microscopy platform (Olympus, Australia).

Table 2.1. Oligonucleotide probes used for FISH on whole bacterial suspension and FFPE fish tissue sections

Probe name	Sequences (5'-3')	Target	rRNA position[d]	Reference
GBS-Cy3[a]	GTA AAC ACC AAA CMT CAG CG	*S. agalactiae*	16/67	1
GBS-Cy5[b]	GTA AAC ACC AAA CMT CAG CG	*S. agalactiae*	16/67	1
Eub338-FITC[c]	GCT GCC TCC CGT AGG AGT	*Eubacteria*	16/338	5
NonEub338-Cy3	ACT CCT ACG GGA GGC AGC	None[f]	None	5

a Cy3 fluoresces in the yellow-green region (550/570 nm)

b Cy5 is fluorescent in the orange-red region (650/670 nm)

c Probe Non-EUB338, complementary to EUB338 was used to detect nonspecific binding of the oligonucleotides to microorganisms.

d First number, type of rRNA (16S); second number, target sequence position of first base of each oligonucleotide.

Immunocytochemistry (ICC) and Immunohistochemistry (IHC)

To confirm that any bacteria observed during histology were GBS, IHC was performed on a selection of 5 μm FFPE archival tissue sections collected during the challenge trials. Optimisation was performed by ICC on pure culture of GBS and tested for specificity against other related streptococci (*S. iniae*, *S. pyogenes*), other Gram-positive (*Lactococcus lactis*, *Bacillus pumilus*, *Staphylococcus epidermidis*) and Gram-negative bacteria (*Photobacterium damselae* subspecies *damselae*, *Vibrio harveyi*), to be finally applied on FFPE fish tissue samples by IHC. Briefly, WBC suspensions were fixed in 4% PFA for 1 hr, rinsed in PBS and 15 μL of each suspension was immobilized onto coverslip (15 x 15 mm) coated with gelatin to 0.5% (v/v) and chromium potassium sulfate to 0.02% (w/v). The histological sections were deparaffinised by dipping slides successively in xylene and 100% ethanol for 10 min twice each, then in ethanol 85%, 70% and distilled water for 5 min each. Both WBC and FFPE sections were then permeabilised in PBS/0.3% Triton X-100 (PBST) complemented with 10% goat serum to block non-specific binding sites for 3 hr at 25°C. Slides were probed with a commercial primary rabbit polyclonal IgG antibody against GBS (Abcam, ab53584) diluted 1:400 in PBST and incubated overnight at 4°C. Primary antibody reacts with type-specific carbohydrate on the surface of GBS. Thereafter, samples were washed six times in PBS for 5 min to remove excess antibodies. Due to high level of autofluorescence of fish tissues when excited at wavelengths in the green, yellow and orange portion of the visible spectrum, detection of the primary antibody with minimum background was achieved with a goat secondary antibody anti-rabbit IgG (Abcam, ab150083) (1:400 in PBST) coupled with a photostable far-red fluorescent dye Alexa Fluor® 647 for 2 hr at room temperature. Tissues were counterstained with DAPI (1:2500) for 10 min and rinsed thoroughly six times in PBS for 5 min before being mounted with an antifading polyvinyl alcohol mounting medium with DABCO®. Coverslips were sealed with nail polish and allowed to cure overnight on a flat surface in the dark at 4°C before being imaged by microscopy.

Imaging

Entire slide tissue sections were scanned using an upright epifluorescent microscope Axio imager Z2 (Zeiss) equipped with a fully motorised X-Y-Z stage/automated robotic arm for loading slides from a feeder platform (Meta Systems). High-quality imaging automation was performed using a digital high-resolution progressive scan CCD camera (Cool Cube, Meta Systems). Slides were pre-scanned with a 5X (0.15) air objective (exposure time (et): 80 ms; camera gain: 16.0). A focus map for each slide was created before the slide was scanned with a 20X (0.8) air objective (camera gain: 7.0). Three-color images at each position were obtained using an ultra-fast colour LED illumination system (Meta systems) with the appropriate fluorescent filters: DAPI: nucleus in blue (et: 0.5 ms), Alexa 647: *S. agalactiae* in red (et: 33.3 ms) and Alexa 488: tissue autofluorescence in green used to contrast with other dyes and to visualize tissue structures (et: 7.8 ms). Tiled images were assembled with the V-slide software (Meta Systems) and saved as an imaris file (.ims).

2.3 RESULTS

Injection challenge

Injection challenge of juvenile Queensland grouper with *S. agalactiae* strain QMA0285 resulted in 100% mortality within four days, at all six doses ranging from 10^3 to 10^8 cfu fish^{-1} in both replicate systems with the exception of the low dose (10^3 cfu fish^{-1}) in replicate 2 that resulted in 62.5% mortality (Fig. 2.1 A, B). There were some small differences in mortality rates between the replicate systems. In replicate 1 (Fig. 2.1 A), fish started to die after 42 hr post injection at the higher challenge doses and all fish were dead in the other treatment doses after 81 hr (Fig. 2.1 A). In replicate 2 (Fig. 2.1 B), the first fish to die (27 hr) had been injected with the highest dose (10^8 cfu fish^{-1}) and a very clear dose effect was noted in terms of mortality times (Fig. 2.1 B). In replicate 1, fish from the high dose treatment all died within 72 hr, followed by all the other treatments groups 10^7, 10^6, 10^5, 10^4 and 10^3 cfu fish^{-1} were all dead 81 hr post-injection. In replicate 2, there were three surviving fish in the lowest treatment dose group (10^3 cfu fish^{-1}). These animals did not present any external clinical signs of the disease four days following the final mortality and were euthanized.

Immersion challenge

In system replicate 1, there was 100% survival in the low, medium and sentinel groups; 87.5% survival was recorded for the high dose group (10^6 cfu mL^{-1}) with a single fish dying 169 hr post immersion (seven days). A consistent 100% mortality was recorded in the control-injected group used to check that challenge strain (QMA0285) had not lost its virulence; with the first fish dying 67 hr post challenge and last one 92.5 hr, thus confirming the high virulence of the inoculum used (Fig. 2.1 C). In replicate system 2 (Fig. 2.1 D), a 87.5% survival was recorded in the low dose groups, with a single fish dying 82 hr post challenge; 75% survival was recorded in fish treated with the medium dose with first mortality occurring 62 hr post immersion; 37.5% survival was recorded in the high dose treatment with first mortality at 73 hr and the last mortality seven days post-immersion. Biofiltration in replicate tank system 2 was less effective than system 1, and level of ammonia increased up to 1–2 ppm over a period of four days. All handling control fish survived and the immersion challenge was concluded after 15 days, eight days post-final mortality.

Oral challenge

Oral challenge by admixture with feed resulted in development of clinical symptoms only in ~10% fish challenged with the highest dose (10^6 cfu fish^{-1}). In replicate 1, a single fish was moribund and immobile at the bottom of the tank. After examination it had severe unilateral exophthalmia and, following euthanasia ascitic fluid was found in the peritoneal cavity. In replicate 2, a single fish was swimming in a disoriented manner, with darkening and loss of appetite.

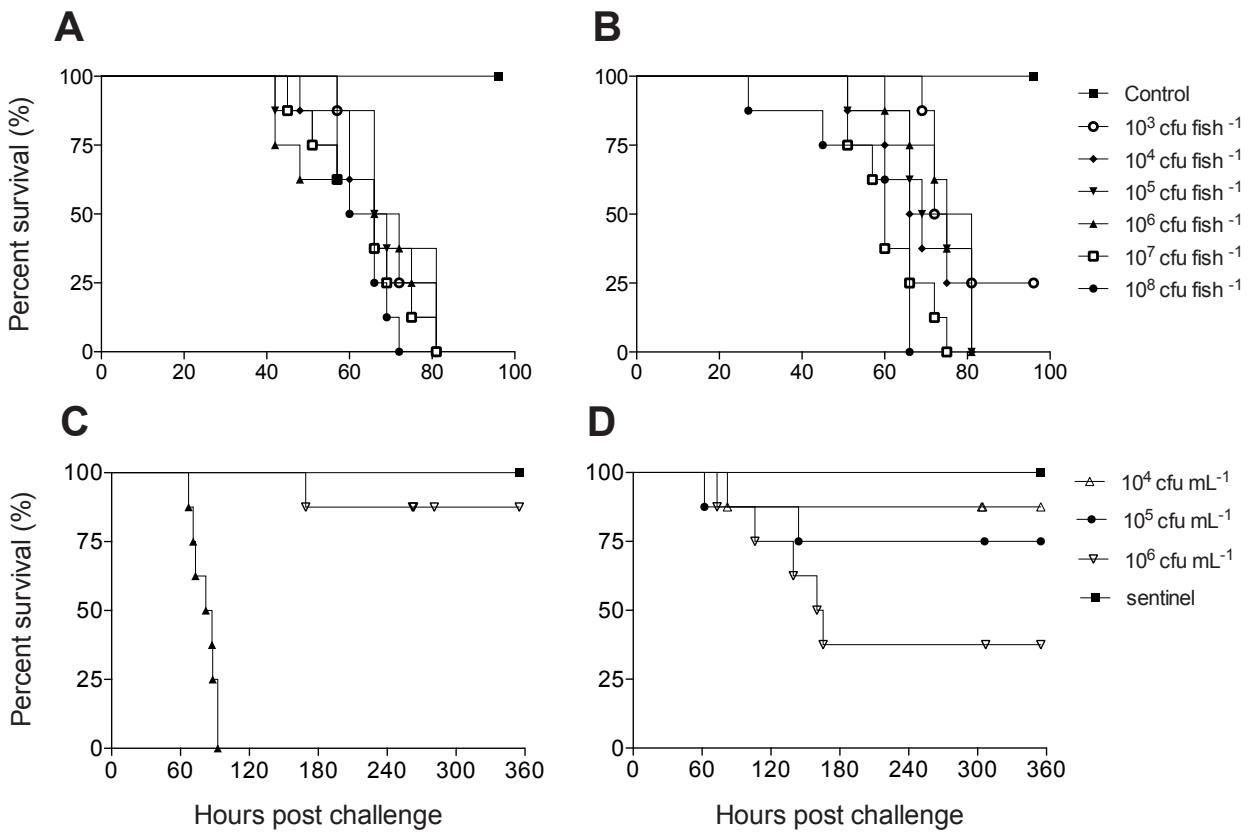

Figure 2.1. Survival curves from injection and immersion challenges using ***S. agalactiae*** (QMA0285) (Kaplan-Meier survival curves). A. Injection (replicate 1), QMA0285 inoculated to juvenile Queensland grouper ***Epinephelus lanceolatus*** by intraperitoneal injection; B. Injection (replicate 2). Six doses were used in the challenge ranging from 10^3 to 10^8 cfu injected per fish. Cohorts of eight fish were injected per treatment. Comparison of survival curves using log-rank (Mantel-Cox) test showed that curves were significantly different and therefore confirmed a dose effect (replicate 1: $P = 0.0031$ and replicate 2: $P < 0.0001$). C. Survival results from immersion challenge (replicate 1). D. Replicate 2. Three doses were used in the challenge ranging from 10^4 to 10^6 cfu mL^{-1}; sentinel fish were unchallenged; cohort of eight fish were immersed per dose. Curves were significantly different for system 1: $P < 0.0001$ but not significantly different for system 2: $P = 0.2129$ (Mantel-cox).

Bacteriology and PCR

Injection challenge

In each replicate and treatment, organs (brain, kidney, liver and spleen) were screened for bacteriology and then by PCR from both bacteriology and tissues samples. GBS was successfully recovered on SBA from all four organs targeted for bacteriology from the fish tested (Table 2.2). Regardless of the six treatments used, ranging from 10^3 to 10^8 cfu injected per fish, all tissue samples showed typical GBS phenotype on SBA. Positive PCR results using the GBS-specific primer pair AgaF/AdyR were obtained in all organs tested (Table 2.2). The three surviving fish (replicate 2) injected with the lowest inoculum (10^3 cfu $fish^{-1}$) were negative by PCR and culture. Bacteriology and PCR tests from all control fish were negative (Table 2.2).

Immersion challenge

In replicate system 1, organs from fish immersed at low, and medium doses, were negative by bacteriology (Table 2.2). However, bacteria were recovered from the brain, kidney, liver and spleen from all positive control fish and from 25% of sampled fish immersed in the high dose in replicate 1. Similarly, all organs were also positive by PCR from all positive control fish, and from 50% fish sampled from high dose in replicate 1, for GBS (Table 2.2). In replicate system 2, all low dose fish were negative by PCR from bacteriology but 75% of sampled fish were positive when PCR was performed on tissues. GBS were recovered by bacteriology from 50% of fish sampled from medium dose (replicate 2), and 100% were PCR positive from organs. All sampled fish immersed at the highest dose from replicate 2 were positive for *S. agalactiae* by bacteriology and PCR. Most fish that were positive for GBS by bacteriology and PCR displayed clinical signs of exophthalmia, skin lesions and erratic swimming behaviour. Remaining fish were all survivors with minor external clinical signs of streptococcosis. Bacteriology and PCR tests of both sentinel and negative control groups were negative in replicate system 1 (Table 2.2). In replicate system 2, GBS was not isolated in culture from any fish, but all of the fish tissues sampled were positive by PCR.

Oral challenge

33% of sampled fish fed with high dose (10^6 cfu $fish^{-1}$) from both replicate 1 & 2 were positive by bacteriological culture (Table 2.2). Sensitivity of detection increased when PCR was performed on tissues; 100% and 83% of the sampled fish fed high dose tested positive respectively in replicates 1 and 2.17% of sampled fish fed low dose (10^3 cfu $fish^{-1}$) were positive by PCR (bacteriology) in replicate 1, none in replicate 2. However, 100% of sampled fish were positive by PCR (tissues) from both replicates. GBS could not be detected by culture from any of the control fish (both replicates); but 33% (replicate 1) and 17% (replicate 2) of sampled fish were positive by PCR when performed directly on organs.

Gross pathology

Fish presented with various forms of unusual behaviour, including disorientation and erratic swimming indicating central nervous system (CNS) involvement. Juvenile Queensland groupers infected with *S. agalactiae* became anorexic and lethargic (depending on the treatment group). Fish injected with the highest doses (10^8 cfu) of *S. agalactiae* started dying within 24 hr post infection. These behaviours were typically observed in nearly all treatment groups of the injection challenge, with marked effects seen in the higher doses (10^7 & 10^8 cfu). Fish in the high dose cohort of the immersion challenge also showed these clinical signs. Juvenile grouper (depending on the treatment group) developed either unilateral or bilateral exophthalmia (Fig. 2.2 B) compared to control fish (Fig. 2.2 A). Some fish showed opacification of the eye (Fig. 2.2 D). The eyes of fish also displayed intraocular haemorrhage, visible in fish with severe exophthalmia (Fig. 2.2 C). Other organs displaying haemorrhages included the gills and skin. The gills were pale and had multifocal petechial haemorrhages of the gill filaments (Fig. 2.2 I) compared to normal gills (Fig. 2.2 J). The skin was often hyperaemic or ulcerated on the

Table 2.2. Bacteriology, PCR and histology results from ***S. agalactiae*** transmission challenges (injection, immersion and oral) using Queensland juvenile grouper ***E. lanceolatus*** as a model. R1 = replicate 1; R2 = replicate 2.

	Bacteriology		**PCR organs**		**Histology**	
Treatments	% sampled fish positive		% sampled fish positive		% sampled fish Gram-Glynn positive	
Treatment dose cfu $fish^{-1}$(no. fish/treatment)	R1	R2	R1	R2	R1	R2
Injection						
10^3 (n = 8)	100	63*	100	63*	100	63*
10^4 (n = 8)	100	100	100	100	100	100
10^5 (n = 8)	100	100	100	100	100	100
10^6 (n = 8)	100	100	100	100	100	100
10^7 (n = 8)	100	100	100	100	100	100
10^8 (n = 8)	100	100	100	100	100	100
Negative Control (n = 8)	0	0	0	0	0	0
Immersion						
low: 10^4 $cfu.mL^{-1}$(n = 8)	0	0	50	75	50	75
medium: 10^5 $cfu.mL^{-1}$(n = 8)	0	50	50	100	25	75
high: 10^6 $cfu.mL^{-1}$(n = 8)	25	100	50	100	50	100
(+) control (injection) 10^6 cfu $fish^{-1}$ (n = 8)	100	/	100	/	100	/
Negative Control (n = 8)	0	0	0	0	0	0
Sentinel fish (n = 8)	0	0	0	100	0	50
Oral						
High dose 10^6 $cfu.fish^{-1}$ (n = 10)	33	33	100	83	17	50
Low dose 10^3 $cfu.fish^{-1}$ (n = 10)	17	0	100	100	83	0
control (n = 10)	0	0	33	17	0	17

mouth, lower jaw, base of the pectoral fins, perianally, or at the point of inoculation (Fig. 2.2 E, F, G), occasional subcutaneous oedema was observed and skin lesion with ulcerative dermatitis, and haemorrhage on the flank of fish from immersion challenge (high dose) (Fig. 2.2 H). Many fish had marked hyperaemia on the lateral side of the body, and congestion around the anus or genital papilla was also observed (Fig. 2.2 F). The clinical signs of the disease were delayed when the pathogen was administered orally. Fish fed with high dose treatment began displaying signs of infection on the 25th day. Indeed, at day 26, anorexia was observed in two of ten fish fed high dose in each replicate, fish ceased eating and stayed at the bottom of their tank until the end of experiment. In contrast, those fed on the low dose treatment showed no signs of infection for the duration of trial.

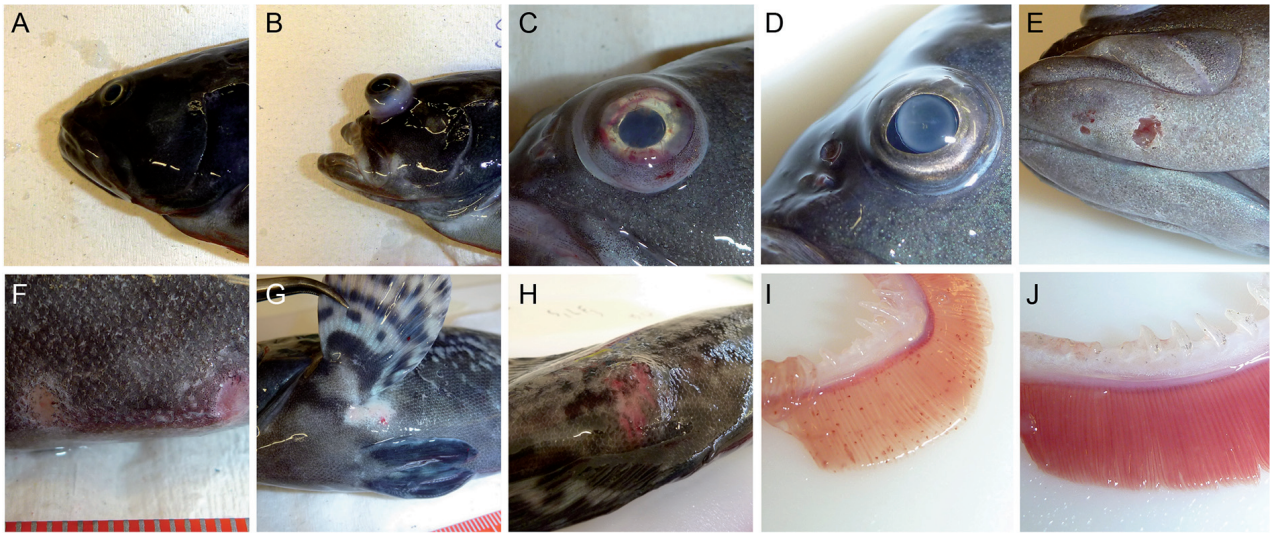

Figure 2.2. External clinical signs of streptococcosis in juvenile Queensland grouper. A. lateral view of a normal eye (control fish); B. lateral view of an eye with severe exophthalmia; C. eye with intra-ocular haemorrhage; D. opacification of the eye; E. skin ulceration and dermatitis of the lower jaw caused by *S. agalactiae*; F. intraperitoneal injection point with ulceration, hyperaemia and abnormal congested anus; G. skin ulceration and haemorrhage at the base of the pectoral fin; H. skin lesion with ulcerative dermatitis, dermal oedema, and haemorrhage on the flank of a fish from immersion challenge (high dose); I. gills filaments with petechial haemorrhages; J. normal gills filaments from a control fish (absence of petechial haemorrhages).

Internal signs of streptococcosis in Queensland grouper

The stomach and gut of the fish were empty when dissected; the gall bladder was often distended indicating the fish were not eating. The stomach and intestine of some fish were filled with a yellowish exudate. There were signs of bacterial septicaemia, including haemorrhage of the spleen and kidney and splenomegaly. Compared with the control fish, moribund fish had distended abdomens with ascites. Injected fish often had a protruding hyperaemic anus accompanying ascites. In contrast, fish from the oral challenge displayed fewer internal signs of bacterial infection. Typical gross pathology observed included petechial haemorrhages on the liver or spleen, but with no observed hepatomegaly or splenomegaly.

Fluorescence *in situ* hybridization: sensitivity & specificity

In order to check if hybridization occurred correctly and for ease of visualisation of samples at higher magnification, bacterial suspensions were incubated with DAPI, which stained all bacterial DNA (Fig. 2.3). Signal intensity obtained from hybridization with the Eub338-FITC probe on pure culture of GBS and *S. iniae* was quite low when compared with the DAPI micrographs (Fig. 2.3). No signals were observed for *S. iniae* with the GBS-Cy3 probe confirming specificity of the probe to *S. agalactiae* (Fig. 2.3). However, as for the Eub338-FITC probe, the level of hybridization with GBS-Cy3 on pure GBS culture was also very low suggesting low accessibility of the probes to their 16S rRNA targets regardless of the enzymatic treatments used. Only a few positive GBS bacteria were visible for Eub338 and GBS-Cy3 after a 12 hr hybridization period at 46°C (Fig. 2.3). Multiple attempts using various enzyme treatments and varying formamide stringency failed to give satisfactory results on 5 μm formalin-fixed and paraffin-embedded (FFPE) tissue sections from Queensland grouper organs, so this approach was abandoned. Alternatively, immunohistochemistry was optimised using a readily available commercial polyclonal antibody that reacts with type-specific carbohydrate on the surface of GBS.

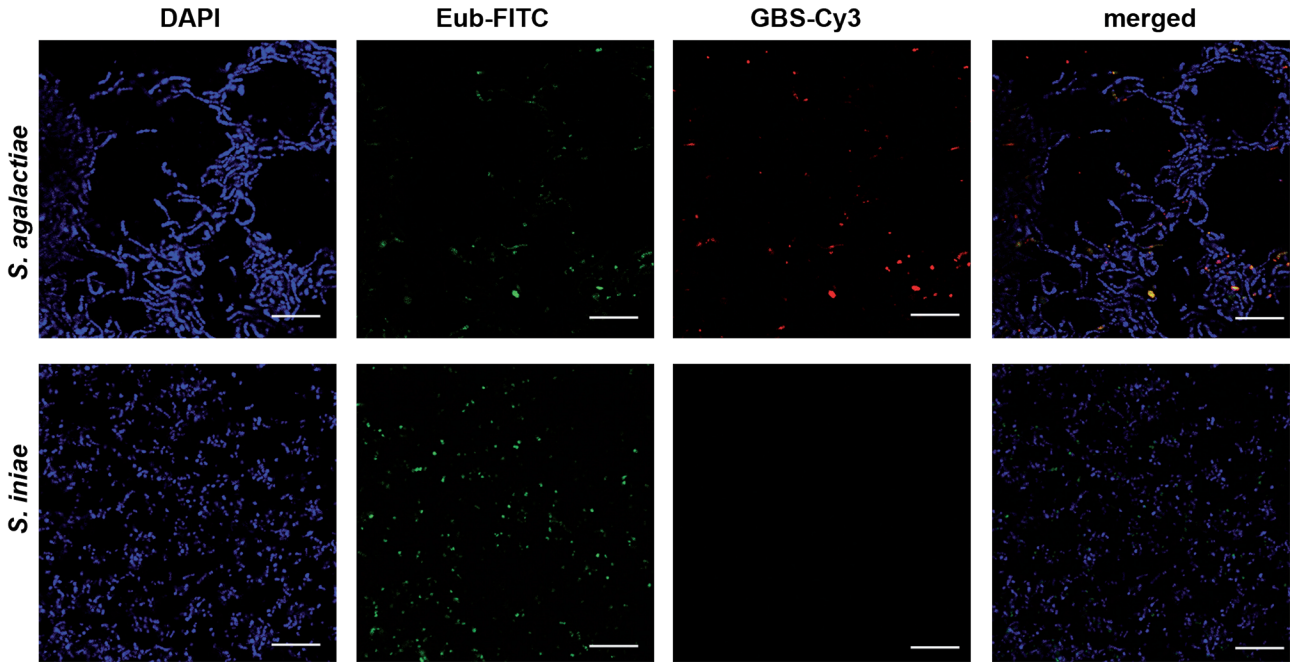

Figure 2.3. Fluorescence microscopy of streptococci after fluorescence ***in situ*** hybridization (FISH) with various oligonucleotide probes. *S. agalactiae*, QMA0285 (top row) and ***S. iniae***, QMA0246 (bottom row) were treated for 30 min with a enzymatic cocktail containing mutanolysin and proteinase K. Bacteria were stained with DAPI (blue signal; staining of DNA) following FISH with probe Eub338-FITC (green signal; specific for all ***Eubacteria***), and probes GBS-Cy3 (red signal; specific for ***S. agalactiae***). Magnification 100X, scale bar = 10 μm.

Autofluorescence of fish tissues

Most cells and tissues have endogenous proteins and compounds that fluoresce naturally. It is an important consideration to keep in mind when imaging samples so there is no ambiguity between the autofluorescence of the sample with the signal of the marker of interest. Different tissues fluoresce in

different ways, in the case of *E. lanceolatus* most organs are autofluorescent, an example is presented in Figure 2.4 with gills from Queensland grouper that fluoresce from the UV through to the red spectrum and can easily be confused with DAPI, Alexa488 and Texas-red labelling (Cy3) dyes (Fig. 2.4 A, B, C respectively). Autofluorescence decreased considerably in the far-red (Cy5) when the gills are excited at 647 nm (Fig. 2.4 D). Therefore detection of GBS in Queensland grouper tissues with minimum autofluorescence will be performed using a secondary antibody coupled with a far-red fluorescent dye whose excitation and emission spectrum is in the infrared.

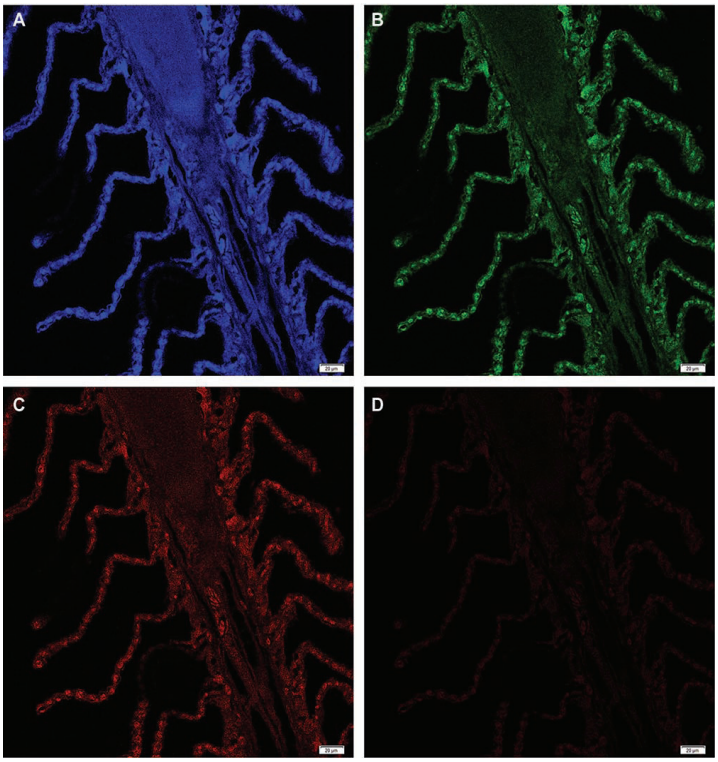

Figure 2.4. Autofluorescence of ***E. lanceolatus*** gills excited with different lasers on the BX61 Olympus confocal microscope. Probes and DAPI were omitted. Note the strong autofluorescence in the blue DAPI/UV 358 nm (A); in the green FITC/488 nm (B); in the red Cy3/550 nm (C) and the much lower autofluorescence in the far-red Cy5/IR 647 nm (D). Exposure time: 200 ms, magnification: 10X, scale bar = 10 µm.

Immunocytochemistry (ICC) validation on pure bacterial culture

The specificity of the primary antibody was validated on pure bacterial suspensions by immunocytochemistry. All negative controls *S. iniae, S. pyogenes, L. lactis, B. pumilus, S. epidermidis, V. harveyi* and *P. damselae* were not recognized by the primary antibody anti-carbohydrates (data not shown). In contrast, fish (QMA0285) and human (QMA0355) GBS isolates were positive (Fig. 2.5). Bacteria present in the bright field phase contrast micrographs of both GBS isolates tested were positive by fluorescence imaging appropriate to the secondary antibody goat anti-rabbit IgG coupled with a far-red fluorescent dye Alexa fluor 647 (Fig. 2.5). Once ICC was validated and optimized on pure culture, primary and secondary antibodies were used on 5 µm FFPE tissue sections. Both new and archival histological sections from diseased and healthy Queensland groupers were analysed by immunohistochemistry.

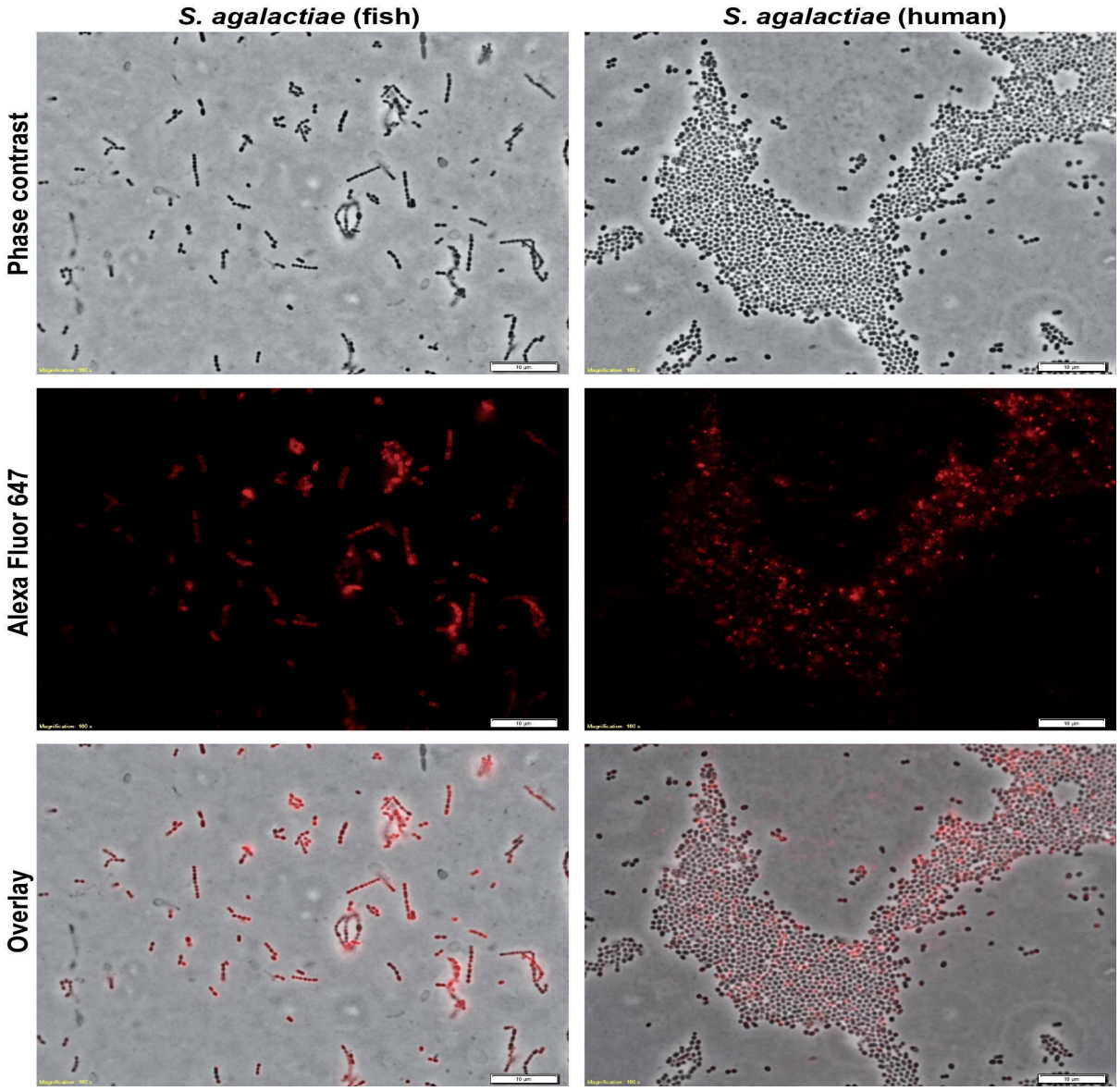

Figure 2.5. Fluorescence microscopy of microorganisms after immunocytochemistry with primary antibody (ab53584) and secondary antibody (ab150083). ***Streptococcus agalactiae*** fish isolate QMA0285 (left) & human isolate QMA0355 (right). Phase contrast, Alexa Fluor 647 and overlay images of the former two were obtained for each bacteria. Both fish and human GBS isolates are positive for Alexa Fluor 647. Note how bacteria can be arranged either in chain or in individual coccus. Magnification: 100X, scale bar = 10 μm.

Histology

Pathological analysis of juvenile Queensland groupers examined from the injection trial displayed consistent pathology. All fish from treatment groups examined had evidence of a systemic bacterial infection, with multi-organ involvement. Fish from all six-treatment doses had evidence of bacterial septicaemia. A predominantly granulomatous inflammatory response, consisting of aggregations of macrophages containing Gram-Glynn-positive coccoid bacteria, was visible in all organs and tissues examined, indicating widespread organ involvement. The brain, eye, gills, heart, head-kidney, caudal kidney, spleen, liver, pancreas, stomach, intestine, visceral and parietal peritoneum, swim bladder, skin and muscle were all affected. Fish had ophthalmitis, exophthalmos, keratitis, meningitis, branchiitis, splenitis, interstitial nephritis, hepatitis, gastritis, enteritis, pancreatitis, peritonitis, myositis and ulcerative and hyperaemic dermatitis.

The meninges of the brain (Fig. 2.6 A) were thickened with macrophages containing Gram-Glynn-positive bacteria. Colonies of Gram-Glynn-positive bacteria were detected in circulating macrophages in the single layer of specialised brain microvascular endothelial cells (BMECs) (Fig. 2.6 A). The gills (Fig. 2.6 B) showed a generalised multifocal branchiitis, with proliferation and hyperplasia of gill lamellar epithelium and fusion of adjacent gill lamellae, thickened with a granulomatous inflammatory response. There was a generalised rare necrosis of gill lamellar epithelium involving a few gill lamellae, and sometimes along the lengths of entire gill filaments. Branchial blood vessels of the gill lamellae and gill filaments were dilated and congested, containing Gram-Glynn-positive bacteria either in colonies or within circulating macrophages (Fig. 2.6 B). The eyes of many fish examined had exophthalmos, with varying degrees of severity of granulomatous inflammation, mainly in the choroid rete, choroid space and lamina choroid capillaries (Fig. 2.6 C). Gram-Glynn-positive cocci were detected in the anterior chamber of the eye in some fish, but were often free in the choroid space, and within macrophages in most parts of the eye. The cornea was often ulcerated and with thickened stromal layers (Fig. 2.6 C). The heart (Fig. 2.6 D) showed a severe haemorrhagic pericarditis, the pericardium of the ventricle and bulbus arteriosus most often affected, compared to the atrium. The pericardium was thickened with infiltrations of macrophages containing Gram-Glynn-positive cocci (Fig. 2.6 D). The intestines of all fish examined had congested blood vessels, sloughing, necrosis and haemorrhage of the mucosal epithelium. There were colonies of Gram-Glynn-positive cocci in the intestinal lumen. The serosa was markedly inflamed with a diphtheritic-like membrane made up of inflammatory cells, and macrophages containing Gram-Glynn-positive bacteria, extending into the peritoneum. The intestinal loops were often dilated with colonies of Gram-Glynn-positive bacteria (Fig. 2.6 E). The spleen (Fig. 2.6 F) was congested and haemorrhagic, with degeneration and necrosis of ellipsoids, depletion of the white pulp (haematopoietic and lymphomyeloid tissue), and with Gram-Glynn-positive cocci bacteria seen forming colonies, and within enlarged macrophages throughout the spleen.

Negative control fish (housed in a separate system away from the experimental fish) displayed some very minor pathology, which was not indicative of bacterial infection, and no Gram-positive cocci bacteria were seen by Gram-Glynn.

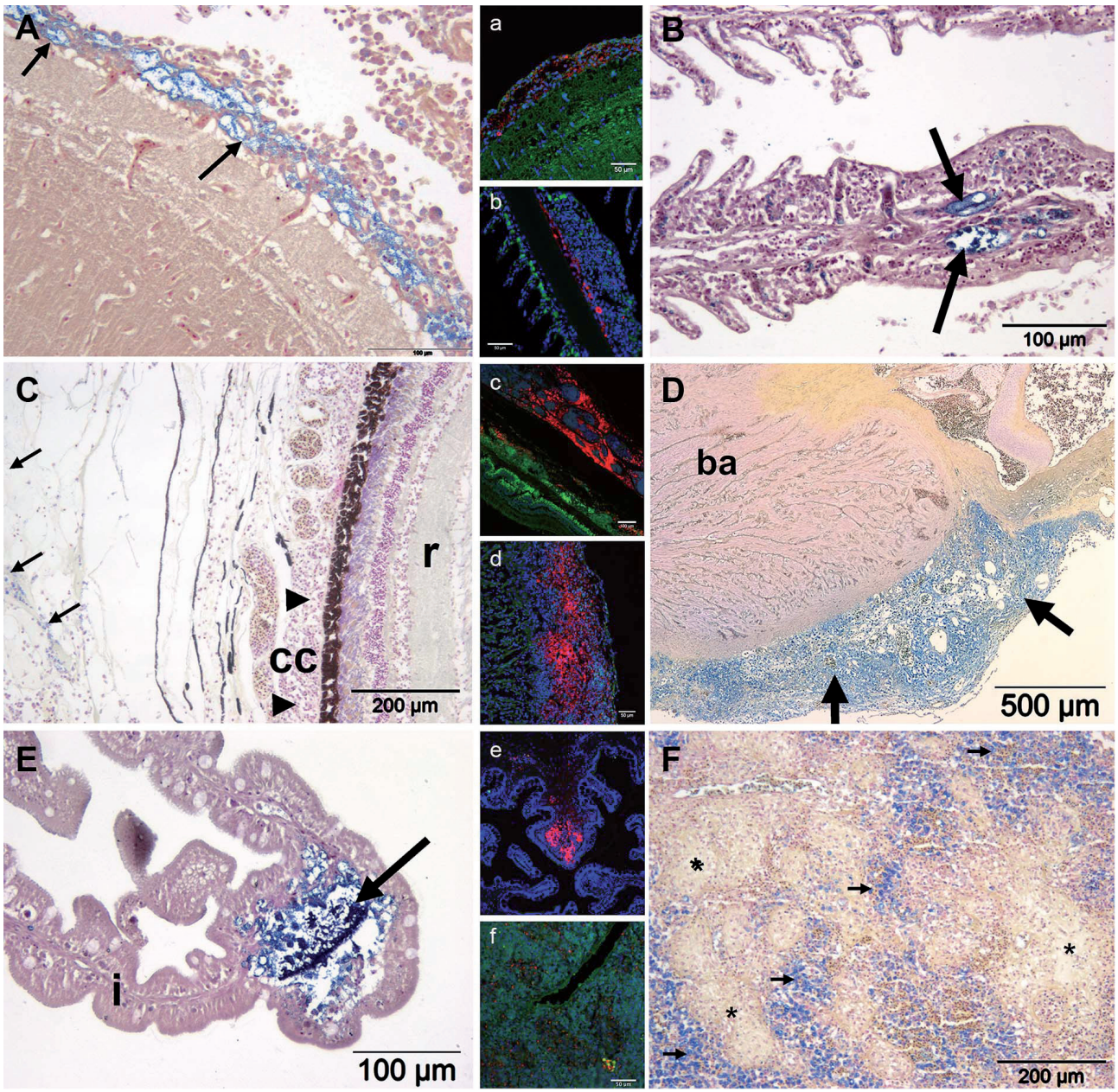

Figure 2.6. Micrographs of histological sections from different organs of ***Epinephelus lanceolatus*** injected i.p with ***Streptococcus agalactiae*** (QMA0285) stained with Gram-Glynn (GG) (A-F) and by immunohistochemistry (a-f). Gram-positive GBS bacteria appear blue with the Gram-Glynn stain (A-F). For IHC pictures (a-f), green is the autofluorescence of the tissue used as a counterstain in the FITC channel; blue is the nuclei DNA stained with DAPI; and red is the immufluorescent positive GBS bacteria (Alexa Fluor 647). Micrographs (a-f) are overlay images of the three channels DAPI, FITC and Alexa Fluor 647, and are there to confirm the identity of GBS and its co localisation with the GG-positive bacteria observed in (A-F). A. Brain, note the meninges of the brain are thickened with macrophages containing Gram-Glynn-positive bacteria (20X, scale bar = 100 µm). B. Gills with multifocal branchiitis, proliferation and hyperplasia of gill lamellar epithelium and fusion of adjacent lamellae, thickened with a granulomatous inflammatory response (20X, scale bar = 100 µm). C. Eye with severe granulomatous inflammation, note GG-positive bacteria in the choroid space (CC). The cornea (C) is ulcerated with thickened stromal layers (10X, scale bar = 200 µm). D. Heart shows severe haemorrhagic pericarditis. Note the pericardium of the bulbus arteriosus (ba) affected with GG-positive bacteria. The pericardium is thickened with infiltrations of macrophages containing GG-positive cocci bacteria (4X, scale bar = 500 µm). E. Intestine with intestinal loop dilated with colonies of GG-positive cocci bacteria (20X, scale bar = 100 µm). F. Spleen with degeneration and necrosis of ellipsoids, depletion of the white pulp *, and with GG-positive cocci bacteria seen forming colonies throughout the spleen (10X, scale bar = 200 µm).

Pathological analysis of juvenile Queensland grouper examined from the immersion trial, displayed pathology indicative and consistent with that of a bacterial infection. A mononuclear, rather than granulomatous inflammatory response was seen in association with Gram-Glynn-positive coccoid bacteria in many organs and within tissue macrophages, indicating that fish were responding to the presence of a bacterial pathogen. Control fish displayed some very minor pathology. Positive control fish (injected with 10^6 cfu *S. agalactiae* $fish^{-1}$) displayed pathology indicating bacterial septicaemia, with widespread organ involvement, similar to that observed for the injection challenge (as outlined earlier). Sentinel fish (negative controls) examined from the immersion trial displayed only minor pathology, but no bacteria were visible.

In the oral challenge, Gram-Glynn-positive bacteria were detected, variously, in the brain, spleen, heart, gills, kidney or spleen. One fish from the high dose (replicate 1), three fish from high dose (replicate 2), and five fish from low dose (R1) tested positive for *S. agalactiae*, respectively by histopathology. One fish from control group (R2) tested positive for *S. agalactiae*, with mild pericarditis, and Gram-positive cocci detected in the ventricle by Gram-Glynn stain.

Immunohistochemistry on Queensland grouper tissues

GBS was detected on 5 μm FFPE grouper tissue sections from organs originated from the different challenge trials (injection, immersion and oral) after validation of the specificity of the antibodies on WBC suspensions by immunocytochemistry. Histopathological observations made by light microscopy on Gram-Glynn stain revealed colonies of typical coccoid bacteria (Fig. 2.6 A-F), which were observed in most organs of infected animals with accompanying inflammation. IHC performed on sections of these same organs confirmed that these coccoid bacteria were indeed GBS (red immunofluorescence) (Fig. 2.6 a-f). Pathological analysis of fish infected with *S. agalactiae* displayed consistent pathology associated with the specifically identified GBS bacteria including meningitis (Fig. 2.6 a), branchiitis (Fig. 2.6 b), ophthalmitis, exophthalmos, keratitis (Fig. 2.6 c), myocarditis and pericarditis (Fig. 2.6 d), enteritis (Fig. 2.6 e), and splenitis (Fig. 2.6 f). Control fish displayed some very minor pathology, but no GBS bacterial cells were detected by IHC in these samples (data not shown).

2.4 DISCUSSION

S. agalactiae in wild Queensland grouper, marine fish and stingrays was first reported in Australia in 2007 (Bowater *et al.*, 2012). The present study explored the susceptibility and pathogenesis of Queensland grouper to infection by GBS via different routes of exposure, and in order to fulfil Koch's postulates and confirm the previous hypothesis that *S. agalactiae* was causative for the wild fish mortality.

Previous studies have demonstrated the susceptibility of Nile and red tilapia (*Oreochromis* spp.) to virulent GBS isolates in experimental injection trials (Abuseliana *et al.*, 2011; Chen *et al.*, 2007; Evans *et al.*, 2002; Evans *et al.*, 2004; Inocente Filho *et al.*, 2009; Pretto-Giordano *et al.*, 2010). Interestingly, three fish survived at low injection dose (10^3 cfu $fish^{-1}$) and had no clinical signs of bacterial infection, with no grossly visible lesions. The detection of the bacteria in these fish was not possible by bacteriology, but was by PCR from tissues and by observation of very rare positive Gram-Glynn-positive bacteria in stained tissues. This suggests that some fish are positive asymptomatic carriers capable of mounting a successful innate immune response to at least contain the bacteria. *S. agalactiae* is frequently carried asymptomatically in humans (Delannoy *et al*., 2013), and asymptomatic carriage has also been reported in Rainbow trout (*Oncorhynchus mykiss*), but only at low water temperatures (Sepahi *et al.*, 2013).

Whilst inoculation by injection even with very low doses resulted in very high mortality, it seems an unlikely route of transmission in wild fish. However, in the wild, Queensland grouper fed on stingrays, crustaceans, small turtles and juvenile crocodiles. In one clinical case in a dead wild Queensland grouper in north Queensland, a stingray barb was recovered from the stomach (Bowater *et al.*, 2012). Injury of the stomach or other internal organs following ingestion of a stingray barb may enable a site of inoculation for *S. agalactiae* that can be used as a portal of entry into the fish (Bowater *et al.*, 2012).

Transmission of GBS via water-born exposure has been previously demonstrated in Nile and red Tilapia (Abuseliana *et al.*, 2011; Mian *et al.*, 2009; Rodkhum *et al.*, 2011). Results from the immersion challenge in the present study showed that *S. agalactiae* can be transmitted by immersion since nearly all the fish from all three-treatment groups showed pathology of internal organs and tissues indicative of bacterial infection. However mortalities compared to injection were much lower, but we observed that they were higher in one of the replicate recirculation systems where a less effective biofilter lead to some accumulation of ammonia. Whilst effect of water quality was not a specific aim of the current study, it was interesting to note that none of the sentinel control fish in replicate 1 became sick or carriers (PCR and Gram-Glynn negative), while some sentinel fish from replicate system 2 with the less effective biofilter were positive by PCR and histology. So whilst we can transfer infection by immersion in a relatively high concentration of bacteria, it appears that better water quality reduces stress in a cohort naïve fish sufficiently to prevent initial colonisation, or reduce shedding in challenged fish. A combination of unfavourable water quality in fish farm can influence

the presence of *S. agalactiae* and the susceptibility of cultured red hybrid tilapia to the bacteria (Amal *et al.*, 2013a). As the environmental drivers for the recent and ongoing mortalities in wild Queensland grouper is unknown this aspect of susceptibility to infection is worthy of further investigation in a controlled study.

When administrated via the oral route, GBS did not cause mortality over the five weeks of the experiment. Only two fish appeared moribund and displayed clinical signs of infection. Many surviving, apparently healthy fish tested positive by PCR suggesting subclinical carriage of *S. agalactiae* rather than pathogen clearance. It is possible that PCR was picking up bacterial DNA that had been phagocytosed by macrophages. Experimental observations support this hypothesis, since although most fish initially showed clinical signs of infection (becoming anorexic and lethargic) early in the experiment, after 2.5 weeks the fish regained appetites, and appeared healthy. However, pathological changes in organs and tissues were detected that were indicative of disease process. It is unknown if fish would have developed the signs of the disease over a longer period of exposure. Busch and Lingg (1975) suggested that asymptomatic rainbow trout (*Salmo gairdneri*) carrier of the enteric redmouth disease were capable of shedding the pathogen with infected faeces and could serve as a reservoir of recurrent infection for long periods of time. Cohabitation with heavily infected and moribund fish has been demonstrated as a route of infection of *S. agalactiae* in Nile tilapia (Mian *et al.*, 2009). However shedding rates in asymptomatic fish are unknown and there is no evidence of transfer from asymptomatic infected fish to uninfected fish to date.

The general clinical signs observed in challenged fish were similar to previous reports of streptococcal infections (Bowater *et al.*, 2012; Chen *et al.*, 2007; Duremdez *et al.*, 2004; Evans *et al.*, 2002; Evans *et al.*, 2006c; Hernández *et al.*, 2009; Pasnik *et al.*, 2009; Plumb *et al.*, 1974; Suanyuk *et al.*, 2008), with fish exhibiting, anorexia, lethargy, erratic swimming, corneal opacity, unilateral or bilateral exophthalmia, periorbital or intraocular haemorrhage, skin ulceration, haemorrhage and dermatitis on the mouth, abdomen, fins, ascites, hepatomegaly, splenomegaly, and cloudy meninges or cerebro-spinal fluid. Queensland grouper examined from the injection challenge had histological lesions consistent with bacterial septicaemia, including multifocal granulomatous inflammatory lesions mainly in the brain, eye, heart, kidney, liver, spleen and gills, similar to previous reports (Baya *et al.*, 1990; Hernández *et al.*, 2009; Suanyuk *et al.*, 2008).

As mortality of Queensland grouper challenged with GBS by immersion and oral challenges was much lower than when challenged by i.p. injection, non-specific gut and skin-associated mucosal immunity may play an important role in the protection against the bacteria (Delamare-Debouteville *et al.*, 2006; Rombout Jan H.W.M *et al.*, 2011; Salinas *et al.*, 2011; Urquhart *et al.*, 2009). Transmission of GBS via water-born immersion or through food exposure most closely mimic natural routes of infections, as they do not compromise the tegument of the fish involved in protection against invading pathogen, which will occur when challenging by i.p. injection (Bricknell, 1995).

Presence of single or multiple aggregations of intact macrophages with phagocytosed bacteria visible

within granuloma-like lesions in organs and tissues such as the choroid of the eye, heart, and brain were reported in wild fish cases suspected of *S. agalactiae* infection (Bowater *et al.*, 2012). Fish from the immersion and oral challenges presented with a mononuclear, and rarely a granulomatous, inflammatory response in the various organs and tissues observed. In many cases Gram-Glynn stain showed bacteria within tissue macrophages, in the eye, intestinal epithelium, and epithelial surface of the skin or muscle. It is possible these fish were attempting to eradicate the bacteria by shedding infected cells (ie. tissue macrophages laden with bacteria). This is in concordance with the critical role that macrophages play in non-specific cellular immunity in fish (Uribe *et al.*, 2011). In wild fish, recorded mortalities spike in winter (Bowater *et al.*, 2012). This is supportive of the role of innate immunity in normal resistance to the disease as the innate immune response is slowed at lower water temperature. Indeed, streptococcal infections in tropical and subtropical species are more intense at lower temperatures (Qiang *et al.*, 2013).

2.5 CONCLUSIONS

While the primary environmental source of *S. agalactiae* is still to be determined, this study demonstrated high susceptibility of juvenile Queensland grouper to piscine GBS strain (QMA0285) by injection and by immersion, with a possible association with water quality. In the oral challenge trial, no fish mortalities were observed, but PCR and histopathology indicated an active disease process. Clinical signs and pathology typical of streptococcal infection with *S. agalactiae* were successfully reproduced and corresponded to what has been previously described in the wild Queensland grouper cases and in other wild and captive fish around the world. Low accessibility of the FISH probes to their target 16S rRNA is probably due to the complex polysaccharide capsule and highly impermeable peptidoglycan-dominated Gram-positive cell wall, which made the FISH approach unsuitable for the detection of GBS in FFPE tissues. The alternative IHC approach target specific epitopes (carbohydrates) that are readily present on the surface of the bacteria using specific antibodies, negating the need for the detection reagent to penetrate capsule and cell wall. Gram-Glynn positive bacteria observed in the tissue sections were confirmed to be *S. agalactiae* by IHC. IHC is a more informative method over routine hematoxylin & eosin stain (H&E) in that it can detect a pathogen in tissues in a very specific manner (e.g. specific organisms within the complex microbiota of the digestive system). PCR performed on fish tissues was consistently more sensitive than bacterial isolation for detecting *S. agalactiae* from the brain, heart, spleen or kidney of fish sampled from all challenge trials including many fish that were alive and apparently healthy. This, when coupled with positive observation of intact cells by IHC, is indicative of subtantial levels of asymptomatic carriage of GBS by challenged Queensland grouper.

Chapter 3

Multilocus sequence typing and capsular operon analysis of Group B *Streptococcus* infecting wild marine fish in Australia indicate that they have not arisen as a result of recent transfer from a terrestrial source

ABSTRACT

Streptococcus agalactiae isolated from wild fish mortalities occurring in north east Australia since 2007 were typed by multilocus sequence typing (MLST) and serotyped in order to infer potential sources of infection of wild fish in this study. All fish isolates from Australia belong to ST-261, a type previously found in Nile tilapia in several countries and thought to have originated from Israel. The marine strains in this study are not closely related to local terrestrial animal or human isolates and are therefore unlikely to have been recently transferred from terrestrial sources. They are unlikely to pose a food safety issue as these sequence types occur in food fish overseas and have not been associated with human illness. Our preliminary data suggest a possible introduction of the strain with Tilapia to north Queensland sometime during the last few decades, concordant with sporadic introductions of Tilapia into the region during the 1970s and 1980s.

3.1 INTRODUCTION

Mortalities in wild Queensland grouper (*Epinephelus lanceolatus*) resulting from infection by *S. agalactiae* (Group B *Streptococcus*, GBS) were first recorded in 2008. Ninety-six adult groupers were reported dead since the index case in 2007, although isolation of *S. agalactiae* and associated pathology were only confirmed in 12 cases (Bowater *et al.*, 2012). Subsequently, several other species were also found to be infected, including wild Javelin grunter (*Pomadasys kaakan*), Squaretail mullet (*Ellochelon vaigiensis*), and Giant catfish (*Netuma thalassina*), along with several species of stingrays from two separate disease outbreaks that occurred at a large commercial display aquarium (Bowater *et al.*, 2012).

Establishing a possible origin and route of spread of *S. agalactiae* is important for several reasons. Firstly, this is the first recorded occurrence of *S. agalactiae* in wild fish in Australia, yet *S. agalactiae* has caused widespread mortalities in several cultured fish species and caused wild fish kills in many countries (Amal *et al.*, 2012; Baya *et al.*, 1990; Evans *et al.*, 2002; Liu *et al.*, 2013c). Determining the source of the Australian outbreak is critical to develop biosecurity measures, to protect Australian aquaculture industries and wild fish stocks. Secondly, *S. agalactiae* is one of the major causes of meningitis in human neonates, and human pathogenic variants have been reported to infect fish (Delannoy *et al.*, 2013; Evans *et al.*, 2008; Evans *et al.*, 2009; Liu *et al.*, 2013a), thus establishing the genetic background of the Queensland grouper isolates is a matter of public safety and reassurance. Finally, an understanding of where the Australian strains of *S. agalactiae* are likely to have arisen can contribute to the development of state and national biosecurity policies, strategies and control measures to assist in protecting native fauna, preventing further infections from entering other wild fish populations in Australia, and identifying where interventions can be made to minimise biosecurity risks to Australian fisheries and aquaculture industries.

Two methods were used for epidemiological tracing of *S. agalactiae*, multilocus sequence typing (MLST) and molecular serotyping. MLST was chosen since it does not require physical collection of bacterial reference strains from around the world, but permits accurate comparison with global databases of bacterial isolates online. A public database was established more than ten years ago (Urwin and Maiden, 2003) (pubMLST.org) that is very rich in sequence-typed *S. agalactiae* strains (Jones *et al.*, 2003) including many fish isolates (Delannoy *et al.*, 2013; Evans *et al.*, 2008; Godoy *et al.*, 2013). The second method used was molecular serotyping, which explores the genes encoding the surface epitope determinants of the capsular polysaccharide (CPS) (Bentley *et al.*, 2006; Honsa *et al.*, 2008; Kong *et al.*, 2008). *Cps* operon sequence typing is also physiologically relevant and mutation can be driven quite rapidly by host response, providing local scale resolution amongst isolates (Millard *et al.*, 2012).

3.2 MATERIALS AND METHODS

Bacterial strains

23 *S. agalactiae* bacterial isolates (including one previously sequenced internal reference, COH1) were selected for genotyping and are presented in Table 3.1.

Genome sequencing

Genomic fragment libraries for whole genome sequencing were prepared at the Australian Genome Research Facility (AGRF) using Illumina TruSeq DNA library preparation protocol. Briefly, 1 µg of DNA was fragmented using Covaris shearing followed by an end-repair (3' > 5' exonuclease and polymerase fill in) to generate blunt ends. Fragments were A-tailed then TA-ligated onto Illumina adaptors. Ligated DNA fragments were size selected by gel electrophoresis and gel excision to create 23 libraries (one per isolate) for subsequent sequencing. Libraries were pooled for sequencing on the Illumina HiSeq 2000 instrument at the AGRF according to manufacturer's protocols. Paired-end 100 bp sequence reads with an estimated insert size average of 370 bp were generated for the 23 isolates, including the previously sequenced strain COH1 (QMA0370) used as an internal sequencing reference. For each isolate, between 5 288 952 and 12 577 340 read pairs were obtained, corresponding to an average coverage of ~670X to ~1,400X. Base calling was processed with Illumina RTA software v1.10.36. De-multiplexing and conversion to FastQ format was performed with CASAVA v1.8.2. FastQC was used to assess the quality of the sequence reads and to look for the presence of contaminating adapters. When required, additional inspection using in-house tools was performed. Due to the excess read coverage obtained for all samples, we sampled down each set of reads to an average coverage of 100X, in order to optimise performance of the subsequent assembly step.

Assembly, annotation and comparative genome analysis

De novo assembly of reads obtained from each isolate was performed using Velvet 1.2.02 (Zerbino and Birney, 2008), following preliminary filtering of sequence reads with a minimum quality of Q30 and selective trimming of low quality 3' ends down to 70 bp maximum. Low contigs number were achieved for most strains, ranging from 21 to 74 contigs, with the exception of the highly repetitive strain QMA0306, totalling 112 contigs. Assembled genomes, which sizes ranged from 1.7 Mb for marine strains to 2.2 Mb for terrestrial strains, were reordered using Mauve (Darling *et al.*, 2004) and were automatically annotated using Prokka (Prokaryotic Genome Annotation System). In addition, assembled reads were also manually inspected with particular attention to the *cps* locus region for each strain to confirm their genotype.

Table 3.1. List of the GBS isolates used in this study. Strains underlined have been isolated from different organs of the same animal.

Isolate	Genus	Species	Host		Year	Geographic origin	Tissue/organ
			Common name	Scientific name			
QMA0284	*Streptococcus*	*agalactiae*	Grouper 1	*Epinephelus lanceolatus*	2008	Cairns	Eye
QMA0285	*Streptococcus*	*agalactiae*	Grouper 2	*Epinephelus lanceolatus*	2008	Cairns	Head kidney
QMA0267	*Streptococcus*	*agalactiae*	Grouper 3	*Epinephelus lanceolatus*	2008	Cairns	Eye
QMA0280	*Streptococcus*	*agalactiae*	Grouper 4	*Epinephelus lanceolatus*	2010	Townsville	Kidney
QMA0281	*Streptococcus*	*agalactiae*	Grouper 5	*Epinephelus lanceolatus*	2010	Townsville	Heart
QMA0368	*Streptococcus*	*agalactiae*	Grouper 6	*Epinephelus lanceolatus*	2010	Topsy creek, Gulf of Carpentaria	Eye
QMA0369	*Streptococcus*	*agalactiae*	Grouper 7	*Epinephelus lanceolatus*	2011	Cape Cleveland, Townsville	Eye
QMA0268	*Streptococcus*	*agalactiae*	Javelin grunter	*Pomadasys kaakan*	2009	Cairns	Caudal Kidney
QMA0287	*Streptococcus*	*agalactiae*	Javelin grunter	*Pomadasys kaakan*	2009	Cairns	Heart
QMA0271	*Streptococcus*	*agalactiae*	Giant catfish	*Netuma thalassina*	2009	Cairns	Heart
QMA0290	*Streptococcus*	*agalactiae*	Giant catfish	*Netuma thalassina*	2009	Cairns	Caudal kidney
QMA0274	*Streptococcus*	*agalactiae*	Squaretail mullet	*Ellochelon vaigiensis*	2009	Cairns	Heart
QMA0275	*Streptococcus*	*agalactiae*	Eastern shovelnose ray	*Aptychotrema rostrata*	2009	Gold Coast	Internal organs
QMA0276	*Streptococcus*	*agalactiae*	Mangrove whipray	*Himantura granulata*	2009	Gold Coast	Internal organs
QMA0277	*Streptococcus*	*agalactiae*	Estuary stingray 3	*Dasyatis fluviorum*	2009	Gold Coast	Internal organs
QMA0320	*Streptococcus*	*agalactiae*	Estuary stingray 5	*Dasyatis fluviorum*	2010	Gold Coast	Brain
QMA0336	*Streptococcus*	*agalactiae*	Crocodile	*Crocodylus porosus*	2010	Darwin	Joint
QMA0370	*Streptococcus*	*agalactiae*	Human	*Homo sapiens*	2006	United States	Infant septicaemia
QMA0355	*Streptococcus*	*agalactiae*	Human	*Homo sapiens*	2011	Townsville	Foot
QMA0357	*Streptococcus*	*agalactiae*	Human	*Homo sapiens*	2011	Townsville	Female genital
QMA0306	*Streptococcus*	*agalactiae*	cow	*Bos taurus*	2005	unknown	Bovine milk
QMA0300	*Streptococcus*	*agalactiae*	dog	*Canis canis*	2008	unknown	Canine urine
QMA0303	*Streptococcus*	*agalactiae*	cat	*Felis felus*	2009	unknown	Feline left forepaw

Multilocus sequence typing

The whole draft genome sequence of each isolate was submitted directly to the MLST database website where a BLAST search was performed to determine each individual allelic profile and sequence type. A novel allelic variation of the *glnA* locus was identified in QMA0368 and confirmed by PCR followed by traditional Sanger sequencing. Two separate PCR reactions from independent DNA templates were performed to amplify the novel variant with the amplification primers glnAAF 5'-CCGGCTACAGATGAACAATT-3' and glnAAR 5'-CTGATAATTGCCATTCCACG-3'. Resulting amplicons (589 bp) were cleaned with shrimp alkaline phosphatase/exonuclease I and sequenced in both directions using sequencing primers glnASF 5'- AATAAAGCAATGTTTGATGG-3' and glnASR 5'-GCATTGTTCCCTTCATTATC-3'. Clonal complexes analysis of all 668 STs available from the MLST database (pubMLST.org; as of December 2013) was inferred using eBURST V3 program (http://eburst.mlst.net; Feil *et al.*, 2004).

Molecular serotyping

Reference sequences for the ten serotypes described in the literature (Ia, Ib, Ic, II to VIII) were retrieved from the GenBank database on the NCBI website (Appendix 1). BLASTn comparisons were then performed to determine the serotype of each isolate. Comparison visualization was undertaken using the Easyfig software on a set of isolates representative of the different serotypes characterised, as described previously (Sullivan *et al.*, 2011).

3.3 RESULTS AND DISCUSSION

All Australian piscine isolates belong to ST-261

The *S. agalactiae* MLST database (pubMLST.org) contains typing sequence information of the following seven housekeeping gene fragments: alcohol dehydrogenase, *adhP*; phenylalanyl tRNA synthetase, *pheS*; glutamine transporter protein, *atr*; glutamine synthetase, *glnA*; serine dehydratase, *sdhA*; glucose kinase, *glcK*; and transketolase, *tkt*. Each different sequence at a locus gives a different allele number. The combination of these alleles gives the allelic profile of the isolate. Corresponding allelic profile of the seven housekeeping genes for each of the ST found in this study are summarized in Table 3.2 and corresponding sequence types (ST) in Table 3.3. All the Australian piscine isolates belong to the ST-261 lineage, a sequence type that was first isolated in 1988 from farmed Nile tilapia (*Oreochromis niloticus*) outbreaks in Israel (ATCC 51487) (Eldar *et al.*, 1994; Eldar *et al.*, 1995). Since then, *S. agalactiae* of this sequence type has been isolated on farms from Nile tilapia (*O. niloticus*) in Indonesia (Lusiastuti *et al.*, 2012); in the United Kingdom, ST-261 was identified during an outbreak in imported freshwater cyprinid doctor fish (*Garu rufa*) from Indonesia (Verner-Jeffreys *et al.*, 2012) used for ichthyotherapy in which fish are employed to remove dead skin as a therapy. ST-261 is not constrained to teleost or chondrychthyan host, and has also been isolated from frogs in the United States (Elliott *et al.*, 1990). However, ST-261 isolates have never been reported in humans or other homeothermic animals, which is probably due to their poor ability to grow at 37°C

and consequently to cause diseases (Delannoy *et al.*, 2013). In Brazil a number of sequence types associated with disease in Nile tilapia (ST-257, ST-103, ST-260, ST-552) and Amazon catfish (ST-552) cluster in the same clonal complex CC 552 (Delannoy *et al.*, 2013; Godoy *et al.*, 2013), with ST-259 and ST-260 isolated from Nile tilapia in the USA and Honduras respectively (Evans *et al.*, 2008). CC 552 is a phylogenetic group distinct from human and bovine isolates (Fig. 3.1).

Table 3.2. Allelic profile for each of the multilocus sequence type (ST) encountered in the present study

Serotype (n)*	**ST**	**Allelic profile**						
		adhp	***pheS***	***atr***	***glnA***	***sdhA***	***glck***	***tkt***
Ib (16)	261	54	17	31	4	26	25	19
III (1)	67	13	1	1	13	1	1	5
Ia (3)	23	5	4	6	3	2	1	3
III (1)	17	2	1	1	2	1	1	1
V (2)	1	1	1	2	1	1	2	2

Abbreviations: alcohol dehydrogenase, adhP; phenylalanyl tRNA synthetase, pheS; glutamine transporter protein, atr; glutamine synthetase, glnA; serine dehydratase, sdhA; glucose kinase, glcK; and transketolase, tkt.

The Israeli piscine isolate (ATCC51487) was initially designated as *S. difficilis* (Eldar *et al.*, 1994), and sequence typed by MLST as ST-246 (Tettelin *et al.*, 2005). Thereafter, the ST of ATCC51487 was determined to be ST-261 identical to ST-246 with the exception of a deletion in the *adhP* fragment (Evans *et al.*, 2008). Both ST-261 and ST-246 form a clonal complex with no predicted founder (Brochet *et al.*, 2006; Evans *et al.*, 2008) (Fig. 3.1). The eBURST analysis confirms that fish strains belonging to ST-261 and ST-246 are closely related to CC 552, indeed ST-261 share two or more MLST alleles with multiple CC 552 members described previously. As ST-261 these STs have until now never been isolated from humans (Delannoy *et al.*, 2013). Although each ST of CC 552 was confined to different geographical locations, it is suggested that they belong to a population of GBS that has a broad worldwide distribution and predominantly causes disease in fish (Evans *et al.*, 2008).

Much less variability was observed in this study with all but one of the piscine isolates belonging to the same clone ST-261, despite being selected from various locations, fish species, infection sites and date range (2008–2011). In fact, only one single locus variant (SLV) was found amongst the Australian fish isolates, in the *glnA* locus sequence of QMA0368 isolated from grouper 6 (*E. lanceolatus*). However, that variability was all relative as traditional Sanger sequencing confirmed it was only differing by one base with the *glnA* allele number 4 defining ST-261. It was also intriguing that none of our strains belonged to ST-6 or ST-7, which are broad-host range strains associated with outbreaks

in farmed fish, dolphin, human and bullfrog (Delannoy *et al.*, 2013; Evans *et al.*, 2008; Rosinski-Chupin *et al.*, 2013). Another group of isolates that belong to clonal complex 283 (Fig. 3.1) have been recently described in tilapia from Vietnam and Thailand and are known to also cause invasive disease in humans (Delannoy *et al.*, 2013).

Table 3.3. Capsular polysaccharide sequence (cps) size and serotype of the different ***S. agalactiae*** isolates used in this study. (§ SLV : Single Locus Variant).

Isolate	**Host**	***cps* sequence size (kb)**	**ST**	**Serotype**
QMA0284	Grouper 1	15616	261	Ib
QMA0285	Grouper 2	15616	261	Ib
QMA0267	Grouper 3	15616	261	Ib
QMA0280	Grouper 4	15616	261	Ib
QMA0369	Grouper 7	15616	261	Ib
QMA0268	Javelin grunter	15616	261	Ib
QMA0287	Javelin grunter	15616	261	Ib
QMA0271	Giant catfish	15616	261	Ib
QMA0290	Giant catfish	15616	261	Ib
QMA0274	Squaretail mullet	15620	261	Ib
QMA0368	Grouper 6	15616	261 §	Ib
QMA0275	Eastern shovelnose ray	15616	261	Ib
QMA0276	Mangrove Whipray	15616	261	Ib
QMA0277	Estuary stingray 1	15616	261	Ib
QMA0320	Estuary stingray 5	15616	261	Ib
QMA0281	Grouper 5	13070	261	Ib
QMA0336	Crocodile	15722	23	Ia
QMA0355	Human foot	15722	23	Ia
QMA0357	Female genital	15722	23	Ia
QMA0370	COH1	15602	17	III
QMA0306	Cow	15487	67	III
QMA0300	Dog	17157	1	V
QMA0303	Cat	17157	1	V

Compared to the first reported case of ST-261 in farmed fish, which occurred 25 years ago in Israel, this new outbreak in Australia affecting new species of teleost and elasmobranch that have never been reported before could suggest that ST-261 is a variant particularly successful in spreading and causing disease in a wide range of fish.

The population structure of the piscine GBS from Australia appears as a homogenous group of strains belonging exclusively to ST-261. To date in Australia, it is unclear whether the true diversity of GBS strains in fish is underestimated. The MLST data confirmed the divergence of all piscine isolates from human, cow, crocodile, cat and dog isolates, as they do not share any allele with their respective STs (Table 3.2).

Unlike the piscine isolates, the population structure of the other *S. agalactiae* strains used in this study displays a greater diversity as represented by the multiple distinct clonal complexes identified (CC 1, CC 17, CC 23 and CC 67) (Skov Sorensen *et al.*, 2010; Tettelin *et al.*, 2005). Contrary to the piscine isolates, the crocodile strain, QMA0336, isolated on a farm in the Northern Territory (Darwin) from a case of necrotizing fasciitis in a captive juvenile *Crocodylus porosus* (Bishop *et al.*, 2007) is unrelated to the ST-261 group. It was identified as ST-23, serotype Ia, as were the two human isolates included in our study, collected from two cases of infection in Townsville, QMA0355 and QMA0357, which all belong to CC 23 (Fig. 3.1). ST-23 is one of the major successful clones identified worldwide, the serotype Ia is associated in humans (Brochet *et al.*, 2006), serotype III in dairy cattle (Brochet *et al.*, 2006; Skov Sorensen *et al.*, 2010), and it has been isolated from Scottish pinniped also serotype Ia, suggesting a possible transmission from human to grey seals via contaminated surface water (Delannoy *et al.*, 2013). Establishing an unambiguous human origin of the Australian ST-23 serotype Ia in juvenile crocodiles remains to be confirmed (Bishop *et al.*, 2007).

ST-67 GBS isolate from the cow (QMA0306) is a ST founder of the CC 67 (Fig. 3.1), and is closely related to the hypervirulent ST-17 COH1 reference strain, as both share four alleles in their allelic profile (Table 3.2). The human ST-17 complex of GBS isolates has arisen from a bovine ancestor (Bisharat *et al.*, 2004); authors postulated that the reoccurring infections in human neonates due to ST-17 could in part be due to a recent transfer from cattle to human.

Both dog (QMA0300) and cat (QMA0303) are serotype V and belong to ST-1 (CC 1), which is known as a successful clone with broad host range spreading worldwide. In Taiwan ST-1 serotype V is the most invasive strain in adult humans (Tien *et al.*, 2011), in France ST-1 serotype II, III, and V have been isolated from human urine, bloodstream, and vaginal samples and ST-1 serotype V from a dog (Brochet *et al.*, 2006). An epidemiological study investigated the high level of livestock exposure with humans, and the possible transmission of GBS interspecies (Manning *et al.*, 2010); stool samples from one couple were both positive and shared the same GBS strain (ST-1) as the bovine (Manning *et al.*, 2010). Acquisition of new virulence factors by lateral gene transfer between human and bovine GBS contributes to the evolution and adaptation of GBS to a wide and diverse niche (Richards *et al.*, 2011; Richards *et al.*, 2012).

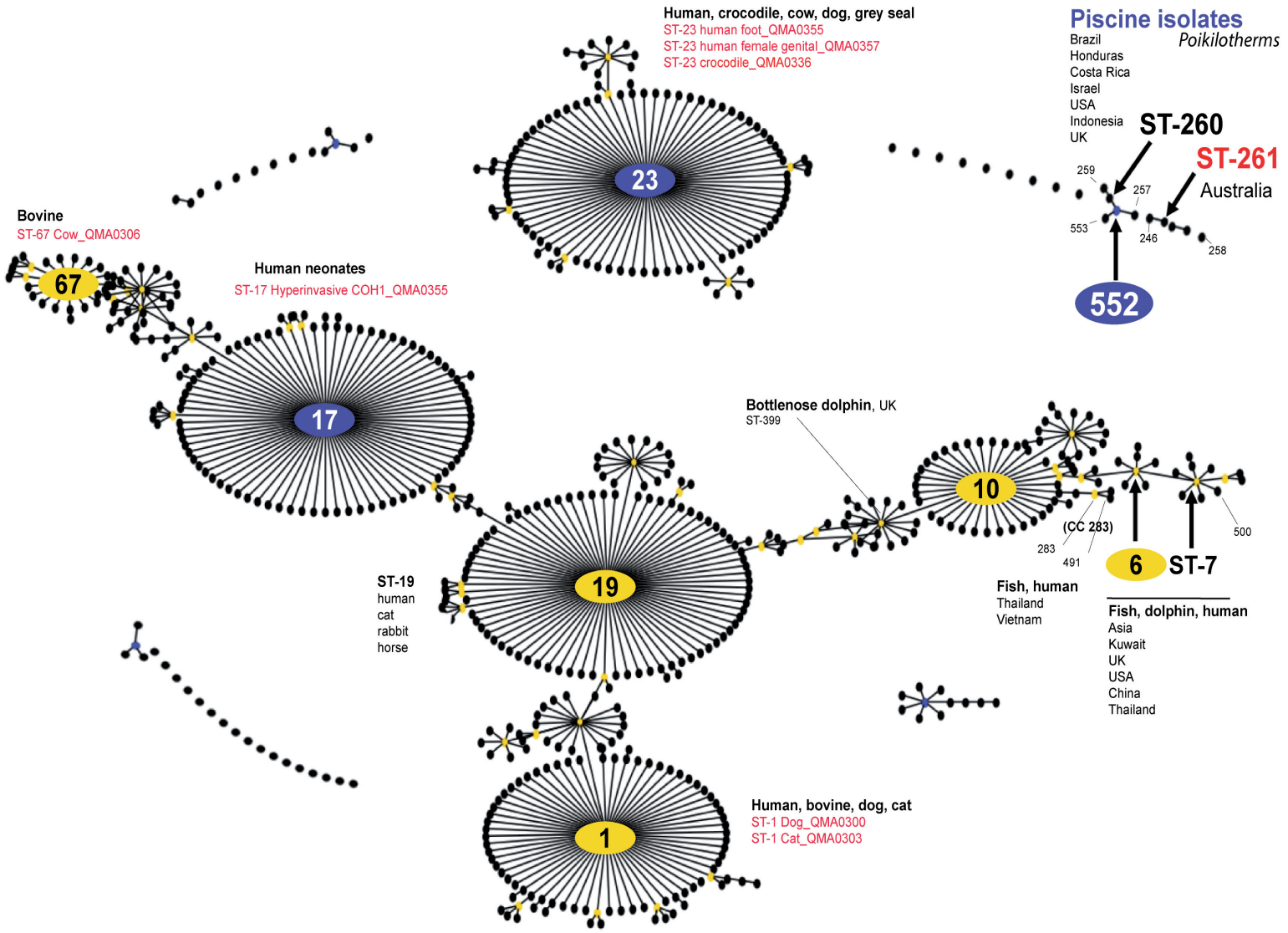

Figure 3.1. eBURST representation of the clonal complexes structure of the STs of ***S. agalactiae*** derived in this study (labelled in red) in the context of all 668 STs available in the MLST database. Each black node represents a unique ST, while each centred node indicates a primary (in blue) or subgroup (in yellow) ST founder. The major CCs are indicated by their number, which corresponds to both ST and CC names. Additional STs relevant to this study were also indicated in the figure.

ST-1 (CC 1) identified from precooked dishes based on seafood products, possibly contaminated via exposure with aquatic GBS, was frequently associated with throat and anal carrier in humans, suggesting a possible transmission via food contaminated product or by direct contact with food handlers during food processing and production (Van Der Mee-Marquet *et al.*, 2009); the authors hypothesised a potential portal of entry for humans blood stream infections.

While in this study, no ST-1 was identified amongst any of the human and bovine samples, it would be interesting to investigate further a possible transmission between human or cattle to dog and cat.

Highly conserved cps in marine strains show signs of undergoing evolution.

Comparative sequence analysis of the *cps* locus of Australian *S. agalactiae* isolates revealed that their serotype was also consistent, as all have been identified as serotype Ib (Table 3.3). There was a high level of conservation amongst the different isolates, with the regions *cpsA* to *cpsD*, *cpsF*-*cpsG*, *cpsL*, and *neuB to neuA* conserved amongst all isolates (Fig. 3.2). The *cps* locus organisation is identical in all piscine isolates with the exception of QMA0281 (grouper 5), which is missing a region of approximately 2 550 bp encompassing part of *cpsB*, *cpsC*, *cpsD* and part of *cpsE* (Fig. 3.2). QMA0368 (grouper 6) harbours minor differences in *cpsE*. The crocodile isolate is the only "marine" (non-terrestrial) related isolate to carry a different serotype (Ia). Interestingly, the *cpsJK* region of the QMA0336 (crocodile) isolate (serotype Ia) is the only significant variable region differing in the *cps* locus of the piscine isolates (serotype Ib), which confirms the close relationship of serotype Ia and Ib, as previously established in the literature. It is likely that QMA0336 is closely related to the human strains included in this study (QMA0355 and QMA0357), as they fall into ST-23 and serotype Ia, which could be the result of a recent transmission from human to crocodile (Bishop *et al.*, 2007). However, these hypotheses remain to be confirmed by whole genome comparative analysis.

ST-67 serotype III from QMA0306 (cow), and ST-1 serotypes V from QMA0300 (dog) and QMA0303 (cat), are nearly identical from a structural point of view (Fig. 3.2) to the previously characterised serotypes III (Glaser *et al.*, 2002) and V (Tettelin *et al.*, 2002). The major differences between these two serotypes are different alleles for the *cpsH* and *cpsJ* genes, and the replacement of *cpsI* by *cpsMNO* in the serotype V (Fig. 3.2). In our study almost all isolates belonging to the same ST had the same serotype, although different serotypes can occur within the same ST (Evans *et al.*, 2008).

The structural analysis of the *cps* locus of QMA0281 (grouper 5) revealed a significant deletion of approximately 2 550 bp of its capsular operon in comparison to the other piscine isolates (Fig. 3.2). We ruled out a possible misassembly of this region by confirming that no sequencing reads were obtained for this region. Besides this deletion, the rest of the *cps* sequence was identical when compared to the representative sequence QMA0284, and likely represents a single mutation event. Reductive evolution of important virulence factors in *S. agalactiae* has been reported in ST260–261 lineages with among others the loss of the *cyl* locus in these non-haemolytic isolates (Rosinski-Chupin *et al.*, 2013).

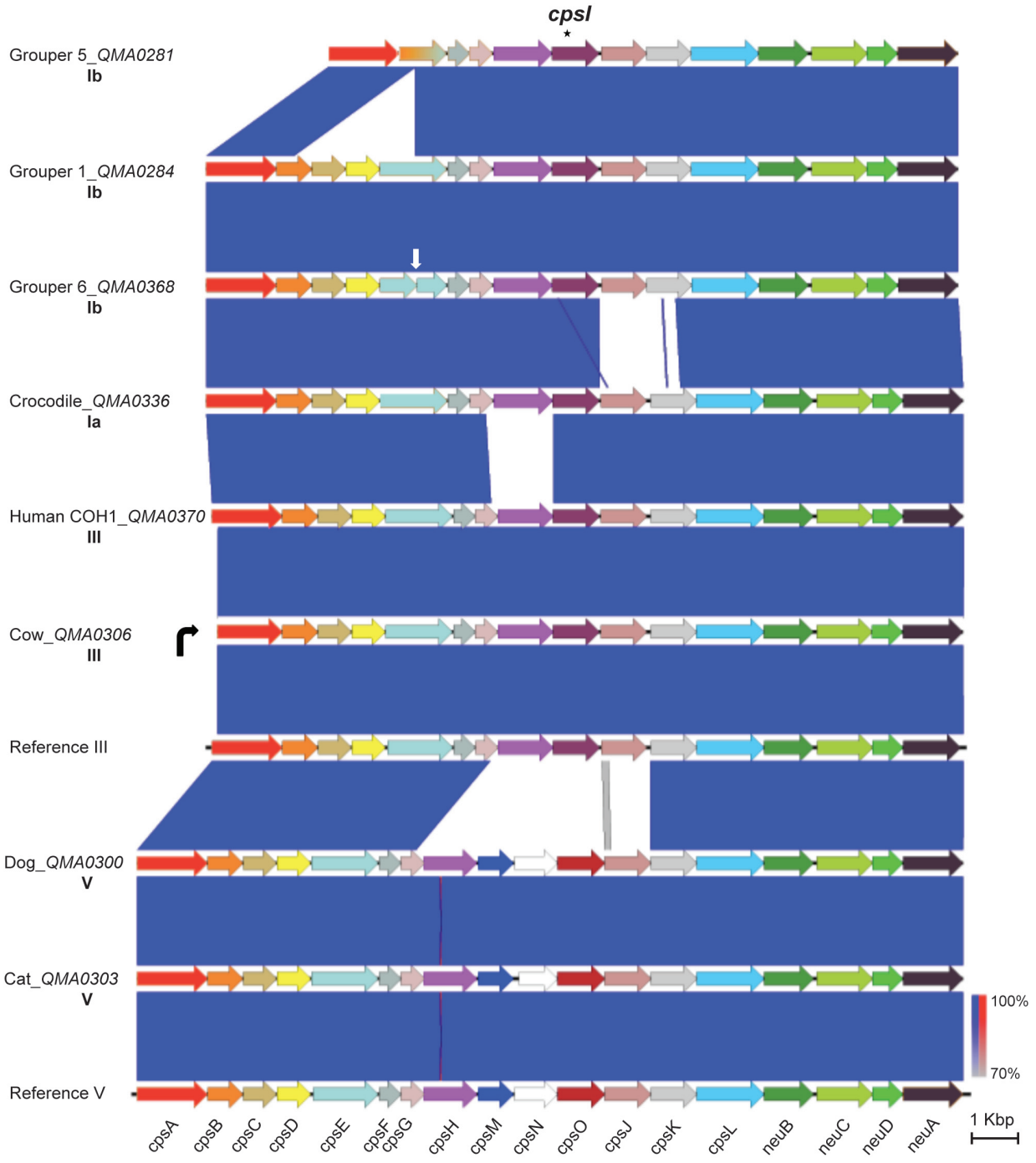

Figure 3.2. Comparison of the genetic organisation of the different polysaccharide synthesis operons identified among the ***Streptococcus agalactiae*** isolates studied. Each sequence is compared to the sequence located below. The nucleotide sequence identity between regions is indicated by a spectrum of blue/red to gray colours depicting a range of 100–70% identity. Each gene of the polysaccharide synthesis operon is shown in a different colour. Of note, a region encompassing part of *cpsB*, *cpsC*, *cpsD* and part of *cpsE* is missing in grouper 5 (QMA0281), resulting in a chimeric fusion gene (colour gradient). White arrow indicates site of insertion (frameshift) and black arrow the missing starting region of *cpsA* (QMA0306). *cpsM, N* and *O* are only present in serotype V (QMA0300 & QMA0303); *cpsI* (not shown on the legend) is located just after *cpsH* for all the other isolates (see black star at the top of the figure). Figure produced with EasyFig (Sullivan *et al.*, 2011).

This significant mutation in the *cps* operon is likely to affect the biosynthesis of the capsule, making this new clone vulnerable to the immune system of the host; and it is unlikely that this clone persists among the population of GBS, but this hypothesis remains to be proved.

Sequence polymorphism analysis of the capsular polysacharride synthesis sequences of all piscine isolates using QMA0284 (grouper 1) as a reference revealed some single nucleotide polymorphisms (SNPs) and insertions which enabled clustering of the isolates. *Cps* loci of isolates from groupers 1 through 4 (QMA0284, 285, 267, 280), grouper 7 (QMA0369), the two Javelin grunter (QMA0268, 287), the two Giant catfish (QMA0271, 290), and the Squaretail mullet (QMA0274) were identical and formed one cluster. Two SNPs occured in the isolates collected from rays: QMA0275, Eastern shovelnose ray (*Aptychotrema rostrata*)*;* QMA0276, Mangrove whipray (*Himantura granulata*); QMA0277 and QMA0320, both from the Estuary stingrays 1 & 5 (*Dasyatis fluviorum*). These two SNPs are non-synonymous and result in an amino acid change, with an isoleucine being replaced by a valine in *cpsC,* and with a threonine being replaced by a proline in *cpsE*. This places the elasmobranch isolates in a single cluster distinct from the grouper isolates. It is possible that rays translocated from Cairns were carrying the bacteria, and would have transmitted it to the other species either by contact or through the recirculation system of the public aquarium. The strain QMA0368 (grouper 6) harbours a small insertion at position 4 531 bp, with two extra thymines, causing a frameshift in the *cpsE* gene.

In terms of the evolution, these differences could suggest an ongoing evolving process, potentially related to host adaptation or pathogenesis. It is intriguing that strains isolated from different species, at different locations and time points, share identical cps sequences, suggesting these isolates originated from a common ancestor. It would be interesting in future to determine whether this pool of strains are undergoing positive selection as a result of specific selective pressures such as adaptive immunity, driving them to diverge from the rest of the isolates. It may also be that there has been translocation of the infection from different regions, possibly through wild fish movement, or through transport of fish as bait.

3.4 CONCLUSIONS

Multilocus sequence typing revealed that all of the piscine GBS isolates from Australia fell into the ST-261 group. This is one of a number of related STs that are commonly found in fish (Delannoy *et al.*, 2013; Evans *et al.*, 2008; Evans *et al.*, 2009; Godoy *et al.*, 2013). Amongst the fish sequence types, ST-261 has first been identified previously in Tilapia, and is identical to the type strain that was recovered in 1988 from Tilapia in Israel (Eldar *et al.*, 1994) and subsequently translocated to the US, Brazil, Honduras, and Indonesia. This is intriguing: of all of the fish sequence types occurring globally, only ST-261 is present in Australia to date, a strain associated with Tilapia, cyprinid doctor fish, and amphibian. Tilapia have been introduced into north Queensland on several occasions since the 1970's around Cairns and Townsville and several species and hybrids are present in many northern rivers (Mather and Arthington, 1991). Whilst it cannot be proven definitively, it is possible that this

GBS ST was introduced along with the Tilapia and has since established amongst the Australian aquatic animal life in the region. One new MLST allele was discovered in a grouper isolate. As the genes used for MLST occur in the core genome and are under positive selective pressure, they evolve very slowly. At this time we are unsure of mutation rates in GBS, but a single allele change amongst the ST-261 type suggests a relatively recent introduction, as there has been insufficient time for major allelic variation.

Cps operon sequence typing corroborates the MLST since the serotypes of the marine isolates are different from local terrestrial isolates. This is reassuring from a public safety perspective as these sequence types have not been found causing disease in humans or terrestrial animals. The *cps* genotyping provided some further resolution of possible strain movement and evolution within the aquatic animals. Identical point mutations have been found in all of the isolates from stingrays suggesting passage amongst these animals. Moreover, five of the Queensland grouper, the Javelin grunter, the catfish and the mullet isolates shared identical *cps* genotype sequences. Bearing in mind the plasticity of the *cps* operon in *Streptococcus* spp., this strongly implicates a shared source of infection amongst these fish. However, for more accurate epidemiological tracing and inference of evolutionary history a larger repertoire of genes needs to be analysed. The hierarchical gene-by-gene approach using the Bacterial Isolate Genome Sequence Database (BIGSdb) as recently proposed by (Maiden *et al.*, 2013) is a logical next step in this analysis.

Chapter 4

Cellular immune functions of Queensland grouper, *Epinephelus lanceolatus*, in response to *Streptococcus agalactiae* strains from marine and terrestrial origins analysed by flow cytometry

ABSTRACT

To better understand how *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) are pathogenic in fish, we investigated interactions between isolates from differing hosts and the cellular immune system of Queensland grouper *Epinephelus lanceolatus* using flow cytometry. Head-kidney leucocytes (HKL) from Queensland grouper displayed two main cell populations with distinct forward and side scatter by flow cytometry. The population of smaller and less complex cells (P1) was composed of monocytes, lymphocytes and thrombocytes, while the population of primarily larger and more complex cells (P2) comprised predominantly of macrophages and neutrophils. The cells in P2 had higher phagocytic index and capacity when incubated with fluorescent latex beads. HKL were activated by phorbol myristate acetate (PMA) but were unresponsive to lipopolysaccharide (LPS) and peptidoglycan (PTG), suggesting the absence of specific receptors on the surface of these cells for these ligands or a requirement for intermediates. All fish isolates of GBS activated a respiratory burst in P2 with a significant production of intracellular reactive oxygen species (ROS). Whilst piscine and terrestrial isolates have different serotypes and multilocus sequence types (STs), the dog and cat isolates also induced significant ROS production in grouper HKL. However, human, crocodile and bovine isolates of GBS did not elicit significant ROS in HKL although this coincided with the highest phagocytic index. This suggests that these bacteria are capable of quenching ROS production. Terrestrial isolates significantly increased mortality of Queensland grouper leucocytes *in vitro*, aligned with a more diverse repertoire of cellular toxins in these strains. Opsonisation of a marine strain and terrestrial strain of GBS with antiserum raised against the marine strain resulted in an increase in ROS production by HKL in both cases although there was low antigenic cross reactivity between the two strains by flow cytometry, reflecting their diverse serotypes (Ib vs III). However, pre-incubation of either strain with normal serum from grouper also increased ROS production of HKL suggesting other opsonins may be involved.

4.1 INTRODUCTION

Total aquaculture production around the world has been growing dramatically to exceed wild fisheries catches by about 70% (Grainger *et al.*, 2012). One of the biggest drawbacks to the development of the industry is the increasing emergence of bacterial, viral or parasitic diseases affecting fish and shellfish production (Ghittino *et al.*, 2003; Woo and Cain, 2013). A better understanding of the biology of the species being cultured, in particular the interactions between the host immune system and the pathogen is central if we want to improve welfare and health management of the animals.

Teleosts (bony fish) are the earliest vertebrate group that have an immune system comparable to higher vertebrates (Uribe *et al.*, 2011). They possess a repertoire of non-specific and specific humoral and cell-mediated mechanisms to resist bacterial diseases (Ellis, 1999). In order to infect the host and disseminate in the organs and tissues, bacterial pathogens must defeat or elude these defence mechanisms. The major interface between host and bacterial pathogen is the non-specific cellular immune system, orchestrated by phagocytic cells, including macrophages and neutrophils that act collaboratively with other leucocyte populations to play a central role in both innate and adaptive immunity (Ellis, 1977, 1999; Whyte, 2007).

The cephalic portion of the kidney (pronephros or head kidney) is a major haematopoietic tissue in fish where progenitor cells, from which many of these leucocyte populations are derived, are present (Rombout *et al.*, 2005). Macrophages are professional phagocytic cells specialised in killing non-self microorganisms. In fish they are found in connective and other tissues and organs and are the predominant phagocytic cells (Secombes, 1990). They play a pivotal role in non-specific immunity, because these cells have evolved a way to utilize pattern recognition receptors (PRRs) to recognize pathogen/microbe associated molecular patterns (PAMPs/MAMPs) such as lipopolysachhride (LPS), peptidoglycan (PTG) and mannose. By binding to these surface molecules, phagocytic cells can ingest and destroy many aquatic pathogens. Following phagocytosis, macrophages enter a state of activation termed respiratory burst, a process where one electron of molecular oxygen is reduced into highly reactive superoxide anion (O_2^-) with the help of the unique macrophage membrane enzyme nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase). Superoxide anion becomes the precursor to the production of other reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH^-$), single oxygen, and hypohalites (Tumbol *et al.*, 2009; Secombes, 1990). This complex molecular mechanism by which this variety of ROS are produced is considered to be one of the most important microbicidal component of the cellular innate defence (Stafford *et al.*, 2002; Secombes, 1990). Phagocytosis and downstream respiratory burst activity have been widely used in a number of fish species as a proxy to measure the intensity of the cellular innate immune response and health status of the organism in response to various stimuli, such as pathogens, immuno-modulators and environmental stress.

Whilst there have been a number of experimental infection studies characterising the cellular immune responses against bacterial pathogens in other teleost species, to date, no work has been done on

Queensland grouper leucocytes.

S. agalactiae in wild Queensland grouper (*Epinephelus lanceolatus*) was first reported in Australia in 2008 and subsequently in other marine teleost and elasmobranch species (Bowater *et al.*, 2012). Susceptibility and pathogenesis of Queensland grouper to infection by GBS via different routes of exposures have been conducted (Delamare-Debouteville *et al*., 2014) and confirmed the previous hypothesis that *S. agalactiae* was causative for the wild fish mortality (see Chapter 2).

Ultimately, most invading bacteria are killed by macrophages, but not always, which may lead to disease. Therefore research should focus on how virulent bacterial pathogens circumvent these cells. In the present study, we use flow cytometry to investigate phagocyte functions in response to GBS by challenging primary leucocyte cultures from Queensland grouper head-kidney (HKL) with different strains of GBS isolated from marine and terrestrial hosts. As marine and terrestrial GBS isolates have differing genetic backgrounds revealed by whole genome sequencing, differences in interactions with the cellular immune system of fish corroborated by genome information, may inform of critical virulence factors and processes involved in colonisation of teleosts and potentially, mammalian hosts.

4.2 MATERIALS AND METHODS

4.2.1 Selection of GBS strains

Strains were selected based on differences observed from multilocus sequence typing (MLST), the capsular polysaccharide (CPS) molecular genotyping results and some preliminary whole genome sequencing data (see Chapter 3). Five marine strains were isolated from wild Queensland groupers and mullet. An additional four terrestrial strains were isolated from mammals including dog, cat, cow and human, and one riparian strain was isolated from crocodile (Table 4.1).

Table 4.1. Marine, terrestrial animals and human strains used in the *in vitro* cellular immune assays

Strain	Genus	species	Host	Scientific name	Year	Tissue/organ	Geographic origin
QMA0284	*Streptococcus*	*agalactiae*	Grouper 1	*Epinephelus lanceolatus*	2008	Eye	Cairns
QMA0285	*Streptococcus*	*agalactiae*	Grouper 2	*E. lanceolatus*	2008	Head-kidney	Cairns
QMA0281	*Streptococcus*	*agalactiae*	Grouper 5	*E. lanceolatus*	2010	Heart	Townsville
QMA0368	*Streptococcus*	*agalactiae*	Grouper 6	*E. lanceolatus*	2010	Eye	Gulf of Carpentaria
QMA0274	*Streptococcus*	*agalactiae*	Squaretail Mullet	*Ellochelon vaigiensis*	2009	Heart	Cairns
QMA0336	*Streptococcus*	*agalactiae*	Crocodile	*Crocodylus porosus*	2010	Joint	Darwin
QMA0355	*Streptococcus*	*agalactiae*	Human foot	*Homo sapiens*	2011	Foot	Townsville
QMA0300	*Streptococcus*	*agalactiae*	Dog	*Canis lupus*	2008	Canine urine	unknown
QMA0303	*Streptococcus*	*agalactiae*	Cat	*Felis catus*	2009	Feline left fore paw	unknown
QMA0306	*Streptococcus*	*agalactiae*	Cow	*Bos taurus*	2005	Bovine milk	unknown

4.2.2 Experimental fish and husbandry

Sibling Queensland groupers (*Epinephelus lanceolatus*), weighing approximately 30 g, were obtained from Northern Fisheries Centre (NFC, Cairns, QLD, Australia) and held in seawater at 35 ppt salinity in 100 L fiberglass aquaria. Aquaria were organized into two banks of four tanks supplied by a common recirculation system comprising of 2 x 250 L sump, 2 x 50 L bio-filter, and a protein skimmer. The temperature was maintained at 28°C by a heating and cooling system with a recirculation rate of approximately 120 L per hour. Fish were fed a maintenance diet comprising a commercial 4 mm floating diet once a day (Ridley Aqua Feed, Narangba, QLD), equivalent to 5% body weight. Water exchange (15%) was performed every week and water quality was checked for ammonium, nitrite, nitrate and pH levels on a weekly basis. Fish were graded by size throughout the experiment in order to prevent aggression, social hierarchies and cannibalism. In the following experiments, no procedures were carried out on live fish. All dissection procedures were conducted on head-kidney leucocytes after fish were euthanized. Fish were euthanized by overdose of Aqui-S (Aqui-S, Lower Hutt, New Zealand), exsanguinated through the caudal vein and immediately transferred onto ice.

4.2.3 Isolation, preparation and culture of head-kidney leucocytes

Head kidney leucocytes were collected from euthanized juvenile Queensland groupers essentially as described by Secombes (1990) with some modifications. Briefly, following euthanasia by overdose of anaesthetic (Aqui-S), fish were completely exsanguinated by bleeding from the caudal vein using a syringe and by sectioning the gills. Subsequently, an incision was made along the lateral line to open the peritoneal cavity. The swim bladder and other organs were removed aseptically in order to expose the head-kidney, which was dissected out and placed in L-15 Leibovitz medium containing phenol red (Invitrogen, Melbourne, Australia), supplemented with heat-inactivated (HI) 2% fetal bovine serum (FBS; Bovogen, Victoria, Australia), 100 units mL^{-1} penicillin streptomycin (P/S) and 10 units mL^{-1} heparin. Head-kidney was cut into small pieces and gently pushed through a 100 μm cell strainer with the plunger of a 1 mL tuberculin syringe into a sterile Petri dish to obtain a single cell suspension in supplemented medium. The resultant disaggregated kidney suspension was layered onto a 34% (1.050 g mL^{-1}) / 51% (1.072 g mL^{-1}) v/v Percoll gradient and centrifuged at 600 x g for 30 min at 23°C. The leucocyte fractions lying at the 34–51% interface and including the upward density layer were collected, washed in L-15 containing 0.1% fetal bovine serum (FBS) (L-15 (0.1%)), centrifuged at 400 x g for 10 min and resuspended in L-15 (0.1%). Total number and percentage of viable leucocyte was determined by propidium iodide (PI) exclusion using epifluorescence microscopy (Texas-red: U-MWIY, excitation filter BP545-580, emission filter 610IF, dichromatic filter 600, Olympus). Viable leucocytes were counted on a haemocytometer then plated at a concentration of 5 x 10^6 cells mL^{-1} in 24 well plates for a minimum of 4 hr in L-15 (0.1% FBS). Leucocytes showed viability of 95% or above for cell samples used in this study. The plates were then washed to remove non-adherent cells and fresh culture medium (L-15 10% FBS) added to the wells. The cells were then incubated at 28°C overnight before being used for subsequent assays the following day.

4.2.4 Cell identification and characterisation by light microscopy

The different leucocyte sub-populations isolated from Queensland grouper head-kidney were characterised based on their morphology using microscopy (cytospin preparations). After separation on a Percoll gradient, both top and bottom layers were carefully collected and cells diluted to give a final concentration of 300 000 cells mL^{-1}. Cell cytospin preparations were prepared on glass slides using 100 μL of cell suspension with a Tharmac, CellspinI cytospin centrifuge (3 min, 1000 rpm). Air-dried slides were fixed for 5 sec in 100% methanol then stained with Hemacolor (Merck). Slides were rinsed in PBS and left to air-dry at room temperature before being mounted with Permount mounting medium (Fisher Scientific) for observation by light microscopy.

4.2.5 Spectrophotometric determination of leucocyte activation

The capabilities of the head-kidney leucocytes to respond to various PAMPs such as LPS, PTG, and the specific activator of protein kinase C (PKC), phorbol myristate acetate (PMA), was measured quantitatively via the detection of intracellular ROS with the cell-permeable non-fluorescent probe 2',7'-dichlorodihydrofluorescin-diacetate (DCFH-DA). Briefly, DCFH-DA diffuses

rapidly into the cells and is de-esterified (deacetylated) by cellular esterases to non-fluorescent 2',7'-dichlorodihydrofluorescin (DCFH), which is oxidised by ROS into the highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF). The fluorescence intensity of DCF is directly proportional to the amount of ROS produced within the cell cytoplasm.

HKL from Queensland grouper were seeded into 96 well black flat-bottom plates (250 000 cells mL^{-1}) and left to attach in L-15 (0.1% FBS) for 4 hr at 28°C. Subsequently supernatant was removed and leucocytes were pre-incubated with DCFH-DA (Sigma, Castle Hill, Australia) (10μM) in L-15 without phenol-red or serum, to minimize extracellular hydrolysis of the dye, and for 30 min, to allow penetration of the dye into the cells. After 30 min, HKL were supplemented in triplicate with the following treatments: LPS 1, 10, and 100 μg mL^{-1}, PMA 10, 100, and 1000 ng mL^{-1}, or PTG 1, 10, and 100 μg mL^{-1} (Sigma, Castle Hill, Australia). The negative control consisted of leucocytes left unstimulated, and supplemented with medium and DCFH-DA. The background noise was obtained with cells incubated without DCFH-DA. When all treatments were added, the plate was incubated at 28°C for 2 hr and relative fluorescence unit (RFU) was measured with a Fluostar Optima plate reader (BMG Labtech, Melbourne, Australia) (excitation: 485 nm, emission: 520 nm).

4.2.6 Flow cytometry

4.2.6.1 Analysis and sorting of HKL by flow cytometry

The BD FACSAria™ II cell sorting flow cytometer (BD biosciences, NSW, Australia) was used to collect and analyse data on cellular functions of specific sub-populations of leucocytes in the presence or absence of different pathogenic isolates of GBS. Samples were prepared as described earlier, and run on the cytometer collecting 10 000 cells in logarithmic mode from each sample using the FACSDiva™ software version 6.1.3 (BD biosciences). Data were collected for forward scatter (FSC) and side scatter (SSC) representing the size and granularity of the cells respectively. Due to the presence of very large cells in our samples, a neutral density (ND) filter in front of the forward scatter was used in order to visualize all the cells in the scatter plot. Gating of the cells was performed to exclude debris and dead cells from subsequent analysis using SyBr green and PI respectively (Life Technologies, VIC, Australia).

Gates around the two main populations observed on the scatter plot (SSC vs. FSC) were created and populations sorted in separate tubes. Cytospin preparations were made with the sorted populations as previously described and stained using Hemacolor stain for morphological analysis.

4.2.6.2 Flow cytometry assay of phagocytosis

The preliminary assessment of HKL phagocytic rate and capacity was performed using fluorescent latex beads by microscopy and flow cytometry. The beads stock (Fluoresbrite ® Yellow Green carboxylate microspheres 0.2 μm; ~5.68 x 10^{12} particles mL^{-1}; 2.65% latex, Polysciences, Inc.) was opsonised using a 10 000-fold dilution in phosphate buffered saline (PBS, pH 7.4; AMRESCO) supplemented

with 10% Queensland grouper HI serum. The leucocytes concentration was adjusted to 2.5 x 10^6 cells per well (500 μL) and the medium was changed to 10%FBS/L-15 containing opsonised beads at a cell/bead ratio of 1:10 per well in 24 well culture plates (Greiner). The plate was then centrifuged at 400 x g, 23°C for 5 min to bring the beads and cells into contact (acceleration 6, break 4), as adapted and modified from Bassity and Clark (2012). Following the 2 hr incubation with beads at 28°C, the cells suspensions were removed and the wells were washed twice with PBS. Adherent cells were loosened by trypsination for 2 min using 200 μL of trypsin-EDTA per well (Invitrogen, Melbourne, Australia). The reaction was stopped by addition of 800 μL of PBS with 10 % serum. The cells were transferred to 5 mL polystyrene falcon tube (BD, NSW, Australia) and put on ice to stop the cell activity until being acquired on the BD FACSAria™ II for green fluorescence (detected with 530/30 bandpass filter; FITC) from 488 nm blue argon-ion laser excitation. Phagocytic rate was expressed as the percentage of total cells with ingested beads, while the phagocytic capacity was defined as the average number of fluorescent beads engulfed per cell. A negative control consisting of a cell suspension without beads was used to determine fluorescence threshold. Cytospin preparations were made and mounted using DABCO anti-fading medium (Sigma, Castle Hill, Australia) for microscopy morphological observations.

Further characterisation of HKL phagocytic capacities was performed using a selection of marine and terrestrial GBS strains (Table 4.1) and analysed by flow cytometry. Briefly, bacterial suspensions in late exponential phase were adjusted to an optic density at 600 nm (OD_{600}) of 1 (~10^8 bacteria mL^{-1}). They were then stained with 0.5μM BacLight Green (BLG) for 15 min at room temperature (RT), rinsed five times in PBS before being diluted 100-fold in 10% FBS/L-15 phenol red. Fluorescently labelled bacteria were added to the cells to give a final multiplicity of infection (MOI) of 1. Plate was spun as described before to ensure contact between HKL and bacteria. After incubation for 2 hr at 28°C, the cells were washed once, trypsinised from the wells, and transferred to 5 mL falcon tubes and put on ice to stop the cell activity until flow cytometry analysis measuring the fluorescence emission at 530 nm using 488 nm excitation laser.

4.2.6.3 Flow cytometry assay of respiratory burst

Cellular oxidative activity of Queensland grouper head-kidney leucocytes in response to different isolates of GBS (Table 4.1) was evaluated by flow cytometry. To avoid overstimulation of the cells and in order to detect maximum activity of the leucocytes in response to the bacteria, a pilot experiment was designed to look at the effect of different multiplicity of infection (MOI) (bacteria to HKL cell ratios) on the production of ROS (using two representative strains of *S. agalactiae,* QMA0285 and QMA0355). The different MOI used were 10, 1 and 0.1 bacteria per cell corresponding respectively to a 1:10, 1:100 and 1:1000 dilution from a bacterial suspension fixed at an optical density (OD_{600}) of 1. In brief, after HKL isolation and incubation overnight at 28°C in 24-well plate, the different GBS isolates were added to the cells at an approximate MOI of 1 in L-15 phenol-red and serum free media with DCFH-DA (10μM). Several controls were included in all experiments for the respiratory burst analyses: leucocytes stimulated with PMA were used as positive controls; unstimulated cells

with added DCFH-DA was used to define the region of oxidative activity in resting cells; untreated leucocytes without stimulants and DCFH-DA served for detection of any possible auto fluorescence from the leucocytes. After 2 hr incubation, samples were put on ice to stop the reaction until being analysed by flow cytometry measuring the fluorescence emission at 530 nm using a 488 nm excitation laser.

4.2.6.4 Flow cytometry assay for viability and apoptosis

Cell viability was assayed using the Alexa Fluor® 488 annexin V/PI dead cell apoptosis kit for flow cytometry (Invitrogen, Melbourne, Australia), according to the manufacturer instructions. Briefly, HKL were incubated with different GBS isolates (Table 4.1) for 2 hr. Positive controls were prepared for both annexin V and PI including camptothecin (Sigma, Castle Hill, Australia) a potent inducer of apoptosis, for annexin V and fixed leucocytes in 4% formaldehyde for PI. Negative control consisted of cells without bacteria or inducing agents. Stained cells were analysed as soon as possible by flow cytometry, measuring green and red fluorescence emissions at 530 and 610 nm using the 488 and 561 nm excitation lasers respectively. Flow cytometry results could be confirmed by viewing the cells under a fluorescence microscope, using filters appropriate for fluorescein (FITC) and PI (Texas Red).

4.2.7 Vaccine preparation, fish vaccination and analysis of sera for specific and cross reactivity antibody response by ELISA.

A formalin-killed bacterin vaccine based on strain QMA0285 isolated from wild Queensland grouper was prepared as previously described (Aviles *et al.*, 2013). Cohorts of fish were anaesthetised with Aqui-S and vaccinated by i.p. injection using a calibrated self-refilling syringe (Socorex, Switzerland) with 100 μL dose per fish of either the vaccine (adjuvanted by emulsifying in a equal volume of Freund's incomplete adjuvant, FIA), PBS or FIA used as controls. After 900 degree-days (32 days at 28°C) to allow development of immunity, all fish were euthanized by lethal overdose of Aqui-S and exsanguinated by caudal venipuncture. Blood was allowed to clot at 4°C overnight, and sera were separated by centrifugation (5 000 x g, 15 min, 4°C) and retained at -20°C for ELISA and flow cytometry assays. Antibody response of vaccinated Queensland groupers (QMA0285) and PBS or FAI injected fish were determined against the vaccine strain (QMA0285) by indirect whole-cell enzyme-linked immunosorbent assay (ELISA) as described previously (Delamare-Debouteville *et al.*, 2006).

4.2.8 Evaluation of antigen exposure by FACS

Evaluation of antigen exposure on the bacterial surface of five marine strains and five terrestrial strains of GBS was performed as described previously (Bensi *et al.*, 2012) with some modifications. GBS culture strains were grown overnight in Todd-Hewitt broth medium. Optical density (OD_{600}) was fixed to 0.5, and then cells washed twice with phosphate-buffered saline (PBS). A volume of 200 μL of bacterial suspension was dispensed in 1.5 mL tube, centrifuged and resuspended in 600 μL

FBS for 60 min at room temperature. Bacteria were then incubated overnight at 4°C with high titre polyclonal sera from fish that had responded positively to the QMA0285 formalin killed vaccine or with PBS immune serum as negative control both diluted 1:50 in dilution buffer (PBS, 20% FBS, 0.1% bovine serum albumin). After centrifugation and washing in PBS and 0.1% BSA twice, samples were incubated for 90 min at 4°C with polyclonal immunoglobulin G (IgG) sheep anti-barramundi IgM (previously shown to cross-react with Queensland grouper serum) diluted 1:200 (PBS, FBS 5%, BSA 0.1%). After centrifugation and washing in PBS and 0.1% BSA twice, samples were incubated at 4°C for 30 min with donkey polyclonal secondary antibody anti-goat IgG (Alexa Fluor 647) (shown to cross-react with sheep anti-barramundi IgM) diluted 1:250 (FBS 5%, BSA 0.1%). Bacteria were washed twice and suspended in a final volume of 200 μL PBS. Control samples with omission of the primary antibodies and with only the tertiary antibody were included to determine the non-specific binding of the secondary and tertiary immunoglobulins. Bacteria alone without antibodies were used as a negative control. For each strain the difference between the mean fluorescence intensity (MFI) of the immune and PBS injected fish sera were defined (ΔMFIs). All samples were kept on ice after incubation until analysis with the BD FACSAria™ II flow cytometer. Forward, side scatters and red fluorescence voltages were set respectively at 250V, 300V and 670V.

4.2.9 Opsonophagocytosis

The effect of pre-opsonisation of bacteria with antibodies on the phagocytosis and respiratory burst activities of HKL was determined in an assay previously described by (Aviles *et al.*, 2013). Briefly, bacteria suspensions were incubated (55°C for 30 min) with HI non-immune serum (from PBS injected fish) or antiserum against QMA0285 vaccine (Ab) (10% v/v) for 1 hr at room temperature. Then the cells were centrifuged at 14 500 rpm for 10 min and resuspended in L-15 medium at a MOI of 1 (bacteria/macrophage). Phagocytosis assay was carried out as previously described with Baclight Green opsonised labelled bacteria, whereas the oxidative activity was performed with bacteria opsonised but not fluorescently labelled.

4.2.10 Microscopy

Cultures were viewed with an Olympus CKX41 inverted microscope and cytospin slides were viewed using an Olympus BX41 microscope. On both microscopes, images were captured with an Olympus DP26/U-CMAD3 camera and optimised with the imaging software CellSens (Olympus Optical Co. Ltd, Japan).

4.2.11 Statistical analysis

Data analysis was performed using GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, San Diego California, USA). Phagocytosis of beads was analysed by a two-tailed unpaired t-test. Data from the oxidative activity, phagocytosis and viability obtained by flow cytometry and spectrofluorometry were analysed by one-way analysis of variance (ANOVA), specific differences between treatments were isolated using Tukey's post-hoc tests. Results are presented as mean ± standard error.

4.3 RESULTS

HKL can be activated by PMA

To determine the activation status of the head-kidney leucocytes, an experiment was conducted to investigate the total redox potential generated by the entire leucocyte population after stimulation with different PAMPs, including LPS, the complex outer membrane carbohydrate of gram-positive bacteria PTG, and the surface receptor-independent protein kinase C activator, PMA. Head-kidney cells produced significantly more ROS in response to stimulation by PMA at 100 and 1000 ng mL^{-1} compared to control unstimulated cells but were not activated at the lowest concentration 10 ng mL^{-1} (Fig. 4.1). Whilst there was a slight increase in relative fluorescence with 10 and 100 μg mL^{-1} PTG, neither LPS nor PTG induced ROS productions that were significantly different from non-stimulated control, and this at all concentrations used (Fig. 4.1).

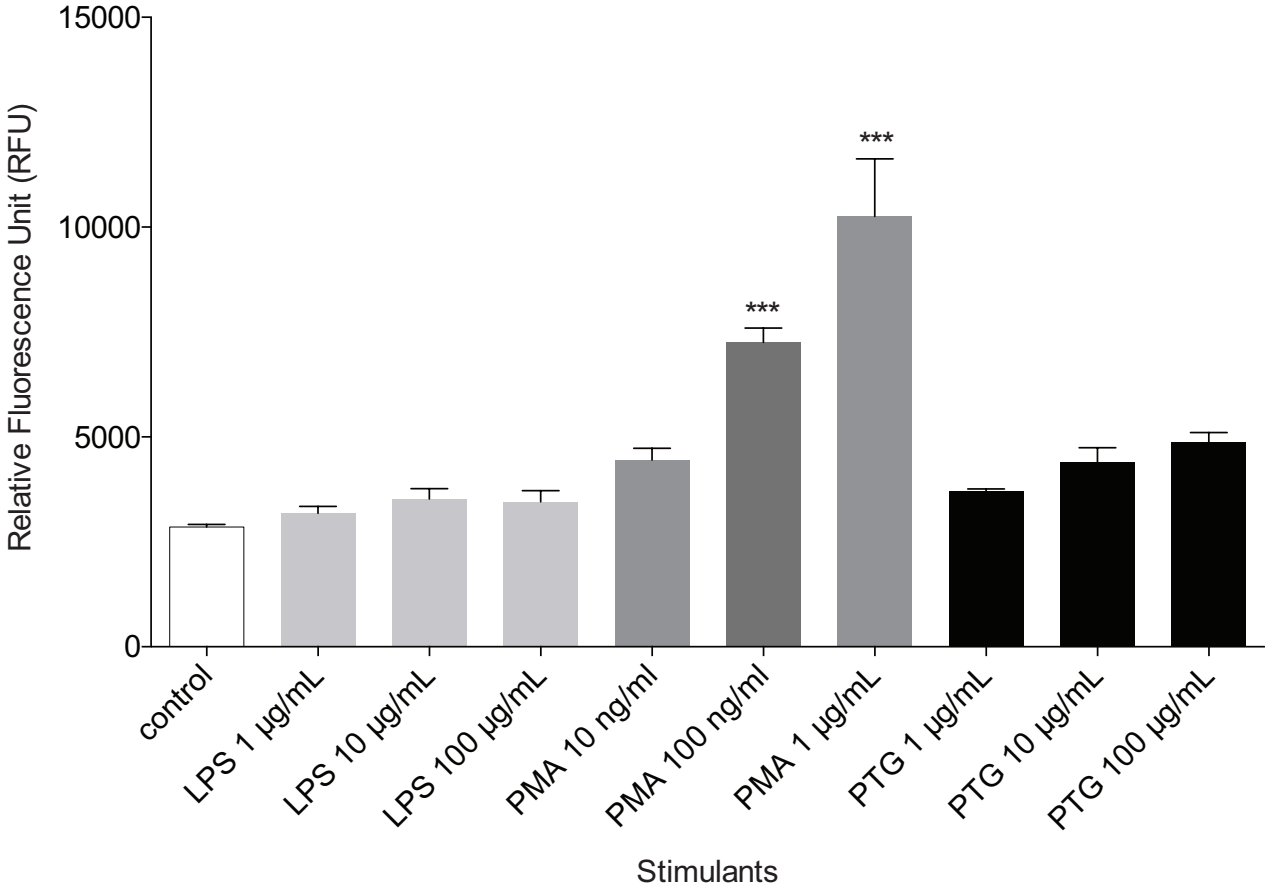

Figure 4.1. Direct stimulation of respiratory burst in Queensland grouper head-kidney leucocyte populations assayed by fluorometry. Percoll-separated HKL (1 x 10^6 mL^{-1}) were pre-incubated in L-15 medium containing 10μM DCFH-DA without phenol-red and FBS for 30 min. Drugs were added for 2 hr and fluorescence (relative fluorescence unit) was measured in a spectrophotometer (excitation 485 nm / emission 520 nm) in plate mode fluorescence. LPS: lipopolysaccharide, PMA: phorbol-myristate acetate, PTG: peptidoglycan. Mean values (± SEM) of replicate assays with leucocytes from three fish are expressed in relative fluorescence unit (RFU). Stars indicate a significant difference with the unstimulated control ($P < 0.05$, one-way ANOVA). *** $P < 0.001$.

Sub-populations of head-kidney leucocytes can ingest fluorescent latex beads

When analysed by flow cytometry, head-kidney leucocytes from Queensland grouper displayed two main cell populations with distinct forward and side scatter (FSC/SSC) characteristics (Fig. 4.2 B). The small and less complex population (low FSC/SSC) was designated as P1 and the population with increased FSC/SSC values as P2 (Fig. 4.2 B).

When analysed for their phagocytic rate and capacity both sub-population P1 and P2 were capable to engulf 0.2 µm fluorescent latex beads (Fig. 4.2 C, D). Number of beads ingested per leucocyte could be determined using a representative histogram of the cell count against green fluorescence (Fig. 4.2 A). The mean fluorescence of one bead was determined by gating the second peaks on the histogram corresponding to the mean fluorescence of cells that had ingested two beads, and divide this value by two. Total percentage of phagocytic P2 was significantly higher than P1 (Fig. 4.2 C) ($P < 0.05$). P2 had a significantly higher phagocytic capacity than P1 (Fig. 4.2 D) ($P < 0.05$), with an average of six beads being ingested compared to two beads by P1.

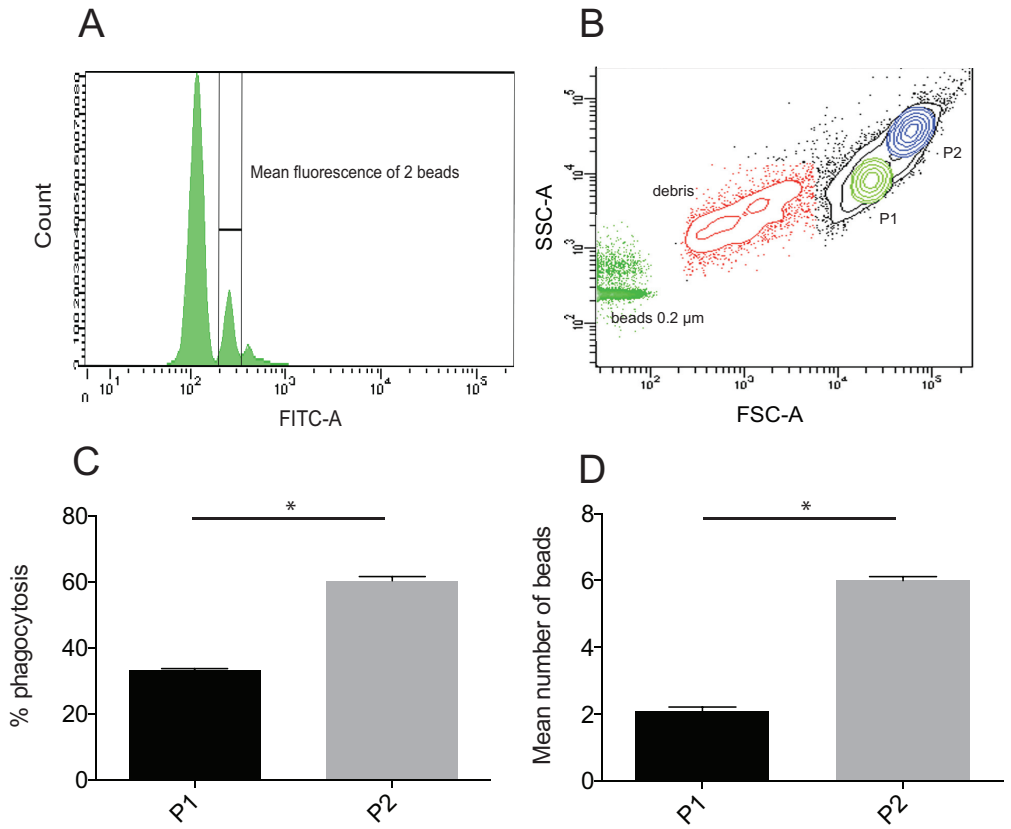

Figure 4.2. Flow cytometric analysis of the phagocytic activity of Queensland grouper head-kidney leucocytes to ingest 0.2 µm beads. A. Representative histogram of cell counts against the green fluorescence (FITC-A) of cells that have phagocytised 1 (1st peak), 2 (2nd peaks) or more beads. B. Representative dot plot analysis of HKL based on the size (FSC-A value) and internal complexity (SSC-A value) of leucocytes collected from the Percoll density gradient obtained by flow cytometry. Two main populations of cells could be differentiated based on their scatter properties: P1 (green) and P2 (blue). The debris were distinct and coloured in red. Note the 0.2 µm beads do not overlap with the leucocytes. C. Percentage phagocytosis (phagocytic rate) of P1 and P2. C. Phagocytosis capacity (mean number of beads ingested) per P1 and P2. Values are expressed as the mean (± SEM) of replicate assays with leucocytes from three fish. Stars indicate a significant difference between cell poplations P1 and P2 ($P < 0.05$, two-tailed unpaired t-test).

P1 and P2 sub-populations are morphologically different

To identify the cells in P1 and P2, these two populations were sorted by flow cytometry based on their different forward (Fig. 4.3 A) and side scatter values (Fig. 4.3 B). The sorted sub-populations were prepared by cytospin and stained with Hemacolor (Fig. 4.3 C, D). The population P1 was dominated by small monocytes (Fig. 4.3 C iii, iv), it also includes a large number of thrombocytes (Fig. 4.3 C v, vi), and small lymphocytes (Fig. 4.3 C i, ii). The population P2 fraction contained primarily larger cells including macrophages (Fig. 4.3 D ii, vi), a small percentage of small monocytes (Fig. 4.3 D ii, v), large lymphocytes (Fig. 4.3 D i), granular cells such as polymorphonucleated neutrophils indicated by high SSC values (Fig. 4.3 D iv, vi), and some dendritic-like cells (Fig. 4.3 D iii).

Since Queensland grouper HK leucocytes were capable of phagocytosis (0.2 μm beads) and ROS production when activated with PMA, their functions in response to five marine and five terrestrial GBS isolates were investigated by flow cytometry measuring their phagocytic, redox potential and viability.

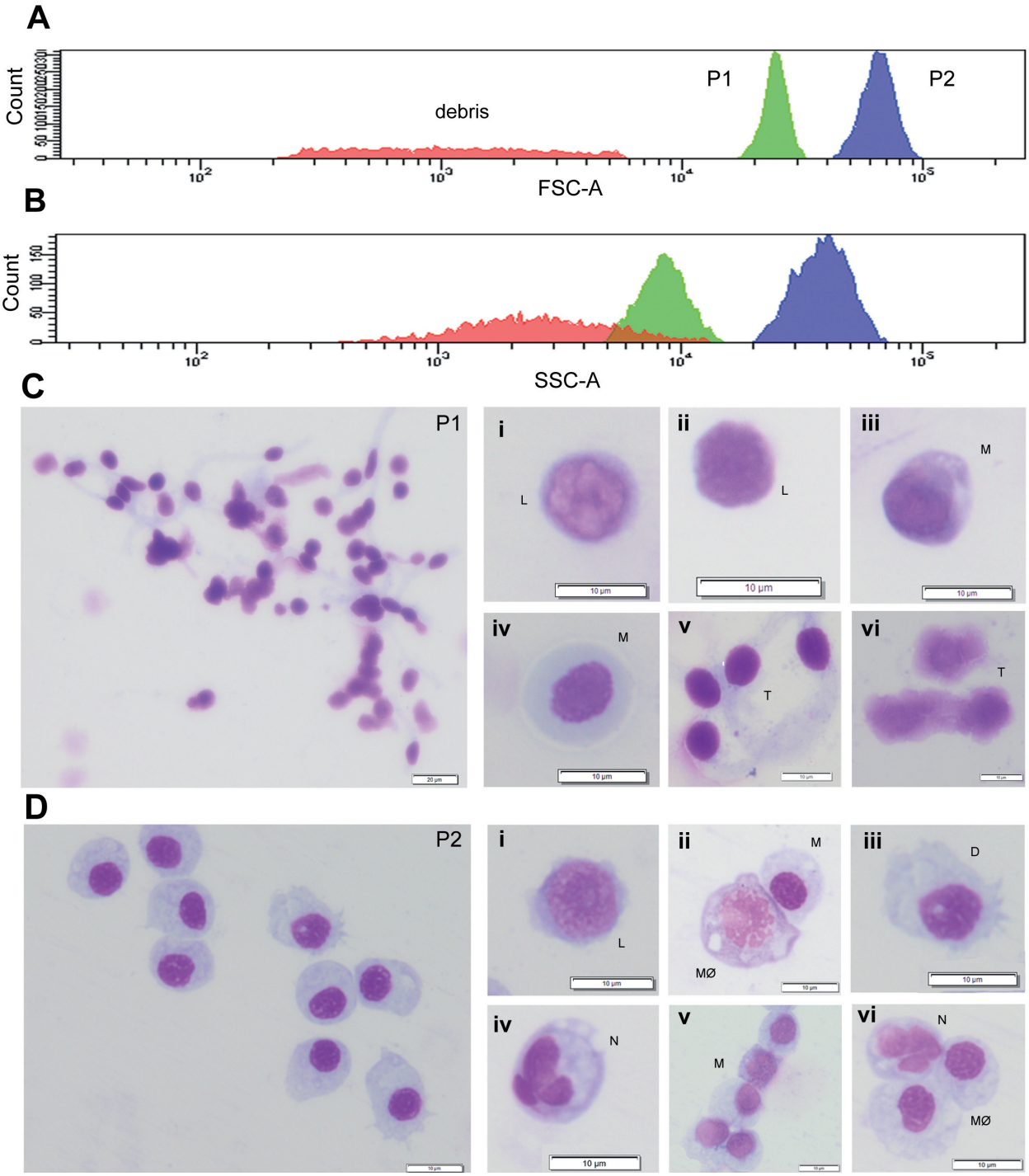

Figure 4.3. Morphological analysis of leucocytes isolated from Queensland grouper head-kidney. HKL were sorted by flow cytometry. A. Representative histograms of cell count against the size (FSC-A) and B. granularity (SSC-A) of cells present in P1 (green) and P2 (blue). Note the debris gate is designated in red. Gated populations P1 and P2 were sorted and cytospin preparations analysed by light microscopy. C. cytospins of P1 and D. cytospins of P2 stained with Hemacolor respectively. The overview photos in C and D (left) and representative single cells (right). In each case, MØ = macrophages; M = monocytes; L = lymphocytes; N = neutrophils; T = thrombocytes; D = dendritic-like cells. Scale bar = 10 µm.

Excessive MOI inhibits ROS production in HKL sub-populations

A pilot study to establish the optimal MOI for subsequent experiments indicated that at a MOI of 10 the ROS production in P1 and P2 were significantly lower than at a MOI of 1 or 0.1 (Fig. 4.4 A, B). The pattern of P1 and P2 are the same, however P2 produced significantly more ROS than P1, almost 10 times more (Fig. 4.4). For all subsequent experiments a MOI of 1 was used.

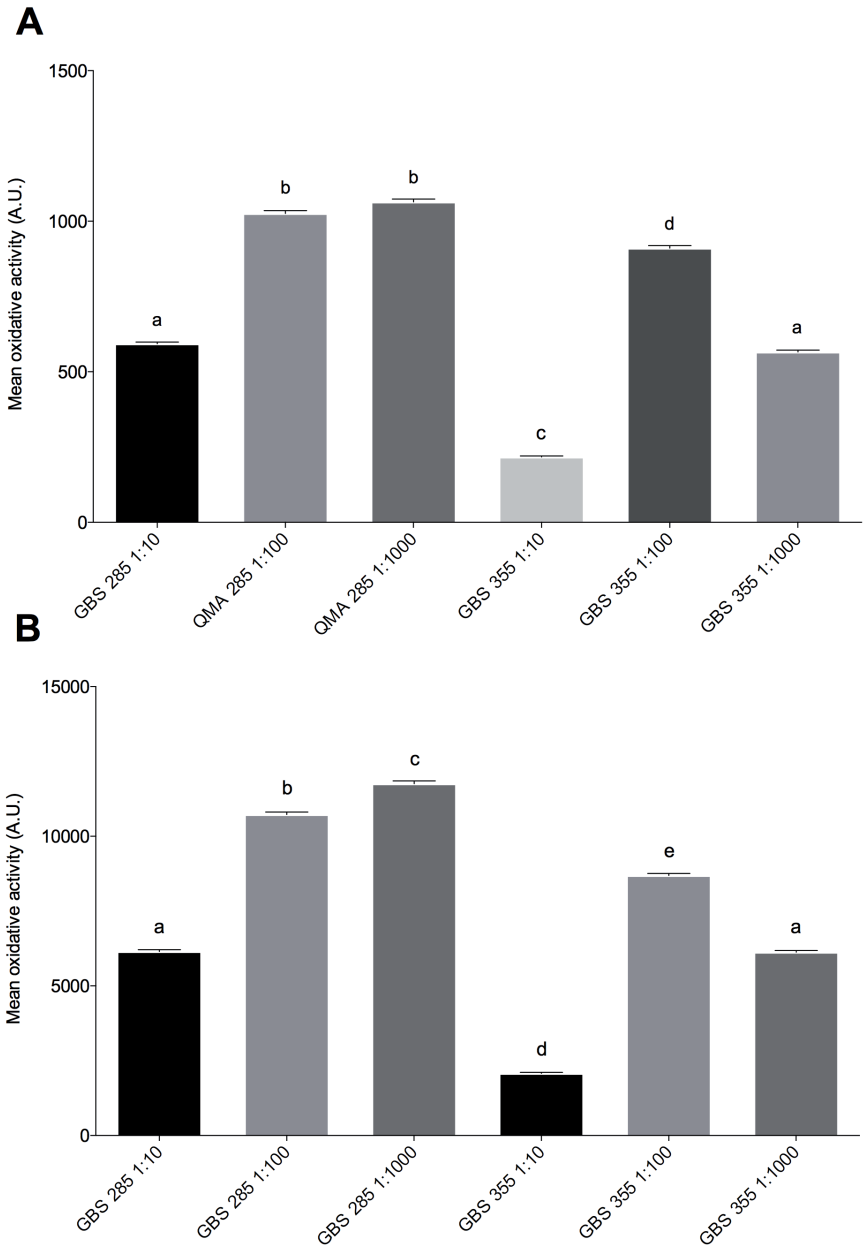

Figure 4.4. Respiratory burst activity of Queensland grouper HKL sub-population P1 (A) and P2 (B) in response to different MOI using strain QMA0285 (grouper 2) and QMA0355 (human foot). HKL (2.5 x 10^6 mL^{-1}) were incubated in L-15 medium containing 10μM DCFH-DA without phenol-red and FBS and the different MOI for 2 hr and then analysed by flow cytometry. All bacteria were fixed at an optical density (OD_{600} = 1), a MOI of 10 corresponds to a dilution of 1:10, a MOI of 1 corresponds to a dilution of 1:100 and a MOI of 0.1 corresponds to a dilution of 1:1000. Mean values (± SEM) are expressed in flow cytometry arbitrary units (A.U) of replicate assays with leucocytes from three fish. Different letters indicate a significant difference between the different treatments and strains ($P < 0.05$, one-way ANOVA).

All piscine and certain terrestrial GBS isolates trigger ROS production in HKL sub-populations

The intracellular oxidative metabolism of the P1 population was significantly different from the control in response to all the marine strains (Fig. 4.5 A). Strains from human foot (QMA0355) and cow (QMA0306) did not significantly induce ROS production in P1 compared to control ($P > 0.05$). A small, but significant increase in ROS production compared to control was observed with the crocodile (QMA0336), cat (QMA0303) and dog (QMA0300) isolates. Mullet (QMA0274) and grouper 6 (QMA0368) induced the highest response in P1. Mean ROS production in HK P1 population when stimulated with 1 μg mL^{-1} PMA was two-fold higher than that induced by QMA0368 (Fig. 4.5 A inset graph). The pattern of activity stimulated by the differing isolates and PMA control between P1 and P2 cell populations were similar, but P2 was metabolically more active with values 10 times higher than P1 (see scales Fig. 4.5 A, B). All GBS isolates had a significant effect on the production of ROS in P2 compared to control except with the isolates from human foot (QMA0355), crocodile (QMA0336) and cow (QMA0306) isolates (Fig. 4.5 B). The strongest activation was obtained with the isolates from grouper 1 (QMA0284), grouper 6 (QMA0368) and mullet (QMA0274) for the marine strains and with the cat (QMA0303) and the dog (QMA0300) for the terrestrial isolates. Overall the effect in response to the different strains of GBS could be categorized as no stimulation, medium stimulation and high stimulation. However, the response to positive control (PMA 1 μg mL^{-1}) was approximately five times greater than the highest response measured with the bacteria (Fig. 4.5 B inset graph).

P2 phagocytic rate and capacity is higher in the presence of terrestrial GBS isolates

When analysed for their phagocytic rate and phagocytic capacity to phagocytose different isolates of *S. agalactiae*, the percentage of positive HK P2 leucocyte for phagocytosis was higher than P1 for all bacteria tested (Table 4.2). However the difference between % positive phagocytic P1 and P2 within each treatment was quite small except with the isolates from grouper 5 (QMA0281) and grouper 6 (QMA0368) for the marine isolates and cat (QMA0303), dog (QMA0300) and cow (QMA0306) for the terrestrial isolates, which were phagocytosed by approximately two times more P2 than P1 cells (Table 4.2). The highest phagocytic rate for P2 and lowest phagocytic rate for P1 were obtained with the isolate from grouper 5 (QMA0281) with respectively 66.6 and 24.4% of cells being phagocytic (Table 4.2).

Overall the phagocytic capacity or index was lower in P1 than in P2 regardless of the isolate used. The phagocytic index for the marine isolates ranged from 2.3 to 3.6 bacteria per cell from P1 and from 15.9 to 27 bacteria per cell from P2. Queensland grouper HKL were generally capable of phagocytising more of the terrestrial GBS isolates compared to the marine strains. The range of phagocytic index for cell from P1 was from 5.7 to 6.6 and from 27 to 49.1 for cell from P2. The marine GBS strain from grouper 2 (QMA0285) resulted in the lowest rate of phagocytosis with 2.3 and 15.9 bacteria ingested per cell from P1 and P2 respectively and the terrestrial crocodile isolate (QMA0336) had the highest phagocytic rate with 6.6 and 49.1 bacteria per cell from P1 and P2 respectively (Table 4.2).

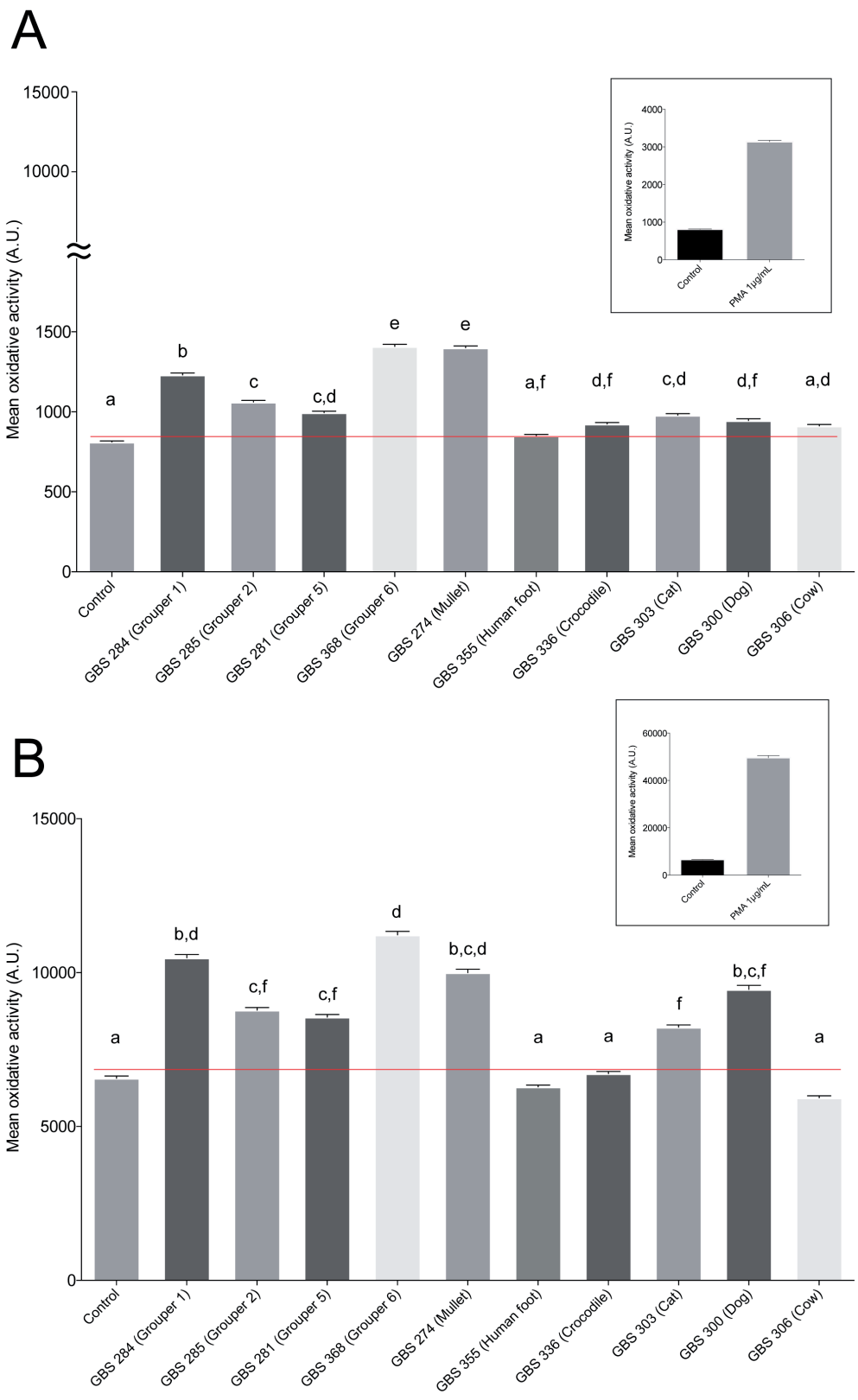

Figure 4.5. A. Mean oxidative activity of Queensland grouper HKL P1 population in response to incubation with marine and terrestrial GBS isolates for 2 hr. B. Mean oxidative activities of Queensland grouper P2 population with the same marine and terrestrial GBS isolates for 2 hr after incubation. Mean values (± SEM) are expressed in flow cytometry arbitrary units (A.U) of replicate assays with leucocytes from three fish. Different letters represent a statistically significant difference between the different strains and the control ($P < 0.05$, one-way ANOVA).

Table 4.2. Phagocytic rate and phagocytic index of Queensland grouper head-kidney P1 and P2 sub-populations of leucocytes after incubation for 2 hr with various GBS isolates (five marines and five terrestrials). Apoptosis and mortality are expressed in fold increase compared to control leucocytes (without bacteria). Phagocytic index was defined as the average number of fluorescent bacteria engulfed per cell and phagocytic rate as the percentage of phagocytosis.

	Treatments									
P1	GBS0284 Grouper 1	GBS0285 Grouper 2	GBS0281 Grouper 5	GBS0368 Grouper 6	GBS0274 Mullet	GBS0355 Human foot	GBS0336 Crocodile	GBS0303 Cat	GBS0300 Dog	GBS0306 Cow
Phagocytic rate (%)	44.8	42.7	24.4	32.8	45.1	41.6	43.6	29.3	36.6	29.9
Phagocytic index	2.6	2.3	3.7	3.1	3.6	5.7	6.6	3.8	6.4	6.0
Fold increase mortality	1.1	1.1	1.1	1.0	1.3	2.0	2.3	1.2	3.5	1.2
Fold increase apoptosis	1.3	1.2	1.4	1.3	1.3	1.0	1.1	1.2	0.9	1.1
P2										
Phagocytic rate (%)	45.5	47.2	66.6	57.7	46.4	49.7	47.1	62.0	52.4	61.6
Phagocytic index	19.2	15.9	17.5	19.9	27.0	38.6	49.1	27.0	45.1	28.1
Fold increase mortality	1.7	1.7	1.7	1.9	1.7	1.7	2.0	1.7	3.0	2.4
Fold increase apoptosis	1.2	1.1	1.3	1.5	1.1	1.4	1.4	1.4	1.3	1.5

Mortality in P1 and P2 is higher in the presence of terrestrial GBS isolates

All GBS isolates induced some mortality in the P1 and P2 populations after 2 hr incubation when compared to control cells without bacteria (Table 4.2). Marine strains only induced a maximum of 1.3 fold increase mortality (QMA0274) in P1, whereas three terrestrial strains: human foot (QMA0355), crocodile (QMA0336), and dog (QMA0300) induced 2.0, 2.3, and 3.5 fold increase mortality respectively in P1. For most strains, mortality in P2 was greater than in P1 except with isolates from human foot (QMA0355), crocodile (QMA0336) and dog (QMA0300) (Table 4.2).

Presence of GBS did not significantly affect apoptosis of either HK cell populations. In the P1 population, fold increase in apoptosis across the marine and terrestrial isolates ranged from 0.9 to 1.4 and from 1.1 to 1.5 in the P2 population.

Confirmation of phagocytosis, respiratory activity and viability by fluorescence microscopy

Phagocytosis, oxidative burst activity and viability of Queensland grouper head-kidney leucocytes in response to various GBS were confirmed by microscopy using the BX41 epifluorescent microscope. Different levels of oxidative burst were observed in leucocytes with some cells having more intense fluorescence than others (Fig. 4.6 A). Group B *Streptococcus* after being stained with the non-nucleic acid Baclight green dye and washed several times before being used in the phagocytosis assay showed bright fluorescence (Fig. 4.6 B). Head-kidney leucocytes after 2 hr incubation with a marine GBS isolate could be seen in phase contrast microscopy (Fig. 4.6 C). Large phagocytic cells with green internalised bacteria were observed after 2 hr incubation (Fig. 4.6 D), as well as with 0.2 μm fluorescent latex beads (Fig. 4.6 E); 0.2 μm latex beads show bright fluorescence (Fig. 4.6 F). After 2 hr incubation with different strains of GBS, mortality and apoptosis in head-kidney leucocytes could be observed by the fluorescence of PI (red) and annexin V (green) respectively (Fig. 4.6 G, H). The nuclei of the leucocytes were stained in blue using DAPI for better observation of internalised bacteria that could be seen inside the phagolysosome (Fig. 4.6 I).

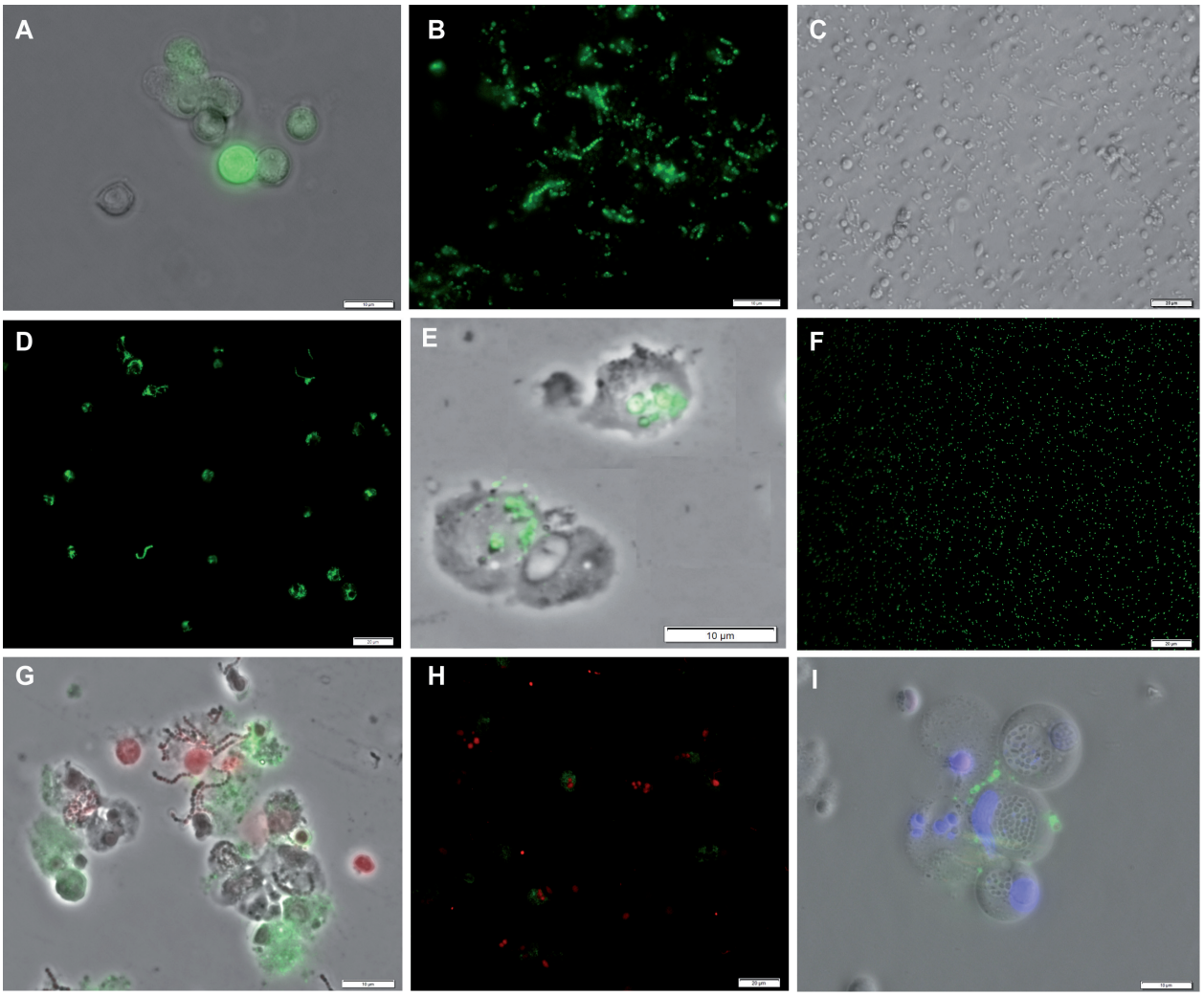

Figure 4.6. Fluorescent microscopy of Queensland grouper HKL cytospin preparations used in the oxidative activity, phagocytosis and viability assays A. Cells that have produced reactive oxygen species are detected with the green fluorescence (DCF). Note the difference in fluorescence intensity and the negative cell on the left. B. Group B Streptococcus (QMA0285) stained with Baclight green stain. C. Phase contrast micrograph of HKL incubated with GBS. D. Macrophages that have ingested fluorescent bacteria. E. Cells with internalised 0.2 µm beads. F. 0.2 µm beads alone. G. Cells viability. Note apoptotic cells (green) and dead cells (red). H. Overlays of apoptotic and dead cells. I. Internalised bacteria QMA0300 in the phagolysosome. Nuclei are stained with DAPI. Note that some apoptotic cells are stained with annexin-V (green).

Specific antibody response of Queensland grouper to *S. agalactiae* vaccine by ELISA

To determine whether fish had responded to the vaccination, serum antibody titres from a cohort of vaccinated and controls fish were determined by enzyme-linked immunosorbent assay (ELISA) (Fig. 4.7 A). After 900 degree-days (32 days at 28°C), fish vaccinated by i.p. injection with a formalin-killed *S. agalactiae* (QMA0285) vaccine produced significantly more serum antibody against QMA0285 than fish injected with PBS or FIA ($P < 0.05$) (Fig. 4.7 A). Only one vaccinated fish with QMA0285 did not respond and had the lowest level of specific antibody produced, and thus was removed from the study as an outlier. Both fish injected with PBS and FIA had low antibody titres when tested against the vaccine antigen (QMA0285) and were not statistically different ($P > 0.05$).

Low antibody cross-reactivity with terrestrial isolates surface antigens

In order to determine whether high titre antisera from fish that had responded to the GBS vaccine (QMA0285) could cross-react with other marine and terrestrial GBS isolates, a flow cytometry assay was conducted using antisera against the QMA0285 vaccine and PBS injected fish with a selection of five marine and five terrestrial GBS isolates (Fig. 4.7 B). The highest reactivity with the antibodies was recorded with the strain used for the vaccine (QMA0285). Immunoglobulins (IgM) cross-reacted with all the marine strains. However, there was low cross reactivity with the terrestrial isolates. Negative ΔMFI values were recorded with the human foot (QMA0355) and the cow isolate (QMA0306) (Fig. 4.7 B).

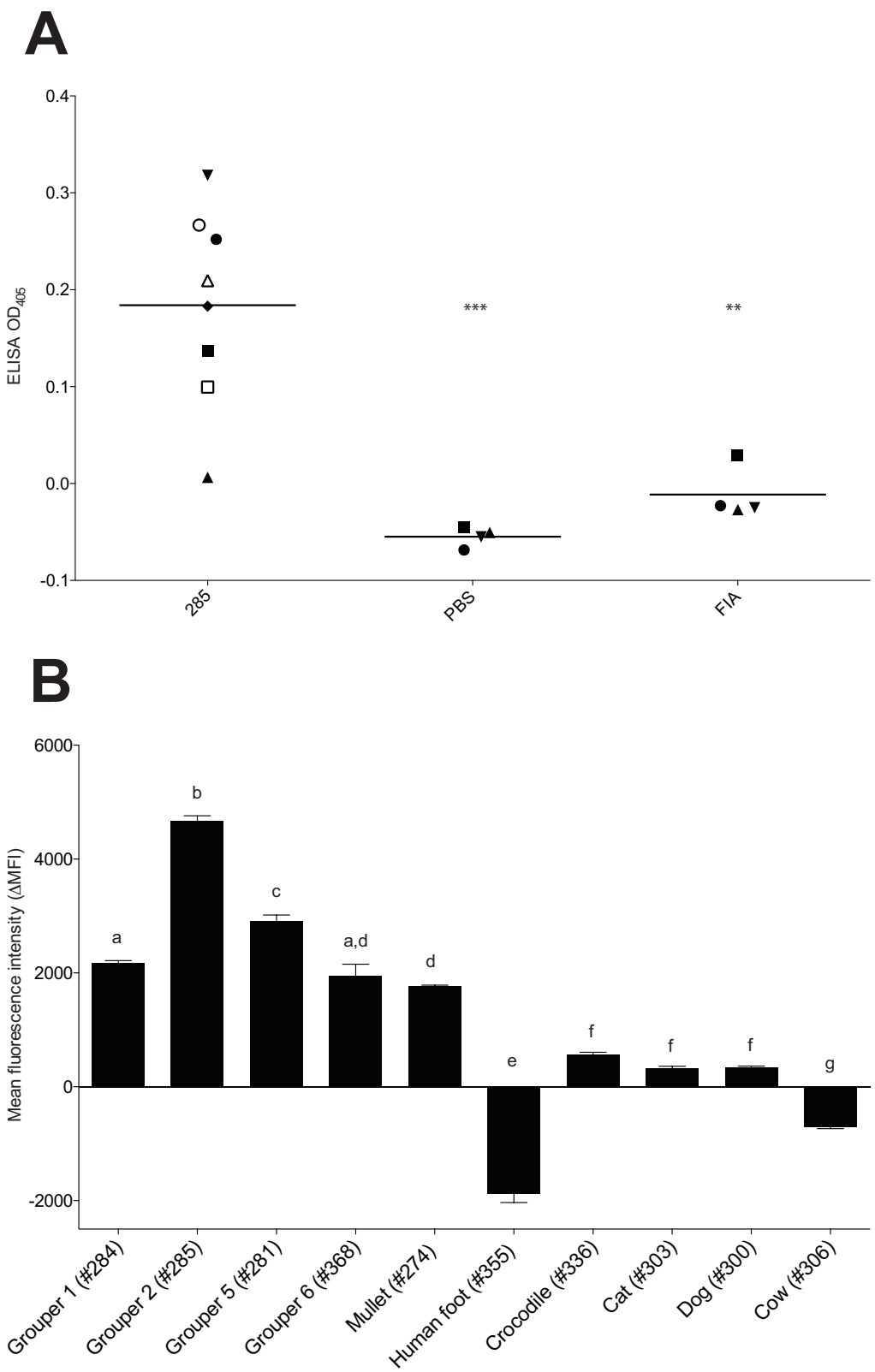

Figure 4.7. A. Serum antibody response by ELISA from vaccinated (n = 8) and control Queensland groupers (PBS injected (n = 4) or Freud's incomplete adjuvant (FIA) injected (n = 4)) tested against the vaccine antigen (QMA0285). B. Surface antigen exposure of five marine and five terrestrial strains evaluated by flow cytometry using a high titre sera antibody produced against the marine isolate QMA0285. Stars indicate significant differences between treatment (vaccine) and controls ($P < 0.05$, one-way ANOVA). ** $P < 0.01$, *** $P < 0.001$. Different letters represent statistically significant differences ($p < 0.05$, one-way ANOVA).

Serum and antiserum increase phagocytosis and respiratory burst of Queensland grouper head-kidney leucocytes

In order to evaluate the effect of antibodies (IgM) on the phagocytosis and respiratory burst activity of Queensland grouper head-kidney leucocytes in response to GBS bacteria, two strains were selected: the marine isolate from grouper 2 (QMA0285) due to its high binding affinity with its homologous sera antibodies (Fig. 4.7 B) and the terrestrial isolate from human foot (QMA0355) because it had the lowest binding affinity with the same sera antibodies produced against QMA0285 (Fig. 4.7 B). As most of the phagocytic and respiratory burst activities were performed by P2, the entire HKL population was considered for this experiment. When both bacteria QMA0285 and QMA0355 were incubated with antiserum from fish vaccinated (QMA0285) and antiserum from fish injected with PBS, there were no significant differences in terms of leucocyte phagocytic rate (data not shown) and capacity between any of the antiserum analysed compared to control (no antiserum) (Fig. 4.8 A)

However, when compared to the control (no antiserum), increasing and decreasing trends were observed for QMA0285 and QMA0355 respectively, in terms of number of bacteria ingested per leucocyte, when both strains were incubated with antiserum from fish vaccinated (QMA0285) and antiserum from fish injected with PBS (Fig. 4.8 A).

A significant increase in oxidative activity of Queensland grouper head-kidney leucocytes was observed when both QMA0285 and QMA0355 were incubated with antiserum from fish vaccinated with GBS vaccine (QMA0285) or with antiserum from fish injected with PBS, when compared to the control leucocytes (no bacteria) and with leucocytes incubated with the bacteria without pre-incubation with antiserum (Fig. 4.8 B). There was significantly more ROS produced in HKL when QMA0285 was incubated with anti-285 antiserum compared to QMA0285 incubated with antiserum from fish injected with PBS (Fig. 4.8 B). Oxidative burst activity was lower in HKL in response to QMA355 opsonised with antibodies from antiserum (QMA0285) compared to QMA0355 incubated with PBS antiserum (Fig. 4.8 B).

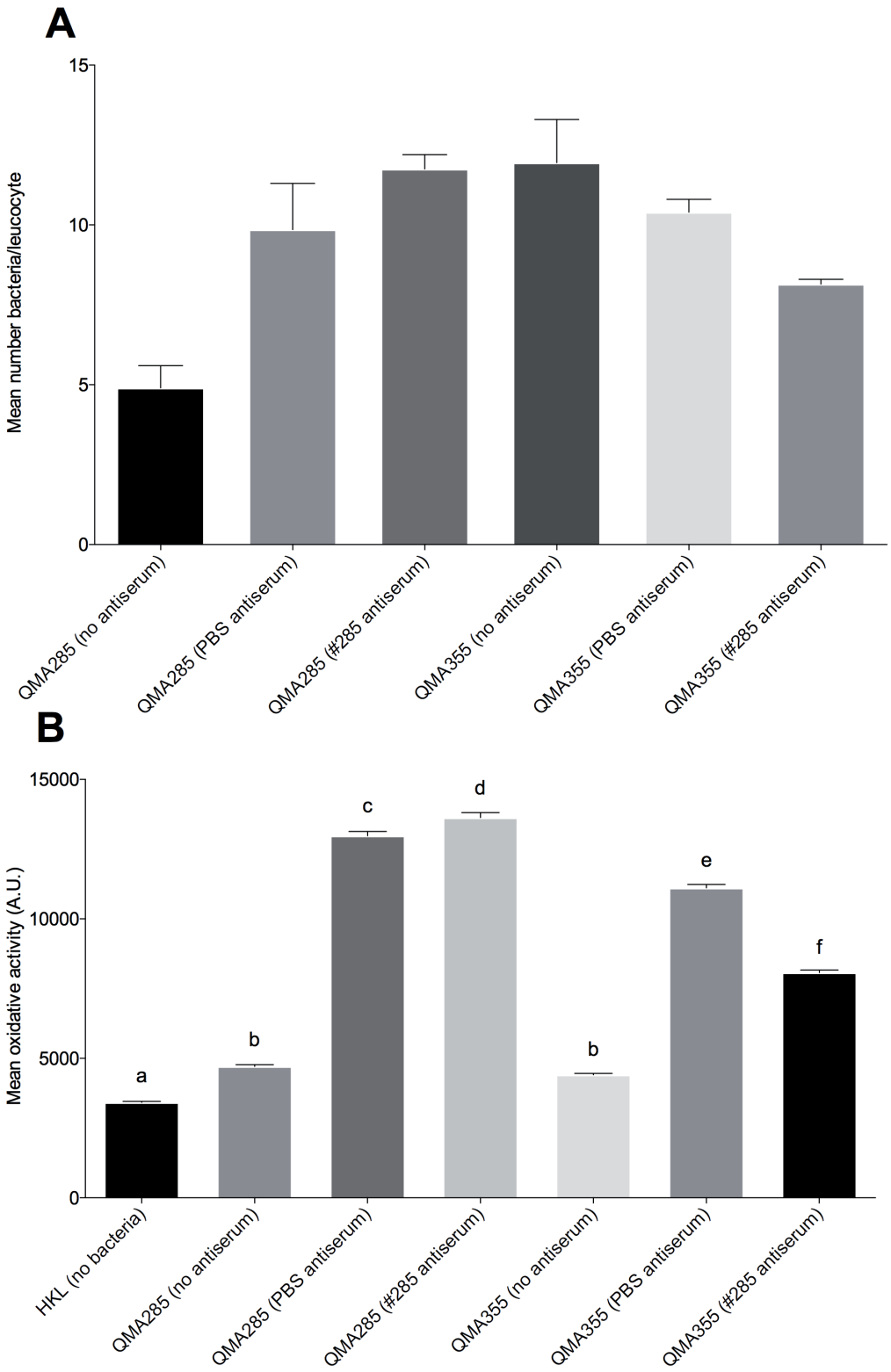

Figure 4.8. Effect of pre-incubation of live QMA0285 (grouper 2) and QMA0355 (Human foot) with antiserum from vaccinated fish (QMA0285) and fish injected with PBS on the mean number of attached or phagocytised bacteria per leucocyte (A) and the mean oxidative activity (A.U.) of Queensland grouper pronephros leucocytes (B). PBS, antiserum from fish injected with PBS; #285, antiserum from fish injected with a formalin-killed vaccine based on strain QMA0285. Mean values (± SEM) are expressed in flow cytometry arbitrary units (A.U) of replicate assays from leucocytes from two individual fish. Different letters represent statistically significant difference between the different treatments and strains ($P < 0.05$, one-way ANOVA).

4.4 DISCUSSION

While virulence of a piscine GBS strain has been demonstrated in *in vivo* infectivity challenges with captive *E. lanceolatus* (Delamare-Deboutteville *et al*., 2014), see Chapter 2, little is known about the ability of this pathogen to cross the interspecies barrier and allow human and other terrestrial isolates to infect other animals such as fish, or *vice versa*.

The objective of this study was to better understand why these strains of GBS are pathogenic in fish, using *in vitro* cellular immune assays with juvenile Queensland grouper head-kidney leucocytes in the presence of different piscine and terrestrial isolates as an *ex vivo* model.

Queensland grouper HKL

Flow cytometry analysis of Queensland grouper head-kidney derived leucocytes purified on Percoll gradients showed two clusters of cells. Cytospin preparations observed by microscopy confirmed the differences in morphology of these two populations. P1 had a lower SSC/FSC profiles and comprised of small lymphocytes, monocytes, thrombocytes and some macrophages. The cells in P2 population, on the other hand, were larger in size and more complex and consisted of more homogenous macrophages, neutrophils and other granulocytes. Similarly, two populations were found in the European sea bass (*Dicentrarchus labrax*) and common carp (*Cyprinus carpio*) peripheral blood leucocytes (Esteban *et al.*, 2000; Nakayasu *et al.*, 1998), both comprising of similar cell types to those identified in the current study. However, three sub-populations were found in leucocytes derived from the head-kidney of barramundi, *Lates calcarifer* (Tumbol *et al.*, 2009), dab, *Limanda limanda* (Skouras *et al.*, 2003) and Atlantic cod, *Gadus morhua* (Ronneseth *et al.*, 2007). These differences in number of clusters and cellular types will depend on the origin of the cells (head-kidney, peripheral blood, peritoneal cavity), but also on the method used to isolate the cells, the age of the animal and its physiological status.

Direct stimulation of respiratory burst in Queensland grouper HKL

In terms of HKL activation by different microbial stimulants, only PMA significantly activated HKL when compared to control, while no effects were measured with LPS and PTG. Similar observations have been reported in barramundi (*L. calcarifer*), Atlantic salmon (*Salmo salar*) and Atlantic cod (*G. morhua*) head-kidney cells (Kalgraff *et al.*, 2011; Tumbol *et al.*, 2009). PMA, which bypasses all normal cell surface receptors, triggers leucocytes respiratory burst activity directly at the intracellular side of the membrane through MAP kinase/protein kinase C activated signal transduction (Kudrenko *et al.*, 2009). In this study PMA was used as a positive control to confirm that these cells could indeed activate a respiratory activity, but it is not a relevant route of activation in terms of determining how these cells interact with putative pathogens.

LPS is an endotoxin, forming part of the outer membrane of Gram-negative bacteria, whose hydrophobic lipid section can induce a strong immune response in normal teleostean leucocytes (Neumann *et al*., 1998; Sarmento *et al*., 2004a; Solem *et al*., 1995). The molecular mechanism by

which LPS stimulates an immune response in fish has been reviewed by (Forlenza *et al.*, 2011). In mammals, two essential accessory proteins mediate the transfer of LPS to its toll-like receptor TLR4 on the macrophage surface: CD14 and LPS-binding protein (LBP) (Mathison *et al.*, 1992; Pugin *et al.*, 1995; Tobias *et al.*, 1995). Genome analyses of many fish species, including Japanese pufferfish (*Takifugu rubripes*) and Spotted green pufferfish (*Tetraodon nigroviridis*) have revealed the absence of a TLR4 homologue and several receptor molecules (LBP, CD14) essential in the recognition of LPS (Iliev *et al.*, 2005; Palti, 2011; Rebl *et al.*, 2010). Whether Queensland grouper possess TLR4 receptor complex or not, the present study shows that HKL were insensitive to LPS at high concentration up to 100 μg mL^{-1}, which suggests the absence of LPS membrane receptors or other factors that are not present in the medium. On the other hand, head-kidney leucocytes of Goldfish, *Carassius auratus* (Neumann *et al.*, 1998), Atlantic salmon, *S. salar* (Solem *et al.*, 1995) and European sea bass, *D. labrax* (Sarmento *et al.*, 2004a), have been shown to up-regulate ROS production upon stimulation with LPS. Altogether, these results clearly show that not all fish respond the same to LPS and that fish-specific ligand recognition by TLRs needs to be deciphered.

The cell wall of Gram-positive bacteria is largely made up of peptidoglycan, a complex carbohydrate polymer that confers rigidity to the cell wall. In mammalian vertebrates, recognition of PTG is mediated by TLR2 (Aliprantis *et al.*, 2000; Schwandner *et al.*, 1999; Takeda *et al.*, 2002). PTG purified from *Staphylococcus aureus* has been shown to elicit the activation of macrophages through carp TLR2 (Ribeiro *et al.*, 2010). TLR1 and 2 have been recently cloned and expressed from Orange-spotted grouper, *Epinephelus coioides* (Wei *et al.*, 2011). In this study the unresponsiveness of *E. lanceolatus* HKL to PTG suggests that sequence homology of TLR2 does not necessarily mean function conservation. In *in vivo* experiments, where PTG was administrated orally or by injection to Bastard halibut, *Paralichthys olivaceus*, it was demonstrated that immune functions such as phagocytic activity, production of ROS, and resistance to injection challenge with *Vibrio anguillarum* were enhanced (Kono *et al.*, 2003; Kono and Sakai, 2001; Zhou *et al.*, 2006). This might suggest that a combination of factors is necessary for PTG to activate macrophages and granulocytes in this fish species.

Other PAMPs such as poly(I:C) and zymozan failed to increase phagocytosis and microbicidal activity in acidophilic granulocytes from *S. aurata* (Sepulcre *et al.*, 2007a). These reports clearly underline the diversity of PAMPs recognition and activation of phagocytic cells in different species of teleost.

Multiplicity of infection effect

In this study when leucocytes were incubated with bacteria at a MOI of 10 the oxidative activity was significantly reduced. Observations by microscopy showed overgrowth of the bacteria and lysis of the cells with terrestrial isolates (data not shown). Boesen *et al.*, (2001) attributed the lysis of macrophages at high MOIs to a toxic effect of the bacteria. Similar results were observed with *Aeromonas salmonicida* on macrophages of Atlantic salmon (Olivier *et al.*, 1992) and *Lactococcus garvieae* with rainbow trout, *Oncorhynchus mykiss* macrophages (Barnes *et al.*, 2002).

Phagocytosis and downstream cellular antimicrobial response of Queensland grouper HKL to GBS

In this study, while both P1 and P2 had phagocytic and radical production capabilities, most of the "heavy lifting" was performed by P2, which contributed to 90% of the metabolic activity and ingested significantly more bacteria than P1. Previous studies have reported that mixed sub-populations of *C. auratus* primary kidney macrophages were differentially phagocytic depending on their stage of maturation (Grayfer *et al.*, 2008; Neumann *et al.*, 2000; Rieger *et al.*, 2010). Flow cytometry and electron microscopy showed that large macrophages and acidophilic granulocytes from *S. aurata* head-kidney were actively ingesting *V. anguillarum* (Esteban *et al.*, 1998).

In Queensland grouper, P2 was mainly composed of mature macrophages and granulocytes, whereas cells in P1 were smaller monocytes, lymphocytes and thrombocytes. Monocytes are precursor cells with moderate powers of phagocytosis until they develop into mature macrophages once they enter the circulatory system and tissues (Ellis, 1977). Mature lymphocytes are non-phagocytic cells capable to respond to only a small number of specific antigens that will trigger a proliferative phase (Ellis, 1977). Thrombocytes are nucleated cells in fish and are the morphofunctional equivalents to mammalian platelets originating from a multipotent myelo-erythroid progenitor (Rombout *et al.*, 2005). Beyond their primary function in homeostasis, it is uncertain if thrombocytes play an immunological role. However, both phagocytic and bactericidal activity have been attributed to *C. carpio* thrombocytes (Stosik *et al.*, 2002), while others suggest an interaction of thrombocytes with peripheral macrophages, erythrocytes, polymorphonuclear cells and lymphocytes in a case of trouts infected with *Candida albicans* (Passantino *et al.*, 2005). These reports in other fish species, as well as the nature of these leucocytes support the different oxidative activity observed in P1 and P2 in response to marine and terrestrial GBS isolates.

All piscines isolates from different fish hosts were capable of triggering an oxidative burst in both P1 and P2 populations, which support that these bacteria are recognized in a specific manner. Recognition of pathogens is mediated through a number of receptors: the aforementioned TLRs, but also macrophage mannose receptor and other scavenger receptors. These receptors have been previously described in teleosts leucocytes and reported to be involved in phagocytosis of Gram-positive and Gram-negative bacteria (Frøystad *et al.*, 1998; Krieger and Herz, 1994; Meng *et al.*, 2012). In Queensland grouper, it is likely that the activation of these cells by GBS is mediated through homologous receptors.

The highest phagocytic rate was observed in P2 when leucocytes were incubated with GBS isolate from grouper 5, which has a large portion of its operon missing (see Chapter 3). While the presence of a capsule in this isolate needs to be confirmed, previous studies have shown that it can greatly impair phagocytic clearance by fish leucocytes and is known as a major virulence factor in pathogenic streptococci (Källman *et al.*, 1998; Locke *et al.*, 2007; Martin *et al.*, 1992).

While, the GBS isolates in this study were genetically diverse and from different host origins as evidenced by different CPS sequences and MLST sequence type (see Chapter 3), two of the terrestrial

isolates, cat (QMA0303) and dog (QMA0300), both serotype V, significantly activated Queensland grouper macrophages in a similar way to some of the marine isolates. These marine and terrestrial isolates probably share common antigens on their surfaces that interact with Queensland grouper leucocytes in a similar fashion.

The highest mortality increase in both P1 and P2 lineages was observed when Queensland grouper leucocytes were incubated with the dog GBS isolate, which suggest that this terrestrial strain may possess specific factors that promote host-cell death, such as toxins member of the *cyl* locus (β-hemolysin/cytolysin) (Los *et al.*, 2013). These toxins are no longer expressed in our piscine isolates as attested by the absence of haemolysis on sheep blood agar. The underlying reasons for this general tendency of the terrestrial isolates to cause higher mortality in P1 and P2 compared to the piscine isolates are still unclear. However, recent studies have shown that some of the major virulence factors found in human and bovine isolates are absent from the ST260–261 GBS piscine lineages (Delannoy *et al.*, 2013; Pereira *et al.*, 2013b; Rosinski-Chupin *et al.*, 2013). This needs to be confirmed in our collection of Australian ST-261 piscine isolates that have been used in this study.

Surprisingly the non-significant production of ROS by P2 in the presence of human, crocodile and bovine GBS strains coincided with high phagocytic index, suggesting that these bacteria possess virulence factors specialised in immune evasion, such as superoxide dismutase (SodA) and other protein members of the *cyl* locus that can detoxify single oxygen and superoxide produced by the host (Poyart *et al.*, 2001; Rajagopal, 2009). In fish, similar inhibition of the leucocyte respiratory burst and apoptosis have been described in *D. labrax* in the presence of *V. anguillarum* (Sepulcre *et al.*, 2007b), which is probably used as a strategy for the pathogen to survive inside the cells and disseminate to other organs and tissues. In view of these findings, the role of grouper immunoglobulin on the phagocytosis and respiratory activity of Queensland grouper HKL was evaluated in an opsonophagocytosis assay.

Antigen exposure and opsonophagocytosis

Antibodies produced against vaccine (QM0A285) cross-reacted with all the marine isolates, suggesting that these strains have common antigens on their surfaces and this is in keeping with the determined molecular serotype (Ib) of all of these marine isolates. In contrast, Queensland grouper antibodies against QMA0285 did not cross-react strongly with the terrestrial isolates of serotypes Ia, III and V. Indeed, a negative ΔMFI was detected for two isolates, QMA0355 (crocodile, serotype Ia) and QMA0306 (cow, serotype III). Previous studies have shown that streptococci are capable of binding immunoglobulin by a non-immune mechanism. Many strains of group A and B streptococci express surface proteins such as M and M-like proteins that can bind human IgA and IgG antibodies via their Fc region (Bessen, 1994; Lindahl *et al.*, 1990; Nordenfelt *et al.*, 2012; Russell-Jones *et al.*, 1984). It is possible that non-immune binding of Queensland grouper IgM by the Fc region prevented recognition by the secondary antibody, but blocked any non-immune binding of the tertiary conjugate and therefore resulted in less fluorescence than the primary antibody free control.

Opsonisation of pathogens by specific antibodies is key to a coordinated response between the cellular

innate and adaptive immune systems. Immune cells use antibodies to differentiate "self" from "non-self" via recognition receptors, leading to immune tolerance and pathogen clearance respectively. However, while opsonisation of QMA0285 and QMA0355 with antibodies raised against QMA0285 (vaccine) significantly increased the ROS productions, there was a decrease in the number of bacteria being phagocytised with the strain QMA0355, which supports a potential binding of IgM by the Fc region, making the antigen-antibodies complex invisible for phagocytosis. Indeed, if the pathogen bound the Fc region of the antibody, the antigens at the pathogen's surface would be dissimulated by the antibody, making the bacterium appears as a "self" cell and thus evades detection from the immune cells. This potential evasion mechanism could have severe consequences, as wild fish populations would not be able to recognize terrestrial pathogens. This is of particular concern when considering agricultural runoffs, dredging activities and the increasing number of urban development projects along the coastlines of Australia, which will all have an impact on the aquatic environment, and could potentially result in the release of terrestrial animal and human GBS isolates into water streams.

In this study the relatively high level of oxidative activity in unstimulated control HKL could be caused by excessive handling of the tissue, and stress of the cells or maybe due to the fact that assays were performed the day after HKL isolation. These results are in accordance with previous studies in European sea bass (*D. labrax*) and barramundi (*L. calcarifer*) macrophages (Sarmento *et al.*, 2004b; Tumbol *et al.*, 2009).

4.5 CONCLUSIONS

In conclusion, the results of these *in vitro* experiments clearly demonstrate the potential for terrestrial animal or human isolates to invade Queensland grouper leucocytes, quench their radical oxygen production and cause a significant increase in cell mortality. There is an increasing body of evidence that describes the presence and virulence of human and bovine GBS isolates in teleosts (Delannoy *et al.*, 2013; Evans *et al.*, 2008; Evans *et al.*, 2009; Liu *et al.*, 2013a; Pereira *et al.*, 2010; Suanyuk *et al.*, 2008).

In future studies on host pathogen interactions, controlled experiments should investigate the susceptibility of *E. lanceolatus* to non-piscine GBS isolates to confirm the premise that regardless of its genetic diversity, GBS can cross interspecies barriers and established itself within fish populations. *Vice versa*, it would be interesting to test human leucocytes, particularly neutrophils and monocytes to determine their cellular responses to piscine and human isolates. This could have major impacts on local seafood industries and consumer health as this pathogen could be transmitted to human via fish products consumption.

Chapter 5

Genetic and phenotypic characterisation of Australian piscine (ST-261) and terrestrial isolates of *Streptococcus agalactiae*. What can the genome tell us about host specialisation and adaption?

ABSTRACT

Whole-genome sequencing analysis revealed a significant genome reduction in the piscine GBS isolates compared to the human and terrestrial strains. This genetic erosion was characterised by the loss of transport, transcription and metabolic functions. Phenotypically, while piscine and terrestrial isolates shared the same enzymatic hydrolysis profile, their ability to ferment carbohydrates differed greatly. Indeed, in many of the marine isolates, we observed reduced metabolic capacities, half of them lost their ability to ferment ribose, 94% lost trehalose, and 81% could not ferment amidon. Only one piscine isolate from Queensland grouper was able to ferment trehalose and glycogen. Intriguingly, three grouper and the bovine isolates were able to ferment lactose. Arginine dihydrolase activity was conserved across all the marine and terrestrial isolates. Genomic mutations observed in the capsular polysaccharide were phenotypically relevant. The loss of critical genes in the regulatory portion of the operon (*cpsB*, *cpsC*, *cpsD*, *cpsE*) or a frameshift in the glycosyltransferase *cpsE* were sufficient to cause the cessation of the biosynthesis of capsular polysaccharide, as evidenced by the lost of cell buoyancy. Marine GBS isolates grew more slowly than terrestrial isolates in rich medium, but this difference, though still significant, was less pronounced at lower temperature. Most virulence factors found in mammalian isolates (toxins, adhesins, immunoreactive antigens, C5a peptidase etc.) were missing from fish strains. However, they have retained critical genes such as the serine-rich repeat (srr) protein, the fibrinogen-binding protein A (fbsA) and a set of adhesins. As the symptoms of GBS include rapid onset of meningitis, these conserved virulence factors are likely to be involved in the pathogenesis of GBS in fish and may provide clues as to how GBS is capable of colonising the central nervous system in both fish and mammals. This study provided intriguing insight into the evolution and adaption of GBS to fish and life in the aquatic environment.

5.1 INTRODUCTION

GBS is a versatile pathogen causing septicaemia and meningitis in neonates, elderly and immunocompromised human hosts (Dermer *et al.*, 2004; Skov Sorensen *et al.*, 2010). It is also a pathogen of ruminants causing mastitis in cattle and camels (Estuningsih *et al.*, 2002; Kinne and Wernery, 2002; Wernery *et al.*, 2008; Younan and Bornstein, 2007), and also causes disease in cold-blooded animals, including crocodile, frogs and lizards (Amborski *et al.*, 1983; Bishop *et al.*, 2007; Hetzel *et al.*, 2003). Since the 1970s it has become an emerging fish pathogen, found in many countries both in the wild and intensively reared fish aquaculture farms (Eldar *et al.*, 1994; Ye *et al.*, 2011; Zamri-Saad *et al.*, 2010).

The advent of the latest next generation sequencing technologies coupled with rapid development of bioinformatics tools have significantly accelerated our understanding of the mechanism of bacterial pathogenesis (Croucher *et al.*, 2013; Forde and O'Toole, 2013; Walker and Beatson, 2012). These techniques are being increasingly used in human epidemiology, and have revealed their power to pinpoint the origin of outbreaks in hospitals, local communities or trace the evolutionary history of a pathogen on a global scale (Cheung and Kwan, 2012; Maamary *et al.*, 2012; Sherry *et al.*, 2013). These technologies are now being used to study microbial ecology outside the human clinical field in highly complex systems such as the marine environments (Kisand *et al.*, 2012; N'Guessan *et al.*, 2012; Wood *et al.*, 2013).

Genome analysis of the major serotypes of GBS causing diseases showed that GBS can be defined by its "pan genome" that includes a "core genome" representing genes present in all GBS strains and the "dispensable genome" comprised of genes only present in a few strains (Tettelin *et al.*, 2005). Comparative genomics of multiple strains of GBS originated from different host species can give great insights into the diversity of the species and how it evolves under selection in the different environments, hosts and their immune systems.

A recent comparative genomic study describes the emergence of an independent lineage of GBS belonging to sequence type ST260–261 that has specialised to the fish host (Rosinski-Chupin *et al.*, 2013). This adaption was correlated with a massive gene inactivation and changes in gene expression with a number of metabolic functions and virulence factors being affected (Rosinski-Chupin *et al.*, 2013).

Since 2007, GBS has emerged infecting wild fish in Australia for the first time (Bowater *et al.*, 2012). Fish mortalities were reported in Queensland grouper (*Epinehelus lanceolatus*) and a number of other wild species including mullet, Javelin grunter and stingrays (Bowater *et al.*, 2012). Multilocus sequence typing and molecular genotyping revealed that all piscine GBS isolates from Australia fell into ST-261 type and were serotype Ib (see Chapter 3). A GBS strain isolated from a wild Queensland grouper was extremely virulent in aquarium challenge models (Delamare-Debouttevillle *et al.*, 2014); indeed 10^3 cfu was sufficient to cause 100% mortality within four days in replicate 1 (see Chapter 2).

Whole-genomes of 16 of the Australian marine isolates of *S. agalactiae* were sequenced and revealed a distinct reductive evolution pattern compared to human and terrestrial isolates (Ben Zakour *et al.*, in preparation). This genome reduction was characterised by a high number of deletions and pseudogenes all indicative of a long-term specialisation and adaption with their host (Ben Zakour *et al.*, in preparation).

Like most other pathogenic bacteria, GBS encodes a repertoire of virulence factors that are indispensable for its aptitude to cause disease (Lin *et al.*, 2011; Rajagopal, 2009). These virulence factors include a number of cell surface proteins that promote adherence, invasion and dissemination within host cells and tissues (Rajagopal, 2009). Others factors promote resistance to antimicrobial peptides or facilitate immune evasion by detoxifying oxygen radicals, preventing recognition of GBS through molecular mimicry of host-cell surface glycoconjugates, or by preventing neutrophil recruitment by cleaving complement C5a during GBS infection (Rajagopal, 2009).

In the current study, we aim to compare Australian marine and terrestrial isolates phenotypically including metabolic activity, growth and interaction with host immunity (see Chapter 3) and determine whether detected functional differences can be explained by changes identified in the genomes. In so doing, we will provide functional evidence to support the contention that marine isolates of GBS have become specially adapted to their environment and host.

5.2 MATERIAL AND METHODS

5.2.1 Strain collection.

A total of 23 isolates from different sources were used in this study, including 16 fish isolates all ST-261, serotype Ib, originated from infections in Queensland grouper (*Epinephelus lanceolatus*), Javelin grunter (*Pomadasys kaakan*), Giant catfish (*Netuma thalassina*), Squaretail mullet (*Ellochelon vaigiensis*), Estuary ray (*Dasyatis fluviorum*), Mangrove whipray (*Himantura granulata*) and Eastern shovelnose ray (*Aptychotrema rostrata*) were kindly provided by Dr Rachel Bowater from the Tropical Aquatic Animal Health Laboratory (TAAHL), QLD, Australia. One isolate from a case of necrotizing fasciitis in captive juvenile saltwater crocodiles (*Crocodylus porosus*) from Darwin (ST-23, serotype Ia) was kindly provided by Dr Cathy Shilton from Berrimah Veterinary Laboratories, NT, Australia. One reference strain from a case of neonate meningitis (COH1) from the USA (ST-17, serotype III) was obtained from the ATCC. Two ST-23, serotype Ia clinical isolates were provided by Townsville hospitals. One isolate from cattle (ST-67, serotype III), one from cat and one from dog (ST-1, serotype V) were provided by the UQ Gatton School of Veterinary Science.

5.2.2 Identification and characterisation of GBS strains.

Isolates were grown routinely on 5% sheep blood agar (Oxoid) as described previously. Biochemical tests, including a series of hydrolysis tests such as the VP, Voges-Proskauer reaction; HIP, hippuric acid; ESC, esculin ferric citrate; PYRA, pyrrolidonyl arylamidase; αGAL, α-galactosidase; βGUR, β-giucuronidase; β-GAL, β-galactosidase; PAL, alkaline phosphatase; LAP, leucine aminopeptidase; and fermentation of carbohydrates including: ADH, arginine dihydrolase; RIB, ribose; ARA, arabinose; MAN, mannitol; SOR, sorbitol; LAC, lactose; TRE, trehalose; INU, inulin; RAF, raffinose; AMD, amidon; GLYG, glycogen, were derived from the API 20 Strep Multi-test system (bioMérieux), following the manufacturer's instructions. β-haemolysis was recorded directly from bacteria grown on sheep blood agar. Serotype and sequence type (ST) were obtained from capsular genotyping and multilocus sequence typing respectively (see Chapter 3).

5.2.3 Identification of species-specific 23S rRNA and universal 16S rRNA genes.

High molecular weight genomic bacterial DNA (gDNA) was extracted using a genomic DNA commercial kit (Genomic-tip 20/G, Qiagen) according to the manufacturer's instructions. GBS identity was confirmed by 1/ amplification of the 16S rRNA gene using universal primers, 29F 5'-AGAGTTTGATCCTGGCTCAG-3' (forward) and 1492R 5'-GGTTACCTTGTTACGACT-3' (reverse), 2/ amplification of the *S. agalactiae* 23S rRNA gene using specific AgaF/AdyR primer set as described by (Kawata *et al.*, 2004). The primer sequences were AgaF 5'-AACAGCCTCGTATTTAAAATGATAGATTAAC-3' (forward) and AdyR 5'-TCCTACCATGACACTAATGTGTC-3' (reverse). The PCR products were analysed by 1% agarose gel electrophoresis, stained with ACTGene DGel Hydra Green (Labpro Scientific) and visualised under a UV transilluminator UVP (BioDoc-it Image system). PCR products were cleaned up for

sequencing using Exosapit (Roche Diagnostics), following the manufacturer's instructions.

5.2.4 Effect of temperature on the growth of GBS

Bacteria were grown first on sheep blood agar, then one colony of each GBS isolate was inoculated into Todd Hewitt Broth (THB) at 28°C for 24 hr. All bacteria starter cultures were fixed at an optical density at 600 nm (OD_{600}) of 1. From these standardised starter cultures, each strain in triplicate was inoculated into THB at a final concentration of 2% (v/v) in 48 well tissue culture plates (Krystal Greiner BioOne). Bacterial growth (OD_{595}) was recorded in a FluoStar Optima plate reader (BMG Labtech, Melbourne, Australia) every 60 min for 24 hr with orbital agitation before each reading. The temperature was set either at 18°C, 28°C or 37°C. Each assay was repeated at least twice.

5.2.5 Virulence genes comparative study by genomic sequence analysis

The virulence factors screening was performed and visualised using a BLAST Ring Image Generator (BRIG) by comparing the assembled genomes of all piscine (n = 16) and terrestrial isolates (n = 7) of this study with 18 other assembled genomes including human, bovine, camel and frog isolates against a list of 52 *S. agalactiae* specific virulence factors sequences collected from the Virulence Factors DataBase (VFDB) complemented by six additional sequences identified in the newly sequenced piscine strains of *S. agalactiae* (ST-6, ST-7, ST-553, ST-260). The comparisons using BRIG was performed as described previously (Alikhan *et al.*, 2011).

5.2.6 Buoyant density assays

Based on the single nucleotide polymorphisms (SNPs) and insertions in the capsular polysaccharide (CPS) synthesis gene sequences (see Chapter 3, Fig. 3.2), a set of isolates representative of the different clusters was selected and a buoyant density assay was determined in a continuous Percoll gradients in order to estimate the quantity of CPS expressed by the different strains as described previously (Locke *et al.*, 2007; Millard *et al.*, 2012). Briefly, mid-exponential-phase THB cultures of each strain were washed once in PBS and resuspended to an optical density at 600 nm (OD_{600}) of 2, and a 0.5 mL aliquot was layered onto standard isotonic Percoll gradients. All gradients were centrifuged at 4 000 x g for 60 min at 4°C with no brake (Eppendorf 5518E). The experiment was repeated using new cultures at least twice.

5.3 RESULTS

Identification of strains as GBS

All isolates in this study were catalase-negative, Gram-positive cocci, appearing in pairs and chains. The marine strains were similar in that they were all non-β-haemolytic, whereas the crocodile, humans, bovine, dog and cat were strongly β-haemolytic. Both the universal 16S and specific 23S rRNA gene fragments were amplified from genomic bacterial DNA of all isolates and GBS identity was confirmed by Sanger sequencing.

Changes in cps genotype are phenotypically relevant

As described elsewhere (see Chapter 3), the piscine and terrestrial isolates were organised in clusters according to SNPs and insertions in their *cps* genes. Only one representative of each cluster is shown on the Percoll buoyant density gradients (Fig. 5.1), as the buoyant density of other strains within the same cluster were very similar. Clusters represented by isolate from grouper 5 (QMA0281) and grouper 6 (QMA0368) travelled all the way through the continuous Percoll gradients compared to strains from grouper 1 (QMA0284) and Eastern shovelnose ray (QMA0275) both of which produced a capsule evidenced by greater buoyancy (Fig. 5.1). The crocodile (QMA0336) and human COH1 (QMA0370) isolates of serotype Ia and III respectively had different buoyant density profiles with the crocodile producing more capsule (Fig. 5.1). The bovine isolate (QMA0306) serotype III, dog (QMA0300) and cat (QM0303) both serotype V have subtle differences in their buoyancy (Fig. 5.1).

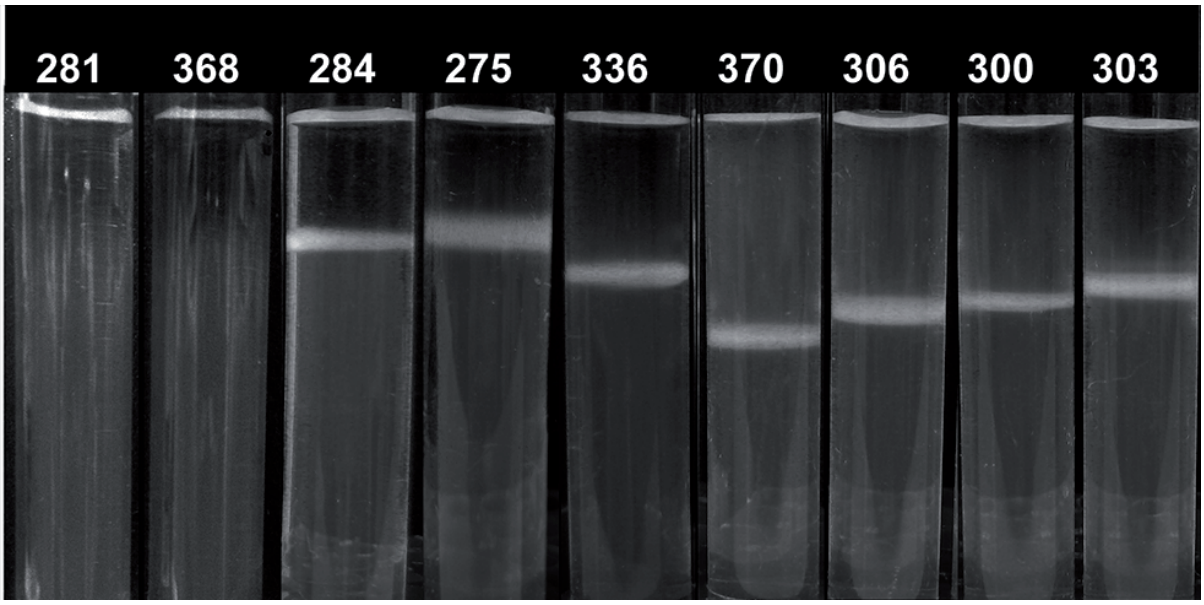

Figure 5.1. Percoll buoyant density assay for grouper 5 (QMA0281), grouper 6 (QMA0368), grouper 1 (QMA0284), Eastern shovelnose ray (QMA0275), crocodile (QMA0336), human COH1 (QMA0370), cow (QMA0306), dog (QMA0300), and cat (QMA0303) isolates.

Carbohydrate utilisation differs substantially between marine and terrestrial isolates.

All the piscine isolates shared the same enzymatic hydrolysis profile with the terrestrial strains (Table 5.1). All strains were positive for the Voges-Proskauer reaction, hippuric acid, β-giucuronidase, alkaline phosphatase, leucine aminopeptidase, negative for esculin ferric citrate, pyrrolidonyl arylamidase, α-galactosidase, β-galactosidase. On the other hand the fermentation of carbohydrates by the marine isolates was quite different to the terrestrial profile. Indeed, only the isolate from grouper 4 (QMA0280) fermented trehalose and glycogen, while the other piscine isolates were unable to ferment these sources of carbon. All the terrestrial isolates had the same phenotypic profile fermenting trehalose and amidon with the exception of the cattle isolate (QMA0306), which was also capable of fermenting lactose. Amongst the piscine isolates only three of the grouper isolates (QMA0285, 267, 280) were able to ferment amidon and lactose. About half of the marine isolates were able to ferment ribose, in common with the terrestrial isolates.

Three isolates from groupers (QMA0281, 368, 369), two from Javelin grunters (QMA0268, 287) and all the ray isolates (QMA0275, 276, 277, 320) were incapable of fermenting ribose. Arginine dihydrolase activity was conserved across all the marine and terrestrial isolates.

Table 5.1. Piscine, crocodile, human, bovine, dog and cat GBS phenotypic properties

VP, Voges-Proskauer reaction; HIP, hippuric acid; ESC, esculin ferric citrate; PYRA, pyrrolidonyl arylamidase; αGAL, α-galactosidase; βGUR, β-giucuronidase ; β-GAL, β-galactosidase; PAL, alkaline phosphatase; LAP, leucine aminopeptidase; ADH, arginine dihydrolase; RIB, ribose; ARA, arabinose; MAN, mannitol; SOR, sorbitol; LAC, lactose; TRE, trehalose; INU, inulin; RAF, raffinose; AMD, amidon; GLYG, glycogen; +, positive reaction; - negative reaction; NT, non-typable. Shaded areas denote differences between isolates.

Isolates	Serotype	ST	CC	VP*	HIP*	ESC*	PYRA*	αGAL*	βGUR*	βGAL*	PAL*	LAP*	ADH†	RIB†	ARA	MAN†	SOR†	LAC†	TRE†	INU†	RAF†	AMD†	GLYG†	β HEM†
Fish																								
Grouper 1 (QMA0284)	Ib	261		+	+	-	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Grouper 2 (QMA0285)	Ib	261		+	+	-	-	-	+	-	+	+	+	+	-	-	-	+	-	-	-	+	-	-
Grouper 3 (QMA0267)	Ib	261		+	+	-	-	-	+	-	+	+	+	+	-	-	-	+	-	-	-	+	-	-
Grouper 4 (QMA0280)	Ib	261		+	+	-	-	-	+	-	+	+	+	+	-	-	-	+	+	-	-	+	+	-
Grouper 5 (QMA0281)	Ib	261		+	+	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Grouper 6 (QMA0368)	Ib	261§		+	+	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Grouper 7 (QMA0369)	Ib	261		+	+	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Javelin grunter (QMA0268)	Ib	261		+	+	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Squaretail mullet (QMA0274)	Ib	261		+	+	-	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Giant catfish (QMA0271)	Ib	261		+	+	-	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
E.S ray 1 (QMA0275)	Ib	261		+	+	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Crocodile																								
Crocodile (QMA0336)	Ia	23	23	+	+	-	-	-	+	-	+	+	+	+	-	-	-	-	+	-	-	+	-	β
Human																								
COH1/REF (QMA0370)	III	17	17	+	+	-	-	-	+	-	+	+	+	+	-	-	-	-	+	-	-	+	-	β
Human foot (QMA0355)	Ia	23	23	+	+	-	-	-	+	-	+	+	+	+	-	-	-	-	+	-	-	+	-	β
Bovine																								
Cow (QMA0306)	III	67	67	+	+	-	-	-	+	-	+	+	+	+	-	-	-	+	+	-	-	+	-	β
Dog																								
Dog (QMA0300)	V	1	1	+	+	-	-	-	+	-	+	+	+	+	-	-	-	-	+	-	-	+	-	β
Cat																								
Cat (QMA0303)	V	1	1	+	+	-	-	-	+	-	+	+	+	+	-	-	-	-	+	-	-	+	-	β

Note: Only one isolate from Javelin grunter, catfish and ray is presented in Table 5.1, as the other strains from those species had identical phenotypic properties.

Marine isolates grow better at lower temperature

A growth curve experiment at two different temperatures (28°C and 37°C) highlights the difference of fitness between marine and terrestrial isolates in nutrient rich media (Fig. 5.2). Indeed, at both temperatures the terrestrial isolates reached late exponential phase faster (Fig. 5.2 A, B) compared to marine strains at the bottom (Fig. 5.2 A, B). Terrestrial strains performed better at 37°C as they reached stationary phase within 5–8 hr compared to 8–15 hr at 28°C. Interestingly, isolates from crocodile (QMA0336) and human foot (QMA0355) both serotype Ia grew to a higher OD compared to other terrestrial isolates at 37°C (Fig. 5.2 B). While growing generally more slowly in nutrient rich medium than terrestrial strains at both temperatures, marine strains seem to be better adapted at lower temperature 28°C (Fig. 5.2 A) compared to 37°C (Fig. 5.2 B). Indeed, grouper 5 (QMA0281) and grouper 6 (QMA0368) did not grow very well at 37°C and plateau at an optical density of 0.2 (Fig. 5.2 B). Growth at 18°C over a 24 hr period was generally low, with cell densities not exceeding OD < 0.2 and OD < 0.1 for terrestrial and marine isolates respectively (data not shown).

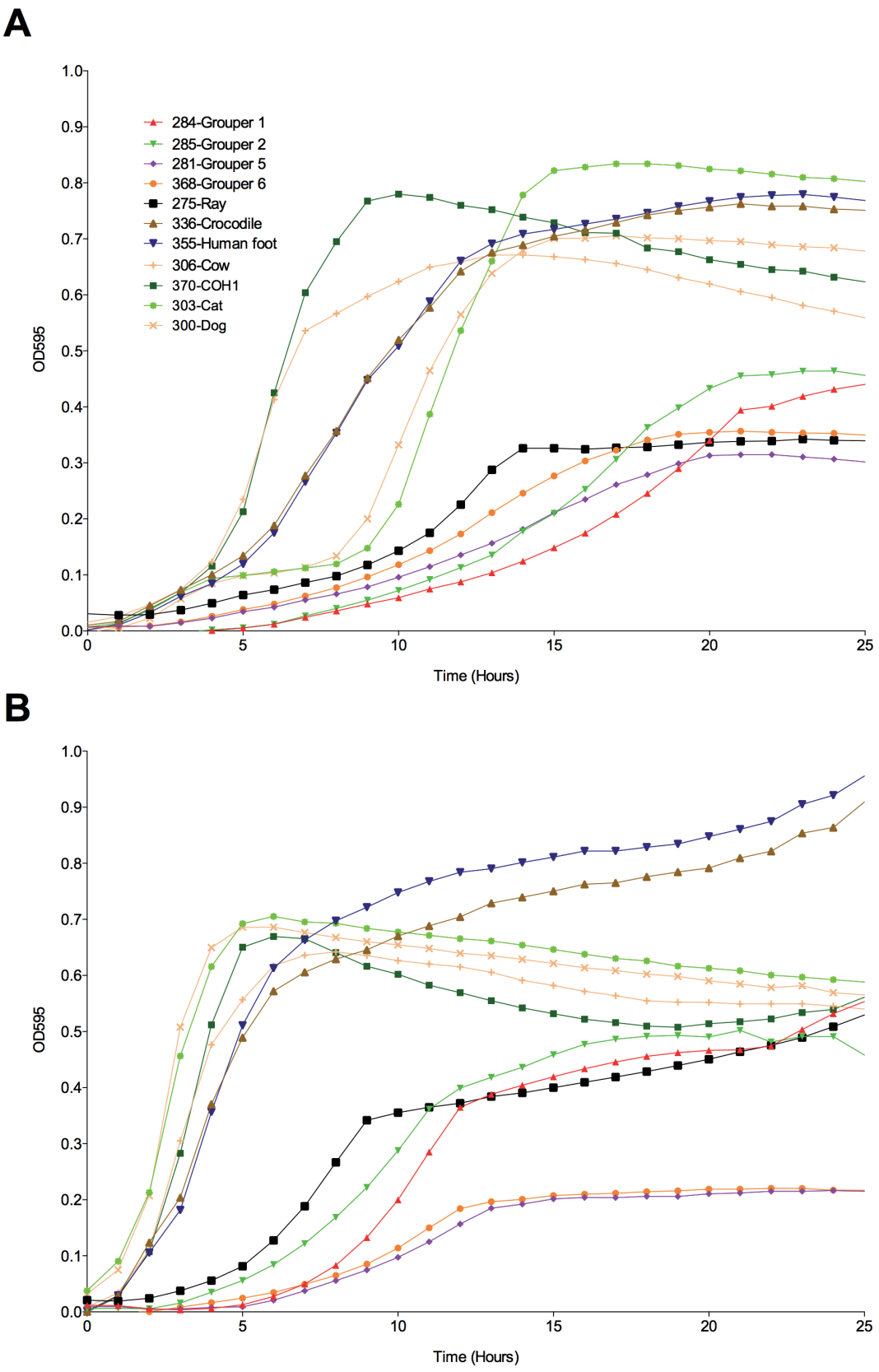

Figure 5.2. Growth curves of different marine and terrestrial GBS isolates grown at two different temperatures. A. 28°C and B. 37°C.

The virulence factor repertoire of the marine isolates is reduced

Approximately 60% of the genes encoding for proteins involved in pathogenesis and that are known virulence factors in mammalian clinical GBS isolates are missing from the piscine isolates. These include the β-hemolysin of the *cyl* locus, which is missing from the genome of the piscine isolates in this study (ST-261) and is in agreement with the absence of detectable haemolytic activity on sheep blood agar plates (Fig. 5.3). Many of genes encoding adhesin proteins implicated in cell-adherence and invasion are absent from the piscine isolates, this includes a number of hypotheical proteins, the components of the pilus (Pilus 1 and Pilus 2), fibrinogen binding protein A (FbsA) and B (FbsB) and the laminin-binding protein (lmb), all present multiple deletions in their genes respectively *fbsA*, *fbsB*, *lmb* (Fig. 5.3). Among the factors involved in immune evasion that are present in terrestrial isolates, the gene encoding for C5a peptidase (*scpB*) is greatly truncated in all ST-261. Most immunoreactive antigens such as the alpha-C protein (bca), the beta-c protein (cba), alpha-like protein (alp2) and rib have been lost in all ST-261 compared in this study.

Some critical virulence factors have been conserved

Some of the major virulence factors found in human and other terrestrial GBS isolates were conserved in the fish strains. The CAMP factor gene (*cfb*) and the fibronectin/fibrinogen binding protein of the PavA family (pavA) are intact in all marine isolates. Six adhesins specific to sequence type ST-261 lineage are represent on the left side of the BRIG (Fig. 5.3), including two serine-rich repeat proteins anchored to the peptidoglycan layers by a LPXTG C-terminal motif (srr), a surface protein from the amidase family, an immunogenic bacterial surface protein belonging to the BibA family (bibA) and two putative peptidoglycan linked protein (LPXTG motif). Finally the capsular polysaccharide which is a major factor for immune evasion was conserved in the piscine isolates. Indeed, ST-261 strains harbor the 16 genes involved in the type Ib capsule synthesis, with the exception of grouper 5 (QMA0281) that is missing part of *cpsB*, *E*, and *cpsC*, *cpsD*.

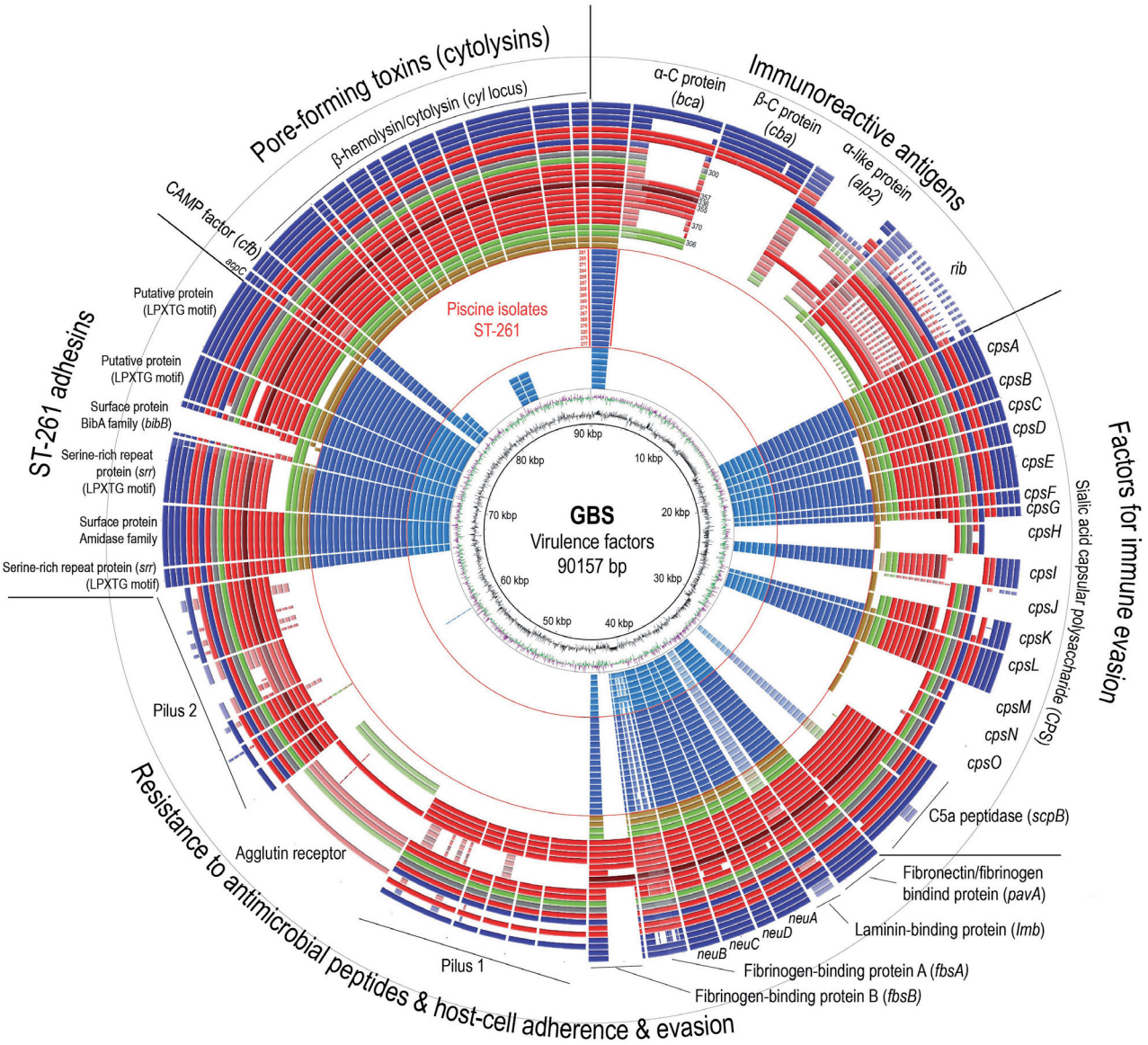

Figure 5.3. BRIG output image of *Streptococcus agalactiae* virulence factors. Figure 5.3 shows a comparison of the total virulence factors of 47 GBS isolates (the full list of the isolates is described in Appendix 2). The innermost rings show GC content (black) and GC skew (purple/green). The remaining rings show BLAST comparisons of the virulence factors of 47 GBS isolates, with the first seven innermost light blue rings showing piscine isolates from previous studies (ST-553, ST-260, ST-261), the next 16 dark blue rings between the two red circles are the piscine isolates from this study (ST-261), and the following 24 rings include terrestrial strains from this study and representative strains of the different sequence-types retrieved from the database. Labels around the outside of the circular image correspond to the GBS virulence factors summarized by their functions with the name of the genes. Gaps within each concentric ring indicate that part or the entire gene is absent for this particular strain. The image is scaled to the nucleotide length of the genes. Long tick marks on the inner circumference of the ring indicate increments of 10 kilobase-pairs (kbp) and short tick marks indicate 2 kbp. Figure produced using BLAST Ring Image Generator (BRIG) (Alikhan *et al.*, 2011).

5.4 DISCUSSION

Reduced metabolic capacities in the piscine isolates

Marine strains have reduced sugar catabolic capacities compared to terrestrial strains (as a consequence of genome erosion and disruption of many metabolic functions) (Ben Zakour *et al.*, in preparation). This is not surprising when considering the host background difference between warm-blooded animals and poikilotherms, but also the drastic changes between the aquatic and the terrestrial environments. Oceanic waters are nutrient-limited environments poor in carbon sources (Cavicchioli *et al.*, 2003). The major fraction of dissolved organic carbon (DOC) in the ocean is made up of carbohydrates (3–30%) (Pakulski and Benner, 1994) which play an important role in marine carbon cycle (Alldredge *et al.*, 1993; Engel *et al.*, 2004; Wang *et al.*, 2006; Wu and Yang, 2013). In primary producers, such as phytoplankton, carbohydrates comprise between 10–70% of the organic carbon (Malinsky-Rushansky and Legrand, 1996). In seawater, carbohydrates can have multiple sources, they can be released directly from organisms by biogenic input (Hama and Yanagi, 2001), originate from organic matter decomposition (Bertilsson *et al.*, 2005; Malinsky-Rushansky and Legrand, 1996), sediment resuspension (Arnosti and Holmer, 1999), or from terrigenous sources such as river runoff (Dittmar and Kattner, 2003; Guéguen *et al.*, 2006). It is still unknown, if GBS is capable of survival in its free-living form or attached to sediment, or if it is an obligate host pathogen. In Australia, GBS have only been isolated from wild tropical moribund or dead marine fish species (Bowater *et al.*, 2012), but has not been detected yet in water or sediment samples during estuarine and coastal environmental surveys. To extract their energy, these bacteria must be adapted to different nutrient sources characteristic of their microenvironments (Ayo *et al.*, 2001). With the exception of fish feeding at low trophic levels including herbivorous and omnivorous species, the majority of fish cannot use non-digestible polysaccharides efficiently because they lack adequate microflora for their digestion (Polakof *et al.*, 2012). While most fish can digest other carbohydrates including glucose or sucrose, carnivorous fish rely mainly on dietary proteins as their energy sources. The GBS piscine isolates in this study were collected from omnivorous and carnivorous fish feeding on a wide range of prey items including crustaceans, cephalopods, fish, molluscs, echinoderms, algae, bacteria, protozoans, detritus and particulate matter. If the bacteria are host specific they will have adapted metabolically to take advantage of the carbon source available from proteins or carbohydrates.

Three piscine isolates all from Queensland grouper were able to ferment lactose. Similar metabolic characteristic were reported from isolates infecting Klunzinger's mullet (*Liza klunzingeri*), Gilthead seabream (*Sparus aurata*) from Kuwait and Nile tilapia (*Oreochromis niloticus*) from Brazil (Evans *et al.*, 2008). While lactate metabolism has not been studied in this species of grouper, it is known to be an active predator that will experience periods of burst swimming activity likely to result in muscle and plasma lactate accumulation as reported for the Silver trevally, *Pseudocaranx dentex* (Wells and Baldwin, 2006). Increase serum lactate was recorded in Malabar grouper, *Epinephelus malabaricus*, following sudden salinity changes (Tsui *et al.*, 2012), and Queensland grouper (this study) are often found in costal water where they are subject to frequent and rapid salinity changes

following tropical and subtropical rainfall. Lactate metabolism has previously been shown to be variable in fish pathogenic streptococci. In a case of *Streptococcus iniae* infection in barramundi (*Lates calcarifer*), molecular epidemiology revealed a novel lactate oxidase, which was confined to a single farm in Northern Territory with extreme tidal flow (Nawawi *et al.*, 2009). The authors suggested an evolutionary advantage for these strains of *S. iniae* to have a more efficient enzyme under conditions where lactate concentration is high (Nawawi *et al.*, 2009). In common with *S. iniae* and other fish pathogenic steptococci, GBS are essentially systemic blood pathogens highly adapted to survive and proliferate within the circulatory system prior to colonisation of the central nervous system (CNS). Therefore, changes in blood physiology, including plasma lactate, are direct environmental cues. Further investigation of lactate metabolism in *S. agalactiae* is merited to determine potential drivers of the variability in the API test observed here.

In the present study, GBS isolates from three of the Queensland grouper, both Javelin grunter and all of the rays were unable to ferment ribose, trehalose, and amidon. Amidon or starch is a polysaccharide produced by most green plants as their energy source. The trehalose (disaccharide) can be found in plants, microorganisms, and invertebrates such as shrimp (Zhang *et al.*, 2012). It is implicated in anhydrobiosis, which is the ability of plants and animals to resist extended periods of desiccation (Cesari *et al.*, 2012; Marunde *et al.*, 2013; Thorat *et al.*, 2012). Ribose is a monosaccharide forming part of the backbone of RNA, it is related to deoxyribose, which is found in DNA, and some derivatives of riboses such as ATP and NADPH play central roles in metabolism. In *Escherichia coli* the molecular genetic basis for ribose catabolic function is under the control of the ribose operon (*rbs* genes) (Cooper *et al.*, 2001); after successive generation (~2000) of *E. coli* grown in glucose minimum medium, the authors identified *rbs*$^{-}$ mutants that had competitive advantage in environment with low concentration of glucose (Cooper *et al.*, 2001). Domelier *et al.*, (2006) showed that D-ribose was one of the four carbohydrates amongst 95 others that enable them to discriminate between high-risk and low risk strains of *S. agalactiae* associated with neonatal meningitis. While the genetic determinants for the loss of some catabolism functions in *S. agalactiae* still need to be confirmed, the cause for these metabolic shifts is certainly due to genetic disruptions and deletions that have improved the fitness of these organism to their microenvironments, either outside or inside the host, where utilisation of these carbohydrates may be unnecessary. Further work should investigate growth in low carbon medium with varying C:N ratios to determine whether the reduced repertoire of carbohydrate catabolic and metabolic genes confers improved fitness on marine isolates in an oligotrophic environment.

Variability in capsular polysaccharide

Like other virulent pathogens, GBS produces a capsule involved in its pathogenesis, survival and dissemination in the host (Cieslewicz *et al.*, 2001; Martins *et al.*, 2010). The capsular polysaccharide is a major GBS virulence factor conferring protection to the bacteria against an arsenal of non-specific cellular and humoral components. It is the main target of antibody-mediated killing, indeed phagocytosis is blocked by inhibition of host activated complement factor C3b that can no longer bind onto the surface of the bacteria, which prevent activation of the alternative pathway and opsonophagocytic

killing by macrophages and neutrophils (Lowe *et al.*, 2007; Marques *et al.*, 1992).

The general genetic organisation of the capsular operon across different species of streptococci is well conserved (Lowe *et al.*, 2007; Martins *et al.*, 2010). It has been suggested that the high selective pressure on pathogens caused by vaccination, host immunity and the environment may be driving forces resulting in the emergence of new serotypes (Millard *et al.*, 2012). The genetic changes in the *cps* genes generally lead to changes in polysaccharide biosynthesis (Sellin *et al.*, 1995; Sellin *et al.*, 2000).

In this study there was a high level of conservation amongst all the different terrestrial and marine isolates, with the region flanking the capsular locus *cpsA* to *cpsD*, *cpsF*-*cpsG*, *cpsL* and *neuB* to *neuA* being extremely well conserved, these results are in agreement with previous studies (Martins *et al.*, 2010; Morona *et al.*, 2004). The genetic mutations across the *cps* operon of the piscine isolates were extremely low and were restricted to a limited number of genes, *cpsB*, *cpsC*, *cpsD* and *cpsE.* One of the surprising finding was the significant 2 550 bp deletion encompassing part of *cpsB* and *cpsE* and the entire *cpsC* and *cpsD* locus of isolate QMA0281 from grouper 5, resulting in loss of the capsule as evidenced by the buoyant density results. Such serious deletion in the operon of QMA0281 is likely to represent a single independent mutation event.

Based on sequence homologies with other species, putative functions have been attributed for these genes. *CpsE* is a glycosyltransferase that initiates biosynthesis of the sugar-repeating unit (Cieslewicz *et al.*, 2001). Upstream of *cpsE* are fours genes *cpsA* to *cpsD*, implicated in CPS regulation. Altogether these five genes are well conserved among the different GBS serotypes and across other capsulated streptococci and Gram-positive bacteria such as *S. thermophilus*, *S. pneumoniae*, *S. iniae*, *Lactococcus lactis* and *Staphylococcus aureus* (Cieslewicz *et al.*, 2001). In GBS *cpsA* is considered to be a transcriptional activator (Hanson *et al.*, 2012). The function of *cpsB* is still uncertain but its inactivation have profound effect on capsule production (Cieslewicz *et al.*, 2001). The next two genes in the *cps* operon are *cpsC* and -*D*, *cpsD* is a auto-phosphorylating protein-tyrosine kinase, acting with *cpsB* and *cpsC* to regulate the activity of *cpsE* in the chain length regulation and export of the exopolysacharide to the surface, thereby loss of one of these proteins would likely result in decrease of virulence and would predictably result in the loss of the capsule. The other interesting result was the insertion in QMA0368 from grouper 6 which caused a frameshift in the *cpsE* gene resulting in the loss of the capsule (evidenced by buoyant density). It seems that a single frameshift mutation in this critical gene is sufficient to cause the cessation of the capsular biosynthesis. A frameshift mutation of the *cpsE* gene that occurred in strains infecting barramundi in New South Wales and South Australia, resulted in a reduction of the capsular polysaccharide production, lower mortality and unusual pathologies including spinal fractures (Millard *et al.*, 2012).

The major difference between the different serotypes of this study occurred in the highly variable region (*cpsG*-*cpsK*) of the central part of the *cps* operon known to be serotype specific (Martins *et al.*, 2010). Modifications in these genes can also alter the final structure and antigenic determinants

of the capsule (Millard *et al.*, 2012). Capsular transformation or switching of capsule-specific genes by homologous recombination or horizontal gene transfers has been demonstrated previously in GBS (Martins *et al.*, 2010). It was interesting to find in this study that *cpsI*, present in all the piscines, humans, cow and crocodile isolates, was replaced by *cpsM*, *cpsN* and *cpsO* in the dog and the cat isolates. *In vitro* assays on capsule production from isolates grown in laboratory media certainly differ from *in vivo* situations, where other factors specific to host immunity are likely to up- or down-regulate the expression of the genes and the production of the capsule in order to maximise the dissemination of the bacteria to tissues during the process of the disease.

Producing capsule during all stages of infection is certainly not an evolutionary advantage for an invasive pathogen, considering the energy cost to synthesise such a complex sugar. In the ideal infection scenario, regulation of the amount of capsule being produced and when to switch on/off the machinery, at critical phase of infection would be an advantage. While over expression of capsule during early steps of colonisation could hide important surface proteins such as adhesins that are critical for host-cell adherence (Morona *et al.*, 2004), its production once in the blood stream is considered important for its survival and dissemination to other organs. Further analysis of the whole genomes is necessary in order to mine the genes involved in sugar metabolism, and confirm their presence or inactivation.

GBS-host interactions with a limited virulence factor repertoire.

Most streptococci have a number of other components on their surface that play an pivotal role in the colonisation of the pathogen by facilitating adhesion and entry to host cells and escaping the protective immune barriers of the host (Lindahl *et al.*, 2005; Moschioni *et al.*, 2010). Although these strains are extremely virulent in fish, including in the Queensland grouper challenge models, they seem to lack most of the virulence factors found in terrestrial strains, including the C5a peptidase, the laminin-binding protein (lmb), the fibrinogen-binding protein B (fbsB), all the immunoprotective surface proteins (α-C protein: bca, β-C protein: cba, α-like protein: alp2, and rib) but also all the pore-forming toxins (β-hemolysin, *cyl* locus), and the component of the pilus have all been inactivated.

Only six adhesins with a LPXTG motif signal for cell-wall peptidoglycan anchor have been conserved in the Australian piscines isolates, including three important GBS virulence factors present in human and other terrestrial animal strains: the fibrinogen-binding protein A (fbsA), the serine-rich repeat protein (srr), the bibA and amidase protein. Rosinski-Chupin *et al.*, (2013) reported the conservation of these virulence factors in their ST260–261 strains from Taiwan, USA, Israel and Honduras.

In southern China, for the past few years, severe outbreaks in cultured Nile tilapia (*O. niloticus*) caused by GBS serotype Ia, ST-7 were confirmed to have the fbsA protein (Zhang *et al.*, 2013). The function of fbsA and the underlying mechanisms by which GBS adheres to different epithelial cells are poorly understood, but many studies using isogenic mutant with deletion of the fbsA were shown to have reduced binding affinity to soluble fibrinogen, adherence and invasion of human epithelial cells (Schubert *et al.*, 2004). In human blood, growth of fbsA-deletion mutant was significantly

reduced (Schubert *et al.*, 2002). FbsA could play an important role in human endocarditis by mediating human platelet aggregation (Pietrocola *et al.*, 2005). The putative role of fbsA is not only to hamper phagocytosis but also to facilitate the colonisation and penetration of epithelial and endothelial tissues, by molecular mimicry, coating fibrinogen onto their surface (Pierno *et al.*, 2006).

GBS in fish as in humans can cause severe meningitis. This was confirmed in our injection model; 48 hr post injection with a GBS strain isolated from a wild Queensland grouper, 100% of the juvenile grouper tested positive for bacteriology and PCR from samples taken for the brain. Histopathological examinations also evidenced inflammation in brain tissues with the presence of Gram-Glynn bacteria that were confirmed to be GBS using a specific immunohistochemistry method (see Chapter 2). To cause meningitis, GBS must cross the blood-brain barrier (BBB) to gain access to the central nervous system (Magalhães *et al.*, 2013; Tenenbaum *et al.*, 2005). In recent studies, members of the serine-rich repeat (srr) protein family have been described to play a mediating role in adhesion and invasion in both an *in vitro* human specialised brain microvacular endothelial cells model and in an *in vivo* murine model (Sorge *et al.*, 2009).

The CAMP factor is another important test that has been used to identify *S. agaclatiae* (Lang and Palmer, 2003; Phillips *et al.*, 1980). While in this study the CAMP factor reaction has not been performed, the CAMP gene (*cfb*) seems to be unaltered in all our ST-261. Rosinski-Chupin *et al.*, (2013) reported that all their ST260–261 were negative for the camp factor reaction, and suggested the reason to be a low gene expression level. Finally, all ST-261 express a fibronectin-fibrinogen binding protein pavA which is considered to be an important virulence factors in many *Streptococcus* species and could also have a role in fish infection (Christie *et al.*, 2002). At this stage only putative functions can be attributed to these virulence factors until functional assays are performed in the lab using isogenic mutants and our piscine model. Also it is unknown if the truncations present in the sequences of virulence genes such as *lmb*, and *fbsB* result in the loss of protein functionality.

Stress adaptation at different temperatures

Capacity to adapt quickly to various environmental stress situations such as rapid changes in pH, osmolarity, and temperature but also in environments deprived of nutrients is a physiological advantage for invasive pathogens such as GBS. Using a simple growth curve experiment we looked at the fitness of marine and terrestrial isolates at two different temperatures. The fish isolates seemed to be better adapted at lower temperatures, which is not surprising when considering the temperature of the aquatic environment where these fish are found and that fish are ectothermic. Sea-surface temperatures during summer for the southern Great Barrier Reef region average between 25–28°C (Weeks *et al.*, 2013). Peak mortalities of the wild Queensland grouper attributed to GBS infection occurred during the winter months (Bowater *et al.*, 2012), thereby temperatures would have probably been below 25°C.

In humans during infection, body temperature can reach 40°C. Indeed, transcription of ß-hemolysin/cytolysin and proteins involved in iron acquisition and nucleotide biosynthesis were all upregulated,

when GBS was grown in laboratory media at 40°C (Mereghetti *et al.*, 2008).

An interesting finding was that the crocodile isolate had very similar growth pattern to the human isolate and that both grew more rapidly than all the other terrestrial isolates at 37°C. An anthroponotic transfer from human to crocodile has been suggested for these isolates, which are both serotype Ia, ST-23 (Bishop *et al.*, 2007). Finally such temperature adaption is arguably consistent when considering human body temperature and the period when the outbreak occurred on the crocodile farm in summer 2005 when the temperature in tropical Northern Territory of Australia would have been high (Bishop *et al.*, 2007).

5.5 CONCLUSIONS

This chapter used whole-genome sequencing to show that genomes of piscine isolates were significantly reduced compared to human and terrestrial strains, and this affected their ability to ferment carbohydrates. While the piscine isolates have reduced fermentation capabilities, future studies should investigate if non-fermenting bacteria are still able to utilise these sources of carbons for growth. It will also be interesting to investigate the fitness of piscine GBS isolates through growth experiments using varying carbohydrate compositions. Because we still do not know where these ST-261 isolates are found outside their hosts (fish and poikilotherm animals), it will be interesting to perform long-term starvation assays to confirm that these bacteria can recover and retain their virulence.

Moreover, mutations in the capsular polysaccharide were phenotypically relevant. Losses of critical genes in the regulatory portion of the operon or a single frameshift were sufficient to cause the cessation of the biosynthesis of the CPS as evidenced by the loss of cell buoyancy.

Although most virulence genes from mammalian isolates were missing in piscine isolates, they were still virulent and had retained some of the critical genes. Such results provide interesting information on the evolution and adaption of GBS in fish. Virulence factors that have been conserved in the ST-261 isolates must play a central role in the colonisation and dissemination, and should be the centre of attention for future development of protective vaccines.

Finally, marine GBS isolates grew slower than terrestrial isolates in rich medium, but this difference, although still significant, was less pronounced at lower temperatures. Indeed, ST-261 isolates grew better at 28°C, which is the preferendum temperature of the hosts in which they are found. Future studies should evaluate piscine isolates ability to invade, colonise and "cause diseases" in *in vitro* assays using terrestrial animals or human's leucocytes (see Chapter 4) at higher temperatures.

Chapter 6

General discussion

6. GENERAL DISCUSSION

Since 2007, 96 wild Queensland groupers, *Epinephelus lanceolatus*, were found dead from Karumba (Gulf of Carpentaria) in Northern Territory to Brisbane in southern Queensland, Australia. In 12 cases, *Streptoccocus agalactiae* was isolated in pure culture from eyes and internal organs. This is the first case of streptococcosis due to *S. agalactiae* in wild fish in Queensland, and to our knowledge, of any fish in Australia. Group B *Streptococcus* (GBS) has been also isolated from other marine species and caused an outbreak in three species of wild stingrays in a public aquarium also in Queensland. These findings have raised public and industry concern over threats to human health from potential transmission between fish and humans, over environmental impacts on other aquatic animals, and over the potential threat posed to the growing Australian aquaculture industry in which, to date, there have been no reported cases of this disease in spite of the devastation caused to the industry overseas (Abuseliana *et al.*, 2010; Geng *et al.*, 2012; Najiah *et al.*, 2012; Pridgeon, 2012). Therefore, my thesis set out to determine whether GBS isolated from dead wild fish in Australia is indeed virulent in Queensland grouper and causative of mortality, to try to identify from where these infections in wild fish originated, to determine potential risk to human health and to other aquatic animals, and to begin to identify mechanisms by which these Australian isolates may cause fatal disease in Queensland grouper. This information may be employed in future risk assessment of potential human transfer, in mitigating further outbreaks in wild fish through understanding origin of infection, and in future development of preventative measures such as vaccination for farmed fish.

In order to confirm virulence of *S. agalactiae* and that it could have been the cause of the mortality in wild fish, infectivity experimental challenges were conducted by injection, immersion and through the oral route. To better understand why these strains of GBS are pathogenic in fish, we conducted a series of cellular immune assays using juvenile Queensland grouper head-kidney leucocytes in the presence of different piscine and terrestrial isolates. Whole-genome sequencing analysis of the piscine and terrestrial isolates provided information on potential origin and potential for transfer between species, and also informed us on critical virulence factors that could be used during pathogenesis of GBS. Moreover, genome analysis gave intriguing insight into the evolution and adaption of GBS to fish in the aquatic environment.

Group B *Streptococcus* is likely to be the cause of the disease in wild marine fish in Australia

Australian GBS isolates were extremely virulent in infectivity challenges in captive juvenile Queensland groupers and are likely to be the cause of the disease observed in wild Queensland grouper and other Australian marine fish species reported between 2007 and present (Bowater *et al.*, 2012). Different modes of transmission of the disease were evaluated in an aquarium model and injection challenge resulted in rapid development of clinical symptoms typical of streptococcal infections including bilateral exophthalmia, skin ulceration and meningitis. Death occurred rapidly within the first four days and the bacteria was recovered from all organs by bacteriology and confirmed to be GBS by PCR thereby fulfilling Koch's postulates. Challenge by immersion resulted in lower mortality with a clear

dose response. Whilst infection was established via oral challenge by admixture of bacteria with feed no mortality was observed during the entire duration of the experiment. Histology revealed typical pathology consistent with wild mortalities (Bowater *et al.*, 2012) in organs from all injected fish, from fish challenged with medium and high doses by immersion, and from high dose oral challenge.

While injection challenge resulted in rapid development of the disease, this is unlikely to reflect the natural route of transmission. Natural infections in the wild are most likely to occur through contaminated water and possibly through the oral route by ingested food (Bowater *et al.*, 2012; Evans *et al.*, 2002; Hetzel *et al.*, 2003; Jafar *et al.*, 2009; Pier and Madin, 1976). It was interesting to note in the immersion trial that mortalities in challenged and unaffected sentinel fish were higher in one of the replicate recirculating systems where a less effective biofilter lead to some accumulation of ammonia. So whilst we can transfer infection by immersion in a relatively low concentration of bacteria, it appears that better water quality reduces stress in a cohort of naïve fish sufficiently to prevent initial colonisation, or reduce shedding in challenged fish.

Outbreaks caused by streptococcal infections in several fish species have been associated with changing environmental conditions, including increased ammonia levels, warm and cold water temperatures, osmolarity changes and low dissolved oxygen (Amal *et al.*, 2013a; Bromage and Owens, 2002; Evans *et al.*, 2006c; Evans *et al.*, 2003; Sepahi *et al.*, 2013). Whilst exposure for 24 hr with a sub lethal dose of un-ionized ammonia UIA (0.32–0.37 mg L^{-1}) did not significantly cause more mortality in Nile tilapia (*Oreochromis niloticus*) after injection with 750 colony-forming units (cfu) of *S. agalactiae* per fish, when exposed to high level of UIA (2.0–4.0 mg L^{-1}) and no subsequent challenge, fish experienced 93–100% mortalities within 24 hr (Evans *et al.*, 2006a). In a long-term environmental study, Bromage and Owens (2009) demonstrated that *Streptococcus iniae* infection was temperature dependant, specifically between 25–28°C, and that in combination with acid water conditions (< pH 6), mortalities in barramundi (*Lates calcarifer*) increased significantly. In rainbow trout, a cold-water species, a clear relationship between temperature and mortalities due to GBS was established (Sepahi *et al.*, 2013). After injection with different doses of GBS, the authors suggested that at the highest temperature of 18°C, the bacterial growth might have been favoured to the detriment of the immune system of the fish outside its optimal physiological temperature range (12°C). Environments with different hydrodynamic conditions can also contributed to the presence of a pathogen. Prevalence of cultured red hybrid tilapia (*Oreochromis niloticus* x *Oreochromis mossambicus*) positive for GBS was higher in lake compared to river, and that unfavourable environmental factors such as ammonia, temperature, water clarity, pH, and dissolved oxygen were positively correlated with the presence of GBS in infected fish (Amal *et al.*, 2013a).

In Australia, most cases of wild fish that tested positive for GBS were found in estuaries close to large urban areas (Cairns, Townsville and Darwin). So the combination of anthropogenic inputs (sewage contaminants, excess nutrient runoff from agriculture, etc.), and high tidal range in this region could have contributed to deteriorated water quality and caused important fluctuation in salinity and temperature respectively. As the environmental drivers for the recent and ongoing mortalities in wild

Queensland grouper are still unknown, these aspects of susceptibility to infection are worthy of further investigation in a controlled study.

Amongst surviving fish challenged by immersion and through infected food, many were asymptomatic carriers as bacteria were isolated from or detected in organs at post-mortem. The conditions required for these fish to contain the infection are not clear, but non-specific gut and skin-associated mucosal immunity may play an important role in the protection against bacteria (Delamare-Debouteville *et al.*, 2006; Rombout *et al.*, 2011; Salinas *et al.*, 2011; Urquhart *et al.*, 2009).

Preliminary environmental surveys in north Queensland failed to identify the primary source of GBS from water and sediments samples. While the ecology of GBS outside its host in the environment is not well understood, previous studies have reported *Streptococcus* species from water bodies and sediments: in Nigeria, surface and groundwater beneath an abattoir were tested positive for many antibiotic resistant bacteria including *S. agalactiae,* which was ubiquitous in all the samples (Adelowo *et al.*, 2012), and could cause serious risk of transmission to animal living in the surrounding aquatic ecosystems, and to the local populations that depend on them. During a disease outbreak in Kuwait bay affecting seven species of fish, GBS was isolated from sewage-water samples and were almost identical to the ones isolated from the kidney and brain of infected mullet, suggesting that the sewage plant could have been a potential reservoir of infection (Jafar *et al.*, 2009). *Streptococcus sanguis* a counterpart member of the pyogenic streptococci was found in abundance in alkaline soil (Gledhill and Casida Jr, 1969). After rainfall pulses, in a brackish estuarine embayment in Chesapeake bay (USA), an increase of faecal *Streptococcus* counts was recorded, and the authors suggested waste from local birds and mammals as the bacterial inputs (Mountford, 1980). These reports strongly support the hypothesis that GBS as a result of biogenic inputs from human or animals can be found in the environment outside its host. The next logical step would be to investigate in a controlled experiment if these fish isolates are able to survive and conserve their virulence after spending an extended period of time in water or sediment under different conditions.

Australian marine ST-261 GBS isolates are not closely related to local terrestrial animal or human isolates

Multilocus sequence typing and the capsular genotyping enabled inference of the relatedness of the piscine isolates to overseas and local human and animal strains of *S. agalactiae*. All piscine isolates from Australia belong to ST-261 and were all serotype Ib. ST-261 was first isolated from Tilapia in Israel (Eldar *et al.*, 1994) in the mid-1980's. More recently, *S. agalactiae* of this sequence type has been isolated on farms from Tilapia in Indonesia (Lusiastuti *et al.*, 2012). In the United Kingdom, ST-261 was identified during an outbreak in imported freshwater cyprinid doctor fish (*Garu rufa*) from Indonesia (Verner-Jeffreys *et al.*, 2012) used for ichthyotherapy in which fish are employed to remove dead skin as a therapy. This has raised some sanitary concerns, as it is possible that, in close contact with humans, this sequence type could adapt and become zoonotic. ST-261 has also been isolated from frogs from Louisiana in the United States (Elliott *et al.*, 1990; Lopez-Sanchez *et al.*, 2012) so

is not constrained to teleost or chondrychthyan hosts. However, ST-261 has not yet been associated with human infections.

Despite the fact that ST-261 is one of many described fish related STs occurring globally (Evans *et al.*, 2008; Evans *et al.*, 2009; Lusiastuti *et al.*, 2012; Ye *et al.*, 2011), it is intriguing that in Australia we did not find any strain belonging to ST-6 or ST-7 which are broad-host range strains often associated with outbreaks in farmed fish (Delannoy *et al.*, 2013; Rosinski-Chupin *et al.*, 2013). Genomic comparisons with Tilapia isolates from Israel and USA revealed that they were extremely similar to our Australian strains (Ben-Zakour *et al.*, in preparation). We know that Tilapia has been introduced on several occasions into North Queensland since the 1970's and several species and hybrids are known to have established amongst the Australian aquatic animal life in the region (Mather and Arthington, 1991). It is therefore possible that the ST-261 genotype was introduced to Australia along with these fish at some point in the relatively recent past.

An interesting finding was a new MLST single locus variant for the *glnA* allele that was discovered in a grouper isolate for the first time. Despite the relatively recent introduction, at this stage we are unsure of mutation rates in GBS, but high similarity with isolates from Israel and USA, and the fact that we found only one new allele amongst our ST-261 isolates suggests that the mutation rate in those strains is either particularly low or introduction has been sufficiently recent such that further substantial evolutionary diversification has not yet occurred. While MLST typing method was able to position the Australian piscine isolates on a global scale amongst a particular clonal complex, finer scale determination of origin requires more extensive analysis of loci that mutate more quickly than the MLST loci employed. A number of genes in the capsular operon mutate at a high rate and therefore can provide some further resolution of possible strain movement and clonal expansion within the aquatic animals (Millard *et al.*, 2012). All Australia piscines isolates were serotype Ib. Fish isolates from different countries have been typed as serotypes Ia, Ib and III (Brochet *et al.*, 2006; Evans *et al.*, 2008; Suanyuk *et al.*, 2008). In the elasmobranch isolates, we found identical point mutations that suggest passage amongst these animals, which is consistent with the fact that these species of ray were translocated from north Queensland to a public aquarium on the Gold Coast. It is unknown if these animals were infected prior transfer or if they acquired the disease in the Aquarium, but the consistent *cps* genotype strongly suggests a direct transmission amongst these individuals. GBS isolates from five of the Queensland grouper, the two Javelin grunters, the mullet and the two catfish shared identical *cps* sequences, which strongly implicates a common source of infection amongst these fish. The spatial and temporal disparity between the isolation of these samples perhaps suggests a common source of infection but subsequent asymptomatic carriage with disease symptoms only becoming apparent at times when environmental conditions favoured disease outbreak.

For greater discriminatory power to detect more genetic diversity within bacterial isolates that are very closely related a larger repertoire of genes needs to be analysed. Recent studies have developed alternative typing scheme to MLST alone. Delannoy *et al.*, (2013) proposed another genotyping approach to characterise human and fish isolates, which in addition to molecular serotyping and

MLST, used a selection of surface protein genes and mobile genetic elements. Although, this approach gave them greater resolution for the majority of the strains used in their study, the technique could not discriminate further ST-261 and ST-260 lineages on the basis of their criteria. Alternatively, Godoy *et al.*, (2013) employed an amended genotyping scheme for Brazilian fish pathogenic GBS using additional virulence genes including *cylE* (β-haemolysin/cytolysin) and *hylB* (hyaluronate lyase) that were not included in the Delannoy's study. This approach resulted in nine genetic profiles that could discriminate isolates from the same ST, allowing them to identify and trace different clones that were involved simultaneously in outbreak cases on Nile tilapia (*Oreochromis niloticus*) and hybrid Amazon catfish (*Pseudoplatystoma fasciatum* x *Leiarius marmoratus*) farms.

In the whole-genome era of microbiology, advances in sequencing technologies have opened the access to considerable amount of genetic information that can be mined to study bacterial diversity. Maiden *et al.*, (2013) proposed a novel hierarchical gene-by-gene approach using the Bacterial Isolate Genome Sequence Database (BiGSdb) that can exploit over 500 loci from whole, core or accessory genomes depending on the resolution required. This approach has the potential to give unprecedented insights into the evolutionary processes of bacterial populations.

Mature macrophages and granulocytes from Queensland grouper head-kidney are the main phagocytes

In addition to understanding routes of infection and environmental origin of GBS infecting Queensland grouper, it is also critical to understand the interaction between these fish pathogenic isolates and the host immune system. Such understanding may help to explain what must be compromised in order for carrier status to develop into fatal pathology.

Recent progress in vaccine development (Liu *et al*., 2013b) has identified immunoreactive proteins as potential vaccine candidate antigens from a haemolytic GBS strain isolated from cultured Tilapia in China (ST-7, serotype Ia). Currently, a protective vaccine from non-haemolytic GBS is commercially available, but does not confer protection against the most common haemolytic strains from Southeast Asia or against important zoonotic infections from fish or fish products (Brochet *et al*., 2006; Delannoy *et al*., 2013). Further research is needed to identify critical bacterial factors that are conserved amongst multiple strains (e.g. adhesins) and required for virulence, enabling guided design of a universal piscine GBS vaccine for aquaculture.

Ultimately, most invading bacteria are destroyed by the phagocytic cells of the innate immune system, so this is a logical starting place for investigation of host bacterial interaction. Leucocytes originating from the Queensland grouper anterior kidney are composed of heterogenic cells including two main sub-populations as evidenced by flow cytometry. The population of large sized cells with high internal complexity identified by flow cytometry was confirmed by morphological characterisation to be composed essentially of mature macrophages and neutrophils, whereas the smaller monocytic lineage comprised monocytes, small lymphocytes and thrombocytes. While both populations of cells

in the presence of pathogenic bacteria were capable of phagocytosis and ROS production, most of the oxidative burst activity was performed by the larger granulocytic cells. In fact they contributed to approximately 90% of the total effort in terms of the reactive oxygen species being produced. These cells were not activated with either lipopolysaccharide (LPS) or peptidoglycan (PTG), but were stimulated when incubated with phorbol myristate acetate (PMA). These findings suggest a differential expression of specific receptors for these ligands. PMA, which bypasses cell surface receptors and acts directly on protein kinase C, was used in this as a positive control to confirm that these cells could indeed activate a respiratory burst, but it is not really a relevant route of activation in terms of determining how these cells interact with putative pathogens. Two accessory proteins mediate LPS binding to its toll-like receptor TLR4 on macrophage surface: CD14 and LPS-binding protein (LBP) (Mathison *et al.*, 1992; Pugin *et al.*, 1995; Tobias *et al.*, 1995). Absence of TLR4 homologue and its associated proteins have been reported in several fish species (Palti, 2011; Rebl *et al.*, 2010). It is not known if Queensland grouper possess or not TLR4, or if a differential expression of the complex receptor and these chaperone proteins is to be expected in other more mature leucocytes. Peptidoglycan is a complex carbohydrate that forms part of the outer membrane of Gram-positive bacteria. In mammals, PTG recognition is mediated by TLR2 (Aliprantis *et al.*, 2000; Schwandner *et al.*, 1999). TLRs homologues have been identified in fish macrophages (Wei *et al.*, 2011) and were implicated in macrophage activation upon stimulation with PTG in carp (Ribeiro *et al.*, 2010). Unresponsiveness of Queensland grouper head-kidney leucocytes (HKL) to PTG suggests that other factors might be necessary for PTG to activate macrophages and granulocytes in this fish species. This could have serious implication in terms of the recognition of major pathogenic bacteria by grouper leucocytes and further investigation is warranted in TLRs and their specificity to pathogen ligands in this species in order to elucidate their function during the infection processes.

The GBS isolates in this study were genetically diverse and from diverse host origins. To explore the effect of this diversity on their potential to infect Queensland grouper, we compared interactions between representative isolates chosen from our collection and the two sub-populations of grouper immune cells. All piscines isolates from different fish hosts were capable of triggering an oxidative burst in both monocyte and granulocyte populations, which clearly demonstrate that these bacteria can be recognised by these highly specialised cells.

Recognition of pathogens associated molecular patterns (PAMPs), such as LPS, PTG, mannose and other carbohydrates, by leucocytes is mediated through specific receptors, such as the aforementioned TLRs, that bind to these surface molecules to ingest and destroy many aquatic pathogens. Macrophage mannose receptor and other scavenger receptors have been previously described in fish phagocytes (Frøystad *et al.*, 1998; Nolan *et al.*, 2006; Seternes *et al.*, 2001). These receptors can bind many different type of ligands, and have been reported to be involved in phagocytosis of Gram-positive and Gram-negative bacteria (Krieger and Herz, 1994). In the absence of direct stimulation of Queensland grouper leucocytes by PTG and LPS, it is likely that the activation of these cells by GBS is mediated through these other types of receptor, perhaps interacting with capsular polysaccharides or sialic acid.

As the major surface structure, the interaction between the pathogen and the host cellular immune system is greatly affected by the presence of a capsule, which dictates the serotype of a particular strain (Cieslewicz *et al.*, 2001; Kong *et al.*, 2008; Manning *et al.*, 2008). We observed the highest phagocytic rate in granulocytes when they were incubated with the capsulate GBS isolate from grouper 5, this is supportive of the role for capsule in phagocytic avoidance in fish pathogenic streptococci (Källman *et al.*, 1998; Locke *et al.*, 2007; Martin *et al.*, 1992) and corroborates the contention raised above that, at least during early infection and spread, fish pathogenic GBS avoid detection through capsular expression. The other interesting finding was that while the terrestrial isolates were not closely related with the piscines isolates as evidenced by different serotype and sequence type, both the cat and the dog induced a significant amount of ROS similar to some of the marine isolates. While, these piscine and mammalian isolates are serologically different, respectively serotype Ib and V, they have a high level of similarity, with the regions *cpsA* to *cpsD*, *cpsF-cpsG*, *cpsL* and *neuB* to *neuA* being conserved in all strains. This suggests that they share immunogenic determinants and are recognised in a similar fashion by grouper leucocytes.

The ability of an invasive pathogen to kill the host phagocytes can be advantageous in some situations. The GBS isolate from the dog lead to the highest mortality increase observed in both the monocytic and granulocytic lineages of grouper leucocytes, suggesting that this terrestrial strain may possess particular virulence factors such as pore-forming toxins that promote invasion of host cells and triggers host-cell lysis and apoptosis (Lin *et al.*, 2011; Rajagopal, 2009). Surprisingly the non-significant production of oxygen radical species by the granulocytes in the presence of human, crocodile and bovine isolates coincided with the highest phagocytic index, which suggest that these bacteria are capable of quenching ROS production. Virulence factors for immune evasion are known for their ability to detoxify single oxygen and superoxide being produced by the host, such as superoxide dismutase (*SodA*) or proteins member of the *cyl* locus (Poyart *et al.*, 2001; Rajagopal, 2009). All these observations clearly demonstrate that these closely related pathogens in order to evade or confront the fish cellular innate immune system must employ different strategies.

Given the results of these experiments, we cannot rule out a potential future transmission from terrestrial animal or human isolates to fish. Indeed, in Kuwait during a mass fish kill, GBS was isolated from seabream, mullet and dolphin, all were part of a clone of serotype Ia ST-7 which has been previously associated with bloodstream infections in neonates in Japan (Evans *et al.*, 2008; Jones *et al.*, 2003). This was further confirmed in 2009 in an injection challenge using the same human Japanese strain serotype Ia ST-7 causing disease and death in Nile tilapia (Evans *et al.*, 2009). Moreover, tilapia has been shown to be susceptible also to GBS bovine isolates, with infected fish exhibiting characteristic meningoencephalitis (Pereira *et al.*, 2010). In Thailand a rare genotype belonging to a subtype of serotype III (III-4) previously identified among invasive human isolates in Hong Kong (Suanyuk *et al.*, 2008) was isolated from Nile tilapia (*O. niloticus*). A recent study demonstrated by genome comparison that isolates from cultured tilapia in China were closely related to the human strain A909 serotype Ia ST-7 (Liu *et al.*, 2013a). Delannoy *et al.*, (2013) identified some sequence types: ST-283 (III-4), ST-7 (Ia) from fish and ST-23 (Ia) from grey seals that are also found in humans. This

increasing body of evidence confirm that these bacteria from human or bovine origins can cross interspecies barriers, and successfully establish themselves within fish populations. If the reverse is also possible, the prevalence of ST-261 in food fishes poses a substantial risk of zoonotic transfers.

For future studies on host pathogen interactions, it will be worthy to use the more mature leucocytes that reside in the peritoneal cavity as these cells compared to head-kidney leucocytes have been shown to be immunologically more relevant (Tumbol *et al.*, 2009).

The marine GBS isolates have adapted to their piscine host and environmental niche

Comparative genome analysis revealed a distinct reduction of the piscine isolates genomes, approximately ~200–300 kbp smaller than the terrestrial isolates, possibly related to host shift (Ben-Zakour *et al.*, in preparation). This reduction was characterised by the presence of numerous deletions and extensive pseudogenization, which suggest a long-term association with their hosts (Ben-Zakour *et al.*, in preparation). Overall when comparing the genome of all strains to a central pan-genome, all marine isolates share most of their genome with terrestrial strains and harbour very few specific regions in their accessory genome (Ben-Zakour *et al.*, in preparation). This massive gene loss is inevitably accompanied by loss of function that have affected genes associated with transport, transcription regulation, and metabolism (Ben-Zakour *et al.*, in preparation). While the fish isolates had identical enzymatic hydrolysis profile by API 20 Strep with the terrestrial ones, it was interesting to note that most of them had reduced carbohydrate metabolic capacities, and have for some of them lost their ability to ferment ribose, trehalose and starch. The way these bacteria ferment these energy sources of carbon must reflect the diet of their host and the niche in which they are found. Glucose metabolism in fish has been extensively studied and suggests unequal opportunities between herbivorous and carnivorous fishes to metabolise some carbohydrates (Polakof *et al.*, 2012).

Another very interesting finding was that changes in the *cps* genotype, were phenotypically relevant. Critical genes that are involved in the early transcription, activation and regulation of the biosynthesis of this complex sugar are conserved across many streptococci species. Point mutations in those key genes resulted in the reduction or the complete loss of the capsule as evidenced by their buoyant density. Genetic mutations and phenotypic differences in the capsular operon of several species of streptococci have been described previously (Cieslewicz *et al.*, 2001; Lowe *et al.*, 2007). Cases of streptococcosis in barramundi (*L. calcarifer*) caused by *S. iniae* were typed and found to carry a frameshift mutation in *cpsE* resulting in loss/reduction of capsule being produced (Millard *et al.*, 2012). Mortalities on farms associated with these strains were reduced, and abnormal pathologies with spinal vertebral deformation were attributed to the bacteria that sought refuge from phagocytic attacks (Millard *et al.*, 2012). Deletion of *cpsB* or *cpsD* in *S. pneumonia* was shown to be avirulent in mice injected via systemic and intranasal routes (Morona *et al.*, 2004). In GBS, mutations in the capsular polysaccharide have been associated in reduced complement deposition and phagocytosis (Martins *et al.*, 2010). GBS *cpsA* deletion mutant produced less capsule and was significantly less virulent than wild type strain in a zebrafish infection model (Hanson *et al.*, 2012).

The fact that isolates from the ST-261 lineage have never been found in warm-blooded animals but are to date exclusively causing disease in fish or frogs (both poikilotherms), reflect their better fitness at lower temperature. In the future it will be worth growing these strains in nutrient poor conditions to confirm the original premise that these isolates have adapted to carbohydrate-limited environments, but also to investigate respiration metabolism that has been shown to contribute to virulence (Yamamoto *et al.*, 2005).

Whilst most virulence factors found in mammalian isolates were missing from the fish strains, these strains were extremely virulent in the challenge model, suggesting they must have eliminated genes no longer required and conserved critical ones for their pathogenesis in fish. An interesting example is the adhesins in human strains, which are adapted to human extracellular matrix proteins, in fish they were replaced by a set of adhesins that were not conserved in all terrestrial strains. We know they lack most toxins, immunoreactive antigens and C5a peptidase that cleaves host complement, so what are the remaining factors that mediate the interactions between the pathogen and the host immune systems and allow them to cause septicaemia and meningitis? That this pathogen is still capable of crossing the blood-brain barrier (BBB) and invading the CNS in spite of its vastly reduced repertoire of virulence factors presents a golden opportunity to understand the underlying mechanisms that allow GBS to cause fatal septicaemias and meningitis. Two very important *S. agalactiae* virulence factors of human strains: the member of the serine-rich repeat (srr) protein family and the fibrinogen-binding protein A (fbsA) are conserved in the piscine isolates and have been described to mediate penetration of the BBB in both an *in vitro* human specialised brain microvascular endothelial cells model and in an *in vivo* murine model (Sorge *et al.*, 2009; Tenenbaum *et al.*, 2005). According to "The Trojan horse effect" theory, apoptotic macrophages with intracellular surviving bacteria can be used as vehicles that are transported into the bloodstream circulation and later unloaded into the central nervous system after BBB transcytosis (Zlotkin *et al.*, 2003). This was supported in this study by histology on brain cross-sections from GBS infected fish, where typical granulomatous inflammatory responses, consisting of aggregations of macrophages laden with fluorescent coccoid bacteria were observed.

Zebrafish (*Danio rerio*) has recently emerged as a powerful vertebrate model for the study of human meningitis (Patterson *et al.*, 2012), so direct mutagenesis against these virulence genes in combination with this model could be used to decipher the pathogenesis and development of invasive GBS in fish.

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APPENDICES

Appendix 1. Serotype specific cps sequences used for molecular serotyping (Chapter 3).

STRAIN	SEROTYPE	ACCESSION NO.
515	Ia	NZ_AAJP00000000.1
2-22	Ib	FO393392
A909	Ic	NC_007432
not reported	II	AY375362
COH1	III	AF163833
CNTC 1/82	IV	AF355776
CNTC 1/82	V	AF349539
NT6	VI	AF337958
7271	VII	AY376403
SMU014	VIII (complete cds)	AY375363

Appendix 2. GBS isolates included in the BRIG images of the virulence factors Figure 5.3 (Chapter 5).

Ring number[1]	Strain	Origin	Country	Year	Source	ST	Accession number
Ring 4	SA20-06	Brazil	Brazil	2006	Nile tilapia	553	CP003919
Ring 5	SS1219	Taiwan	Taiwan	<1990	Frog	260	CAQA00000000
Ring 6	LADL-90-503	USA	USA	1990	Hybrid striped bass		CAPZ00000000
Ring 7	LADL-05-108a	Honduras	Honduras	2005	Tilapia		CATH00000000
Ring 8	STIR-CD_17	Honduras	Honduras	2008	Nile tilapia		ALXB00000000
Ring 9	SS1218	USA	USA	<1990	Frog	261	CAUB00000000
Ring 10	2-22	Israel	Israel	1988	Trout		FO393392
Ring 11	**QMA0277**	Sea World	Australia	2009	Estuary stingray		This study
Ring 12	**QMA0275**	Sea World	Australia	2009	Eastern shovelnose ray		This study
Ring 13	**QMA0320**	Sea World	Australia	2010	Estuary stingray		This study
Ring 14	**QMA0276**	Sea World	Australia	2009	Mangrove Whipray		This study
Ring 15	**QMA0368***	Kowanyama	Australia	2010	Grouper		This study
Ring 16	**QMA0267**	Cairns	Australia	2008	Grouper		This study
Ring 17	**QMA0274**	Cairns	Australia	2009	Squaretail mullet		This study
Ring 18	**QMA0369**	Townsville	Australia	2011	Grouper		This study
Ring 19	**QMA0280**	Townsville	Australia	2010	Grouper		This study
Ring 20	**QMA0268**	Cairns	Australia	2009	Javelin grunter		This study
Ring 21	**QMA0287**	Cairns	Australia	2009	Javelin grunter		This study
Ring 22	**QMA0290**	Cairns	Australia	2009	Giant catfish		This study
Ring 23	**QMA0284**	Cairns	Australia	2008	Grouper		This study
Ring 24	**QMA0271**	Cairns	Australia	2009	Giant catfish		This study
Ring 25	**QMA0285**	Cairns	Australia	2008	Grouper		This study
Ring 26	**QMA0281**	Townsville	Australia	2010	Grouper		This study
Ring 27	ILRI005	Kenya	Kenya	NA	Camel	609	HF952105
Ring 28	ILRI112	Kenya	Kenya	NA	Camel	617	HF952106
Ring 29	FSL_S3-026	USA	USA	2000	Bovine	67	AEXT00000000
Ring 30	**QMA0306**	Australia	Australia	2005	Bovine		This study
Ring 31	GB00112	Canada	Canada	1999	Human	17	AKXO00000000
Ring 32	COH1	USA	USA	1985	Human		AAJR00000000
Ring 33	**QMA0370**	USA	USA	1985	Human		This study
Ring 34	NEM316	USA	USA?	1934	Human	23	AL732656
Ring 35	515	USA	USA	1977	Human		AAJP00000000
Ring 36	**QMA0355**	Townsville	Australia	2011	Human		This study
Ring 37	**QMA0336**	Darwin	Australia	2010	Crocodile		This study
Ring 38	**QMA0357**	Townsville	Australia	2011	Human		This study
Ring 39	18RS21	USA	USA?	1934	Human	19	AAJO00000000
Ring 40	2603V/R	Italy	Italy	>1992	Human	110	AE009948
Ring 41	09mas018883	Sweden	Sweden	NA	Bovine	1	HF952104
Ring 42	**QMA0300**	Australia	Australia	2008	Dog		This study
Ring 43	CJB111	USA	USA	1990	Human		AAJQ00000000
Ring 44	GD201008-001	China	China	2010	Nile tilapia	7	CP003810
Ring 45	H36B	USA	USA?	1934	Human	6	AAJS00000000
Ring 46	A909	USA	USA	1934	Human	7	CP000114
Ring 47	SS1014	USA	USA	<1990	Striped bass	6	CAQC00000000
Ring 48	CF01173	Koweit	UK	2001	Trout		CAQB00000000
Ring 49	ZQ0910	China	China	NA	Nile tilapia		AKAP00000000
Ring 50	GD201008-001	China	China	2010	Nile tilapia	7	CP003810

* The multilocus sequence type of QMA0368 is a single locus variant of ST-261. Isolates of this study are marked in bold.

[1]Strains are listed as they appear of Figure 5.3 (Chapter 5), from innermost to outermost. Rings 1 to 3 correspond to GC content, GC skew (-), and GC skew (+) respectively.