

The humoral immune response of *Lates calcarifer* to *Streptococcus iniae*

**Thesis submitted by
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ABSTRACT

This study characterises various aspects of barramundi (*Lates calcarifer*) humoral immunity, including ontogeny, temperature modulation and kinetics following challenge with *Streptococcus iniae*. It was discovered that Staphylococcal protein A (SpA) was able to efficiently isolate antibody from serum, and that all barramundi Ig found in serum is tetrameric with a weight of approximately 800 kDa. This tetramer is composed of 8 heavy chains (72 kDa) and 8 light chains (28 kDa). Denaturing, non-reducing electrophoresis demonstrated differential disulfide polymerization (redox forms) of the tetrameric Ig which was consistent with those observed with other species. Polyclonal and monoclonal antibodies were produced against the protein A purified barramundi Ig, and various ELISA formats were developed. These serological tools were used to investigate aspects of barramundi humoral immunity.

Examination of ontogeny of humoral immunity, revealed that barramundi possess minimal maternal antibody (<10 µg/ml wet weight) post-hatch, which is depleted rapidly (within 3 days). By day 8 systemic Ig is able to be detected, which continues to increase over the following months. However, it is not until seven week post-hatch that barramundi fingerlings are able to mount a prolonged immune response following vaccination with *S. iniae*.

Environmental temperature was also found to significantly impact the ability of barramundi to respond to vaccination with *S. iniae*. Barramundi maintained at low temperatures (<23°C) displayed a diminished, delayed and highly variable humoral immune response following vaccination, with many of the experimental animals failing to respond to primary vaccination. These responses could be mediated by either administering a booster vaccine or by elevating the environmental temperature.

This study also demonstrated that there was a relationship with specific serum antibody and protection against *S. iniae*, with fish possessing high

levels of specific Ig being protected from lethal challenge, while those with low titres being more susceptible to disease. Specific antibody in barramundi could be generated through natural exposure to the bacterium from the environment or through vaccination. Thus bath vaccination of fish (50,000) held at two facilities resulted in elevated systemic antibody levels and lower observed mortality, when compared to the unvaccinated control fish.

Infections due to *S. iniae* were determined to be associated with elevated water temperatures. Laboratory trials and field data indicated that water temperatures between 24 and 28⁰C resulted in the highest barramundi mortality. A weak association was also determined with low pH and mortality, with fish exposed to low pH's (<6.0) being more susceptible to infection. No association was observed with mortality and salinity.

Four monoclonal antibodies (Mab's) were also generated against a 21 kDa protein from cell wall of *S. iniae*. The Mab's displayed a high level of specificity for *S. iniae*, including those from Australia, Israel and America, and minimal cross-reactivity with other bacterial species tested. The Mab's were used in an immunohistochemical study that confirmed the neurotropic nature of *S. iniae* infections, as well as demonstrating the presence of the bacterium in the intestine of infected fish.

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LIST OF ABBREVIATIONS

PBS	phosphate buffered saline
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
UV	ultraviolet
ELISA	enzyme linked immunosorbent assay
CAGE	composite agarose-acrylamide gel electrophoresis
PVDF	polyvinylidene flouride
ADH	arginine dihydrolase
VNN	viral nervous necrosis
BDS	bovine donor serum
FBS	fetal bovine serum
OPI	oxaloacetate-pyruvate-insulin
PEG	polyethylene glycol
Ig	Immunoglobulin
FIA	Freund's incomplete adjuvant
FCA	Freund's complete adjuvant
blgM	barramundi immunoglobulin
IP	Intraperitoneal
IV	Intravenously
CCB	carbonate coating buffer
ABTS	2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid)
DAB	3, 3'-diaminobenzidine tetrahydrochloride
AEC	amino-ethyl-carbozole
HRPO	horseradish peroxidase
PH	post-hatch
ppt	parts per thousand
ANOVA	Analysis of Variance
RPS	relative percentage survival
HAT	hypoxanthine-aminopterin-thymidine
HT	hypoxanthine-thymidine
DMSO	dimethyl sulphoxide
OD	optical density

CHAPTER 1

INTRODUCTION

The primary species of fish cultured in the tropics of Australia is the barramundi, *Lates calcarifer*. Barramundi culture commenced in Queensland in 1982, and over the proceeding years, production has grown at a rate of 20% per annum (Anon 2003a). With recent advances in culture technology, the species is now grown in all Australian states with industry growth expected to reach 40% per year over the coming years (Anon 2003a). The dramatic increase in the production of barramundi has unfortunately led to the emergence of infectious diseases within these systems, and disease outbreaks are both costly and common.

There are two primary diseases that affect the barramundi industry; viral nervous necrosis (VNN) (Chi *et al.* 2003) and streptococcosis (Bromage 1997). The former is principally a hatchery-related disease, while the latter is of primary importance to growout. Streptococcosis outbreaks are due to *Streptococcus iniae*, a bacterium originally isolated from captive dolphins (Pier and Madin 1976). *Streptococcus iniae* is a unique bacterium, as it displays little discrimination in the species it infects and possesses a global distribution (see Chapter 2). It is recognised as the most problematic bacterial pathogen in intensively cultured tilapia (*Oreochromis spp.*), red drum (*Sciaenops ocellatus*) and striped bass (*Morone spp.*) cultured in Israel and the United States (Perera *et al.* 1997; Camus 2002; Colorni *et al.* 2002). Locally the bacterium causes chronic mortality in marine cultured stocks, and its prevalence is increasing in freshwater systems (Personal observations). Most recently, the loss of \$2 million worth of barramundi at the largest culture

facility in Western Australia (equal to 2002's total production from that state) has highlighted the importance of this disease to the industry (Anon 2003b).

There has been no research into the epidemiology of *S. iniae* outbreaks in barramundi, or any other species. Most publications merely indicate that outbreaks are more common during periods of warm weather or high rainfall (Ghittino *et al.* 1998; Nguyen *et al.* 2002). Research into the environmental stresses that lead to bacterial infections has been imperative to the understanding and control of diseases in rainbow trout culture (*Salmo gairdneri*), such as *Renibacterium salmoninarum* and *Aeromonas salmonicida* the cause of furunculosis (Dalsgaard 1986; Dale *et al.* 1997). Similar research into the key environmental variables that play a role in the susceptibility of barramundi to *S. iniae*, as well as other factors such as the mode of infection, would be both important and timely.

Currently, the only means of managing an outbreak of *S. iniae* is through the use of antibiotics. However, an alternative method of control may be through the development of an efficacious vaccine that can be administered to susceptible fish prior to exposure. Initial trials with barramundi, utilising a whole cell vaccine appeared promising (Bromage 1997), and significant funding is being allocated to the development of *S. iniae* vaccines in striped bass (Anon 2004). Crucial to the successful production of a vaccine is the development of immunological reagents to monitor and assess the shift in immune status of the host (Coll and dominguez-Juncal 1995). Regrettably, these reagents do not exist for barramundi, and the knowledge of their humoral immune response is limited (Bryant *et al.* 1999).

There are three major aims of this thesis; the first is to acquire greater knowledge of the mechanisms that underpin *S. iniae* outbreaks in barramundi through the investigation of both the environmental stresses that are important to establishment of disease and the mode of transmission. The next major aim is to begin to characterise the barramundi humoral immune system, concurrently developing the immunological tools to perform this task. The third aim of this study is to assess the efficacy of the previously developed

vaccine in large scale field trials. It is the hope of the author to provide data that is both important to the successful control of *S. iniae*, and provide the foundation research on the barramundi immune system for others to build upon.

CHAPTER 2

REVIEW OF THE LITERATURE - *STREPTOCOCCUS INIAE*

2.1 Introduction

The genus *Streptococcus* includes a diverse array of pathogenic bacteria that cause disease in phylogenetically disparate groups of animals. Streptococci are of considerable concern to human health (Spencer 1995), land-based agriculture (Hillerton and Berry 2003), and has more recently arisen as a concern in aquaculture. In general, the streptococci are phenotypically described as bacterial cells that are spherical or ovoid, 0.5-2.0 μm in diameter, occurring in pairs or chains when grown in liquid media, and stain Gram-positive (Facklam 1976). A feature of streptococci is they produce haemolysins which lyse red blood cells, with either greenish discoloration (α -haemolysis) or complete clearing (β -haemolysis).

There are a number of streptococci that have been reported as pathogens of aquatic animals (Bromage 1997), but *Streptococcus iniae* has clearly emerged as the greatest global threat. The first appearance of *S. iniae* was in the United States in a captive dolphin (*Iniae geoffrensis*) housed in an Arkansas aquarium (Pier and Madin 1976). The dolphin possessed golf-ball sized lesions on the dorsal surface, from which the bacterium was isolated in monoculture. The disease was resolved in this animal through treatment with penicillin, but a number of years later another dolphin in this facility succumbed to infection with a 2nd isotype of the bacterium (Pier *et al.* 1978).

This fairly non-descript beginning of *S. iniae* has been proceeded by the enormous media attention this bacterium has recently received. The primary cause of media attention was the transmission of the bacterium from infected fish to humans (Litt *et al.* 1997), and more recently the enormous losses of fish in both aquaculture and wild fish stocks (Ferguson *et al.* 2000; Colorni *et al.* 2002). In this respect, *S. iniae* is now lumped into a unique group, the zoonotic bacteria, which are bacteria that are transmitted from an animal host and cause disease in humans.

The bacterium has become widespread throughout the world in wild populations of fish as well as reaching epidemic levels in aquaculture enterprises. The level of knowledge available on *S. iniae* is rapidly developing and this review is attempts to discuss the current knowledge and to expand on future research directions.

2.2 Methods of Isolation and Characterisation

The isolation of *S. iniae* is relatively simple when the bacterium is present in high numbers, for example in infected fish. The typical choice of growth medium is blood agar or brain heart infusion agar. Unfortunately, these agars are not selective and prove inefficient at isolating the bacterium from the environment due to the large numbers of other bacteria present. A selective media was developed that was designed specifically for this purpose (Nguyen and Kanai 1999), which proved successful for the isolation of the bacterium in tropical waters (Bromage *et al.* 1999; Bromage and Owens 2002).

Identification of the bacterium can be accomplished by a number of methods. The standard technique is *via* biochemical characterisation that examines the utilisation of various substrates. This technique is laborious and requires considerable equipment and expertise. Commercial bacterial identification

systems, such as the API 20 Strep, (BioLog, Hayward, CA) are yet to list *S. iniae* in their database, although their kit can be used and manually read.

There are a number of flaws with biochemical characterisation, specifically the strain variation in biochemical reactions. The variability of the biochemical profile of *S. iniae* isolates was previously demonstrated (Bromage 1997). Upon comparison of all the published biochemical profiles of *S. iniae* strains that have caused outbreaks in teleosts, a profile variability of over 15% was observed.

More recently molecular techniques have aided in the identification and classification of *S. iniae*. In a simple but elegant study, Goh and coworkers (1998) demonstrated that *S. iniae* could be identified from closely related species by the chaparonin 60 gene (*cpn60*). In this technique they amplified a 600 base pair DNA fragment, from the highly conserved *cpn60* gene region and performed a reverse checkerboard hybridisation against specific probes. While the authors were able to use this method successfully for identifying *S. iniae* on the mucosal surfaces of tilapia they did not explore the possibility of using this method for identifying the bacterium in mixed samples, such as organ homogenates of the brain or kidney.

Other techniques utilised for the positive identification of *S. iniae* include the 16S RNA gene sequence, pulse field gel electrophoresis, and 16S-23S ribosomal DNA intergenic spacer (Eldar *et al.* 1997; Berridge *et al.* 1998; Marchesi *et al.* 1998; Lau *et al.* 2003). Each of these methods have individual advantages, but do not represent an effective means for screening a large number of isolates due to the cost per sample, the expertise, and equipment required.

Unfortunately there has been no research into antibody based detection and identification of *S. iniae*. The production of these probes has been very successful in monitoring and surveillance of many pathogens (Coll and Dominguez-Juncal 1995; Nicholson 1995; Poulos *et al.* 1998; Jitkasem *et al.* 1999), giving culturists advance warning, and rapid diagnosis in the field.

2.3 Outbreaks of *Streptococcus iniae*

2.3.1 Aquaculture

The impact of *S. iniae* on the successful culture of farmed fish has dramatically increased over the last 20 years, and it currently causes tremendous losses in the industry. The bacterium is not restricted in its host range, or a specific culture environment, as *S. iniae* can be isolated from fresh, salt and brackish water operations.

Streptococcal outbreaks were common in Japan in cultured yellowtail, *Seriola quinqueradiata*, starting as early as the 1950's. The etiological agent of disease was unknown or misidentified for a long time (Hoshina *et al.* 1958; Kusuda *et al.* 1976), with the primary cause of losses only identified over 30 years later as *Lactococcus garvieae* (Muroga 1990; Eldar *et al.* 1996). In 1979 (Minami *et al.* 1979) reported another species of streptococci affecting yellowtail, identifying the bacterium as *S. equisimilis*. The biochemical evidence presented in the published report clearly illustrates that the isolate was *S. iniae*, not *S. equisimilis*, which could have been determined via comparison to already the published reports of Pier and coworkers (Pier and Madin 1976; Pier *et al.* 1978). This was the first time that *S. iniae* had been isolated from cultured fish, and the beginning of major stock losses in Japan.

In the following years, there were numerous reports of streptococcosis due to *S. iniae* in Japan. Two further reports (Minami *et al.* 1979; Ohnishi and Jo 1981) described the concurrent isolation of an alpha and a beta-haemolytic streptococci from diseased ayu (*Plecoglossus altivelis*) and amago (*Oncorhynchus rhodurus* var. *macrostomus*). The authors correctly identified the beta-haemolytic isolate as *S. iniae*. The second report was from a marine cage facility in the Mie prefecture (Kaige *et al.* 1984). In this instance the authors reported that 2.1% of the yellowtail, *Seriola quinqueradiata*, cultured in this facility were suffering from vertebral deformity, specifically vertebral lordosis, kyphosis and scoliosis. The authors speculate that exposure to

S. iniae in the fry stages resulted in the observed deformities and subsequent retarded development.

A broad study on bacterial diseases observed in freshwater fish cultured in Japan (Kitao *et al.* 1981), described identical isolates of beta-haemolytic streptococci infecting tilapia (*Tilapia nilotica*), steelhead trout (*Salmo gairdneri*), and ayu (*P. altivelis*). Despite extensive biochemical and serological characterisation conducted by the authors, they indicated that these isolates were not comparable to any published species of *Streptococcus*. Again, the authors failed to compare their results to the published reports of *S. iniae* (Pier and Madin 1976), resulting in incorrect identification. By the mid 1980's, *S. iniae* was endemic throughout the Japanese aquaculture industry, with further reports of outbreaks occurring in flounder (*Paralichthys olivaceus*), ayu (*P. altivelis*), amago salmon (*Oncorhynchus rhodurus*), rabbitfish (*Siganus fuscescens*), and flounder (*Paralichthys olivaceus*) throughout the country (Ohnishi and Jo 1981; Ugajin 1981; Nakatsugawa 1983; Sugita 1996; Nguyen *et al.* 2002).

The next reported outbreak occurred in a multi-species aquaculture facility located on the northeast coast of Singapore (Foo *et al.* 1985). The unusual aspect of this outbreak was that it only caused significant mortality in one species, the rabbit fish (*Siganus canaliculatus*). The outbreak caused close to 100% mortality but caused limited mortality in barramundi (*Lates calcarifer*) and grouper (*Epinephalus tauvina*). The authors indicated that these two fish species were fed the same feed as rabbit fish, which was thought to be infected with *S. iniae*, and hypothesised that rabbit fish must be more susceptible to the disease. This finding was supported by current evidence that the rabbit fish succumb to the disease at a much lower bacterial load than other species (Yuasa *et al.* 1999; Bromage and Owens 2002).

During the same period as the Singapore outbreaks (1986), *S. iniae* infections became common in Israeli aquaculture (Eldar *et al.* 1994). Initially the outbreaks were focused in rainbow trout (*Oncorhynchus mykiss*) and St. Peter's fish (*Tilapia spp.*), and the bacterium was subsequently determined to

be a different strain to the original isotype isolated in the United States (Eldar *et al.* 1997). In 1996 a new isotype of *S. iniae* emerged in both the Mediterranean and the Red Sea (Colorni *et al.* 2002) in cage cultured Red Drum (*Sciaenops ocellatus*) and seabass (*Dicentrarchus labrax*). This isotype was identical to the American strains, and coincided with the first importation of red drum from the United States. Despite the timing of the outbreak, researchers were unable to establish a definitive connection between the importation and the outbreak of disease (Angelo Colorni, personal communication)

Streptococcus iniae has become established throughout the United States, and is now regarded as the most serious disease threat to the successful culture of tilapines and striped bass (*Morone saxatilis*) in this region (Stoffregen *et al.* 1996; Berridge *et al.* 1998). The first reported isolation (Perera *et al.* 1994) of *S. iniae* appeared to be a biotype of the original type strain isolated in Arkansas. Clearly the bacterium was present in the tilapia aquaculture industry prior to this report, evidenced by the zoonotic transfer of *S. iniae* from tilapia to a human in Texas 1991 (Anon 1996). Subsequent reports clearly demonstrate the fact that the disease has become widespread in the United States, affecting tilapia, striped bass, channel catfish (*Ictalurus punctatus*) and rainbow trout (Stoffregen *et al.* 1996; Berridge *et al.* 1998; Bowser *et al.* 1998; Getchell 1998).

The first occurrence of *S. iniae* in Australia was in a marine-cage facility during summer of 1992 (Bromage *et al.* 1999). The bacterium was responsible for considerable mortality in barramundi, with epizootics occurring every year when the water temperatures increased. Subsequently, the bacterium was isolated from diseased fish at a number of other facilities where barramundi were cultured in both fresh and saltwater. Most recently (2004) the bacterium was responsible for an epizootic in barramundi in that caused an estimated loss of \$2 million (Anon 2004b).

Streptococcus iniae infections have also been implicated in a number of other regions. Since 1994, yearly epizootics have been common in cultured

rabbitfish, *Siganus canaliculatus*, in Bahrain (Yuasa *et al.* 1999) with losses approaching 10% per day when outbreaks occur. Saudi Arabia has also reported mortalities in cultured fish (Al-Harbi 1996). More recently, outbreaks in India have been reported in cultured tilapia (Mukhi *et al.* 2001), again with heavy losses. A presentation at 2nd South American Aquaculture conference 1999, also implicated *S. iniae* as the cause of mortality in cultured tilapia in Venezuela (Clavijo *et al.* 1999), but this has not been reported further.

2.3.2 Isolation of *Streptococcus iniae* from wild fish stocks

Outbreaks of *S. iniae* in wild fish stocks have been uncommon, but the occurrence has increased over the last few years. The first outbreak that could be attributed to *S. iniae* occurred in the tributaries of the Chesapeake Bay (Virginia, United States), while the authors speculate that it was group B streptococci (Baya *et al.* 1990). Subsequent work at the Virginia Institute of Marine Science has indicated that the bacterium was *S. iniae* (present author). More recent surveys have determined that approximately 30% of the striped bass captured in the bay are carriers of the bacterium (Martha Rhodes, VIMS, personal communication). The effect of *S. iniae* on these stocks is currently undetermined, and is likely overshadowed by the concurrent infection with *Mycobacterium* spp a disease with significant impacts on these fish (Rhodes *et al.* 2001; Rhodes *et al.* 2003).

A massive fish kill around the southern Caribbean islands of Trinidad, Barbados, Grenada, and St Vincent in 1999 was attributed to *S. iniae* (Ferguson *et al.* 2000). Many species were affected in the outbreak, including pelagic and reef fish and losses were estimated at 30,000 kg. The occurrence of these fish kills in this region has now become a cyclic event with events reported in 2000, 2001, and 2002 (June Roach, Veterinary Diagnostic Laboratory, Barbados, personal communication). The source of the infection is unknown, but it has been speculated that the bacterium was originally transported to the region in a lens of freshwater that arose from heavy river outflow from the Orinoco and Amazon rivers following a significant rainfall

event in 1999 (Siung-Chang and Lum-Kong 2001). However the more recent outbreaks can not be associated with a similar event and it is likely the bacterium is now resident in local populations of fish.

An enormous epizootic occurred in Kuwait bay, a semi-enclosed embayment of the Arabian Gulf, throughout August and September 2001. It was estimated that 3,500,000 kg of mullet were killed during this period, and the media coverage incorrectly attributed this mortality to *S. iniae*, following a publication by the United Nations Environmental Programme (Anon 2002) who had incorrectly cited a source. This inaccuracy was subsequently corrected (Glibert *et al.* 2002) and the fish kill attributed to *Streptococcus agalactiae*.

Israeli scientists have been isolating *S. iniae* from ever increasing numbers of species of fish from the Mediterranean and Red Sea fish (Zlotkin *et al.* 1998; Colorni *et al.* 2002), and divers have been collecting dead or dying fish in various localities in the Gulf of Eilat (Zlotkin *et al.* 1998). All the current marine isolates grouped into one homogeneous cluster by restriction fragment length polymorphism (RFLP) ribotyping, although they were isolated in different years (since 1995), from different species, wild (Red Sea) as well as cultured on both the Mediterranean and Red Sea (Angelo Colorni personal communication, submitted article).

The isolation of *S. iniae* from numerous wild fish that were collected in and around barramundi enclosures was the first reported infection in the wild fish in Australia (Bromage and Owens 2002). A brief report of an epizootic that caused extensive mortalities in wild barramundi was also reported in 1999 (Bromage 1999). The author suggested that the cause of the outbreak was *S. iniae*, due to the isolation of the bacterium from 94% of morbid and moribund fish.

2.3.3 Human infections

The cause of much of the media attention directed to *S. iniae* came from the isolation of the bacterium from patients in North America. The first incidence of *S. iniae* in humans was reported from a single patient in Texas in 1991 (Anon 1996), however the original source for this report is never cited and unfortunately little information is available. It was a number of years later before *S. iniae* was isolated again, this time in a Toronto hospital (Ontario, Canada) from four patients (Anon 1996). The majority of patients reported a puncture injury while preparing fish for consumption, and had external signs of cellulitis at the point of penetration. One elderly patient had well developed symptoms of endocarditis, meningitis and septic arthritis. A further nine patients in Toronto had positive blood cultures for *S. iniae* over the following years, all with a history of handling fish (Weinstein *et al.* 1996; Weinstein *et al.* 1997).

More recently, *S. iniae* was isolated from two patients in Hong Kong presenting with similar symptoms to those in Canada (Lau *et al.* 2003). The authors make a point of emphasising that all infected patients in North America and China were of Asian origin, but state that the association between *S. iniae* infections and ethnicity as a risk factor is probably an overrepresentation. They correctly identify that the risk factor is more likely based on cultural aspects of food preparation. Asian populations are more likely to purchase whole or live fish, on a more regular basis, than other cultures. Thus the likelihood that they will be exposed to the pathogen is increased.

2.4 Symptomology

2.4.1 External signs of disease

The presentation of the disease in fish is fairly uniform despite the diversity of species that it affects. The most aggressive outbreaks of the disease cause mortality within 24 hours of exposure with minimal external signs of the disease. This rapid occurrence of disease has been reported extensively in laboratory trials as well as cultured fish stocks, primarily from the rabbit fish family (*Siganus* spp.), as well as barramundi (Foo *et al.* 1985; Sugita 1996; Bromage *et al.* 1999; Yuasa *et al.* 1999; Bromage and Owens 2002). In most other species, the infections are usually protracted over a number of days allowing more developed signs of disease to appear (Eldar *et al.* 1994; Evans *et al.* 2000; Colorni *et al.* 2002).

The most commonly reported external signs of the disease are changes to the eyes of infected fish, including bilateral exophthalmia, corneal opacity and in severe cases ocular decomposition (Nakatsugawa 1983; Ghittino *et al.* 1998; Perera *et al.* 1998; Bromage *et al.* 1999). In such cases the bacterium can be isolated readily from the eyes of these infected fish. Less common external signs of the disease are ulcerous lesions on the skin and haemorrhaging around the base of the fins, operculum and anus (Ohnishi and Jo 1981; Eldar *et al.* 1999; Mukhi *et al.* 2001). It is uncertain whether *S. iniae* is responsible for these clinical signs, as they were not reproduced in laboratory trials.

A classic characteristic of *S. iniae* infection is the erratic swimming behaviour of infected hosts. This has given rise to the colloquial expression 'mad fish disease' to describe infection with *S. iniae*. This phenomenon has been reported in wild fish, cultured fish, as well as in laboratory infected fish (Bromage *et al.* 1999; Evans *et al.* 2000; Shelby *et al.* 2002). The cause of this unusual behaviour is believed to be from the degeneration of brain tissue, as well as meningitis which is commonly reported in infected animals (Perera *et al.* 1998).

2.4.2 Internal pathology

The visible internal signs of the disease are usually minor but there have been reports of ascites, as well as enlargement of the liver, spleen, and kidney (Ugajin 1981; Perera *et al.* 1994; Yuasa *et al.* 1999). In severe cases of the disease the bacterium can be observed in blood smears (Foo *et al.* 1985; Evans *et al.* 2001), however it is more likely to be recovered from the spleen, kidney and brain (Sako 1998; Bromage *et al.* 1999; Eldar *et al.* 1999). Histological examination reveals the full impact of the disease, with most organs displaying some pathology.

The liver and the kidney are probably the least affected organs following infection. Cellular infiltration, consisting mainly of macrophages and lymphocytes, is reported within the architecture of both organs, and bacterial dissemination throughout the organs is common (Chang and Plumb 1996; Bromage *et al.* 1999). In the kidney, hyaline droplet degeneration has been reported (Miyazaki *et al.* 1984; Bromage *et al.* 1999). Glomeruli are frequently invaded but little pathology is associated.

More substantiative changes are often observed in the spleen. Frequently there is considerable fibrin deposition within the microcirculation (Perera *et al.* 1998; Bromage and Owens 2002), indicating disseminated intravascular coagulation (DIC). Haemosiderin deposition is usually increased in infected fish (Perera *et al.* 1998), which is likely due to the bacterium's haemolytic ability (Kawahara *et al.* 1991; Fuller *et al.* 2002). Again the bacterium is disseminated throughout the splenic tissue, and cellular infiltration is common.

The heart is often invaded by large numbers of the bacterium, and pericarditis, myocarditis and epicarditis are common findings (Perera *et al.* 1998; Bromage *et al.* 1999; Eldar and Ghittino 1999). Fibrin deposition has been observed, and small granulomas are sometimes present.

In the eyes, severe degenerative changes have been observed. Bacterial and cellular infiltration is pronounced, and inflammation of the periorbital tissue is common. The infiltration of macrophages and neutrophils into the infected tissue, leads to the production of abscesses. In the most severe cases capsular and lenticular necrosis and ulceration of the cornea are observed (Miyazaki *et al.* 1984; Chang and Plumb 1996).

Bacterial meningitis and massive cellular infiltration are reported as the major pathology of the brain (Perera *et al.* 1998). Bacterial laden proteinaceous fluid surrounded by an inflammatory exudate, indicating the possibility of a cranial oedema is reported in barramundi (Bromage and Owens 2002). The brain also appears to be the only site in which the bacteria maintain residence long after the infection is resolved in other organs.

2.5 Mode of transmission and progression of disease

The mode in which *S. iniae* infects susceptible hosts has been the focus of a number of reports. Theories of the route of infection include; through the nasal canal (nares), oral infection, and waterborne exposure with, or without, epidermal scarification. These hypotheses can be further clustered into 2 distinct groups, which are that the fish are exposed to the bacteria from (a) the water or (b) food items. However it should be noted that these two categories are not mutually exclusive.

Waterborne exposure to *S. iniae* has been able to initiate the onset of signs and cause mortality in susceptible species (Perera *et al.* 1997; Shoemaker *et al.* 2000; Bromage and Owens 2002). The lethal dose of bacteria that causes 50% mortality (LD₅₀) varies considerably between species, as does the time until the onset of disease. Perera and co-workers (1997) required 10⁶ cells/ml to achieve a LD₅₀ in tilapia (*Tilapia nilotica* x *T. aurea*), with the first deaths occurring at day 7 and the mortalities continuing over a three week period. This is in contrast to the data reported by Bromage and Owens (2002; Chapter 8) who only required 3.2 x 10⁴ cells/ml in saltwater and less in

freshwater to achieve the LD₅₀ for barramundi. The progression of the disease was more rapid in this species as well, with the first mortalities occurring within 24 hours of challenge, and no further mortalities observed after day 6. These types of variances in susceptibility are expected, and can be attributed not only to the different species of fish, but strain variations of the bacterium as well.

Epidermal abrasions caused from handling, sorting, or other fish have also been hypothesised as a point of entry of *S. iniae* into a host (Foo *et al.* 1985; Bromage *et al.* 1999; Bromage and Owens 2002). However upon examination of this route of entry, it did not appear to increase the susceptibility of host, when compared to control animals (Bromage and Owens 2002).

Evans and coworkers (2000, 2001) examined waterborne exposure in greater detail, looking specifically at whether the bacterium enters through the eyes or the nose. Their data indicated that the nares represented a portal of entry into tilapia (*Oreochromis niloticus*) that led to a lethal infection. This was the first time this route of exposure had been proposed for fish, but was a logical development considering the nares provides direct access to neurological tissue which has been shown to be extensively affected by *S. iniae*.

The pitfall with this waterborne entry is that there are no supporting reports that indicate that *S. iniae* can be isolated from waterways in the concentration required for infection. Thus, this route of exposure may not be the sole source of infection.

Oral infection was indicated as an alternate mode of entry into tilapia and barramundi (Shoemaker *et al.* 2000; Bromage and Owens 2002). Shoemaker suggested that cannibalisation of experimentally infected fish was responsible for the spread of infection. In barramundi, it was determined that the bacterium could be isolated from the faeces of infected fish and that numerous wild fish located in and around aquaculture facilities carry the bacterium (Bromage and Owens 2002). These two findings and the carnivorous nature of barramundi led to experimental research that

demonstrated that ingesting as few as 100 cells could cause mortality in barramundi.

2.6 Virulence factors of *Streptococcus iniae*

The term virulence is a quantitative expression that describes the capacity of a pathogen to cause disease to its host, or its degree of lethality. Virulence factors are a complex interaction between host and parasite and can include factors that promote the entry, colonization, growth, and transportation of the pathogen within the host, as well as those that allow the pathogen to evade the immune system.

There has been little published research on the specific virulence factors of *S. iniae*, but new information is emerging on this important area. One of the most obvious virulence factors is the production of extracellular toxins that cause the complete or partial lysis of red blood cells. This is a key feature of many streptococci, and the compounds are collectively known as streptolysin's. The suffix O is given for partial (alpha) haemolysis and S for complete (beta) haemolysis (Wannamker 1983). In certain culture conditions, *S. iniae* demonstrates both alpha and beta haemolysis (Pier and Madin 1976; Foo *et al.* 1985; Sako 1998; Bromage *et al.* 1999). Recently, the gene sequence responsible for encoding one of the streptolysin's was characterised (Fuller *et al.* 2002). This gene sequence shared significant genetic similarities (74%) with the streptolysin S recovered from Group A streptococci. The authors further speculated that it is the regulation of this haemolysin, depending on culture conditions (oxygen deprived or supplied), which is directly responsible for the alpha/beta haemolysis observed on blood agar, rather than two separate toxins being produced. By mutating the gene responsible for encoding the streptolysin (streptolysin S deficient strain), Fuller and co-workers (2002) also demonstrated that this protein is responsible for some of the tissue pathology observed in artificially challenged

mice. However even in these mutated strains, the bacteria evaded whole-blood killing, and a lethal bacteremia still developed in these mice.

The ability to evade being killed by serum proteins is also a common feature of many streptococci. This usually occurs through the use of external structures such as the M-protein that aid in this function. In his doctoral dissertation, Camus (2002) designed primers to regions of the M-protein that are believed to be conserved throughout all streptococcal species. His findings revealed that no M-protein was present in *S. iniae*, but electron microscopy revealed the presence of a capsule. This later finding was supported by the findings of Barnes and co-workers (2003), who determined that all strains of *S. iniae* they tested, possessed a polysaccharide capsule. The presence of a capsule is vital to virulence of many streptococci, and specific antibodies directed against the capsule can confer protection in the host (Chaffin *et al.* 2000).

The potential significance of *S. iniae*'s capsule is beginning to emerge. New serotypes are becoming evident in infected and vaccinated populations of fish (Bachrach *et al.* 2001; Barnes *et al.* 2003). Clearly, vaccination of the fish with serotype I have led to a shift in the capsular composition resulting in a new serotype (Type II), which is able to evade the protective response developed from vaccination with serotype I strain.

Recently, it was demonstrated that *S. iniae* possesses a surface factor that is able to bind rainbow trout antibody by the Fc-region (Barnes *et al.* 2003). This antibody binding capability is another important virulence factor utilised by many streptococci (Boyle *et al.* 1990). This Fc-binding factor behaves similar to the M-protein in that it enables the avoidance of phagocytosis, and therefore being killed. However a recent publication indicated that *S. iniae* are readily phagocytosed, but in an unusual twist the bacterium was able to survive and multiply within the macrophage (Zlotkin *et al.* 2003). Clearly the bacterium has evolved a mechanism for survival within the host that may utilise binding of the antibody, resulting in phagocytosis, but without initiating the complement cascade that would result in killing. This may also represent

a very effective mechanism for the transportation of the bacterium throughout the host.

A potentially important finding of Camus (2002) was that it was unclear whether streptokinase-like activity was present in *S. iniae*. The bacterium was able to delay but not completely inhibit clot formation in tilapia plasma (*Oreochromis sp.*). Tilapia are not subject to the acute mortalities (<24h) observed in rabbitfish and barramundi upon challenge, and it was hypothesised that a form of anaphylaxis, similar to streptococcal shock syndrome, may be involved in the rapid onset of mortality in barramundi (Bromage 1997). Streptokinase is a potent proteolytic enzyme that is associated with the risk of anaphylaxis in mammals. It would be interesting to test whether streptokinase activity is enhanced in the species that are acutely susceptible to *S. iniae* infection.

2.7 Future research directions

The emergence of antimicrobial resistance among streptococci is making them among the most difficult bacteria to treat in aquaculture situations. As the efficacy of antibiotic therapy rapidly wanes, attention must be focused upon new approaches to controlling infection (Lim *et al.* 2003). Vaccines are one of the few protective measures that can potentially save money and improve output of farming operations. Research should be focused on determining the immunogenic components of *S. iniae*, and developing combination vaccines (Klesius *et al.* 2000), that may prevent future infections by mutated strains of the bacteria. Alternatives to injectable and adjuvant-based vaccines also need to be developed, for example, orally and mucosally-delivered vaccines (Ototake and Yoshiura 2000; Nakanishi *et al.* 2002). There is also significant potential in augmenting 'natural' defences through dietary supplementation (Lim and Klesius 2001; Sealey and Gatlin 2002).

A new approach for controlling streptococci may offer promise for combating many other bacterial species, including those which have developed antibiotic resistance. The treatment involves the use of enzymes created by bacteriophages, tiny viruses that infect bacteria, replicate within them, and then use enzymes to cut through the bacterial cell walls and escape (Fischetti 2003). One of the most exciting benefits of this new technique is that it prevents transmission of bacteria between susceptible hosts.

An additional focus of research should involve the examination of exotoxins produced by *S. iniae*. The effects of streptococcal toxins on the mammalian immune system are well documented. These toxins are responsible for complications such as toxic shock syndrome, necrotizing fasciitis, and forms of psoriasis and atopic disease (Leung *et al.* 1998; Ginsburg 1999; Molinari and Chhatwal 1999; McCormick *et al.* 2001). However this research is in its infancy with *S. iniae*, despite being an important facet of bacterial pathogenesis.

Other possibilities from research beyond the treatment of diseased animals must include the development of diagnostics for rapid identification of infections within populations. Early recognition of the disease within a population will allow preventative management strategies to be enacted, resulting in a reduction in mortality.

CHAPTER 3

GENERAL MATERIAL AND METHODS

3.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Methods

A mini Protean II apparatus (BioRad, Australia) was used throughout for all electrophoresis according to the manufacturer's directions. Gels were produced according to the methods of (Harlow and Lane 1988) without modification. Gels were made to a thickness of 0.75 mm, and were typically a 12% resolving gel and 4% stacking gel. Protein samples were loaded at a concentration of 2 µg per lane for silver staining (Sigma, St Louis, MO) and western blotting, or 10 µg per lane for Commassie Blue staining (BioRad, Australia). Gels were run at 125 V until the dye front reached the bottom of the gel, and immediately removed from the cassette for staining or transfer.

3.2 Blood Processing

Blood was collected into sterile plastic tubes (Nunc, Australia) and allowed to clot for 4 h at room temperature. The clot was dislodged from the side of the container and broken up using the end of a pipette and placed at 4⁰C for a further 24 h. The solution was then centrifuged at 2000 g, the resulting serum was removed, and aliquoted into small units and stored at -20⁰C until required.

3.3 Western Blotting

Protein samples were separated using either sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) or sodium dodecyl sulphate composite agarose gel electrophoresis (SDS-CAGE). The transfer of gel

contents occurred according to the methods of (Towbin *et al.* 1979). Basically, 1 L of transfer buffer (Appendix 1.1) was chilled to 4⁰C and placed in a large casserole dish. Four pieces of filter paper, cut to a size slightly larger than the gel were placed in this solution and allowed to soak for 30 minutes. Just prior to assembly of the clamping apparatus the gel and a piece of methanol-activated polyvinylidene flouride (PVDF; Millipore, Australia) were placed in the buffer and allowed to equilibrate. The transfer apparatus (MiniProtean II; BioRad, Australia) was assembled according to manufacturers instructions, with care taken to ensure no bubbles are formed in the sandwich. The transfer was conducted at 100 V for 1 h, with constant chilling, before the PVDF was removed and allowed to air dry.

3.4 Monoclonal Antibody Production

The fusion technique used was derived from the methods of Zola (Zola 2000) and Harlow and Lane (1988). The following description was not the method used for the first fusions performed in this thesis but represents the final method utilised which provided the best production of viable hybridomas.

One week prior to the fusion, one vial of myeloma cells (Sp 2/0 ag-14; in-house stock) were rapidly thawed in a 37⁰C water bath. The vial was then centrifuged at 500 *g* for 5 minutes and the media removed. Cells were resuspended in 8 ml of growth media (CSL MDM; TropBio, Townsville, Australia) supplemented with 10% fetal bovine serum (FBS; Strarrate Pty Ltd, Wagga Wagga, Australia) and cultured in a flat bottom 25 cm² polystyrene flask (Nunc, Denmark). The cultures were grown in a humidified incubator, supplemented with 5% CO₂, maintained at 37⁰C. Two days prior to the fusion the cells and media were transferred into a 75 cm² flask (Nunc, Denmark), and CSL-MDM with 20% FBS and 1 x oxaloacetate-pyruvate-insulin (OPI; Sigma, St Louis, MO) were added to make the total volume 20 ml. On the day immediately prior to the fusion, 4 ml of the cells were transferred into five, 25 cm² flasks, and fresh media added (CSL-MDM, 20% FBS, 1 x OPI) to make a total volume of 8 ml. On the day of the fusion, cells were pooled into

a 50 ml conical bottom tube (Falcon, USA), and viability was checked by the exclusion of trypan blue. If the viability of the SP2/0 cells was greater than 95% the fusion was performed.

The spleen cells were recovered from a previously vaccinated mouse (Chapter 5.2.2.2 and 10.2.1.3), immediately prior to the fusion. All procedures were conducted aseptically and unless otherwise stated, all solutions were maintained at room temperature. The mouse was euthanased by CO₂ asphyxiation, and the spleen removed through a small incision in the upper left side of the peritoneal cavity. The spleen was placed into a Petri dish containing 20 ml of CSL-MDM, and splenocytes were purged from the capsule by injecting growth media into the capsule drawn using a 5 ml syringe with a 22 gauge needle. Utilising bent forceps, the capsule was gently massaged to break up the remaining spleen cells clumps. The capsule was again flushed with media to remove the remaining cells, and finally the capsule was discarded.

The media containing the cells was repeatedly aspirated through a 25ml pipette until a single cell suspension was achieved, and transferred to a 50 ml conical bottom tube (Nunc, Australia). The Petri dish was washed with fresh CSL-MDM to recover any remaining cells, and added to the tube. Bovine donor serum (BDS) (2-3 ml) was carefully added to the bottom of the tube (underlay) and the cell suspension was allowed to settle for 5 minutes. Cells on or above the BDS interface were collected, and the clumps below discarded.

The two tubes containing the myeloma and spleen cells were then washed three times in CSL-MDM, through the subsequent use of centrifugation (500 g for 5 minutes) and resuspension in fresh media (50mls). A cell count was performed on each vial, and the spleen cells were added to the myeloma cells at a ratio of 10 to 1 and centrifuged (400 g for 10 minutes) to form a loose pellet of cells. The day old media from the myeloma cells was kept (conditioned media), and utilised in the final step of the fusion.

In order to perform the fusion, 1 ml of hybridoma tested polyethylene glycol (PEG; Sigma, St Louis, MO) was rapidly added to the cell pellet. This solution was gently agitated for 1 minute by flicking of the bottom of the tube to resuspend the cell pellet. Following this step, the fusion was slowly stopped through the drop-wise addition of 3 ml of CSL-MDM over 10 minutes, followed by 7 ml over a further 10 minutes with constant agitation. The resulting fused cells were then gently centrifuged (300 g X 10 minutes), media removed, and resuspended into a volume of selective media that gives a final concentration of 2×10^5 myeloma cells/ml (CSL-MDM, 20% FBS, 1 X OPI, and 2 x hypoxanthine-aminopterin-thymidine (HAT; Sigma, St Louis, MO)). To this solution an equal volume of conditioned media is added (giving a final solution of CSL-MDM, 20% FBS, 1 X OPI, 1 X HAT) and cells allowed to rest for 1 h at room temperature. The cells were then resuspended and 200 μ l of this solution was added to each well of a 96 well tissue culture plate (Nunc, Denmark) and placed in a 37⁰C humidified incubator.

Ten days following the fusion the plates were examined for colony development, and if present, the cell culture supernatant were collected and tested for specific antibody production. When cells chosen for further evaluation reached 25% confluence, they were transferred into one well of a 24 well plate (Nunc, Denmark), supplemented with CSL-MDM, 20 % FBS, and 1 X hypoxanthine-thymidine (HT; Sigma, St Louis, MO). Cells were then cloned (Chapter 3.7) or expanded and stored frozen (Chapter 3.6).

3.5 Cryopreservation of Hybridoma Cells

Rapidly growing hybridoma cells were pelleted via centrifugation (500 g X 10 minutes), and resuspended into fresh growth media, containing 20% BDS, to a concentration 2×10^7 cell/ml. To this solution ice cold dimethyl sulfoxide (DMSO; Sigma, St Louis, MO) was added to achieve 10% v/v. The solution was placed into a 1.5 ml cryopreservation tube (Nunc, Denmark) which was then wrapped in cardboard and placed at -80⁰C overnight. Following this step the vial was placed into liquid nitrogen for long term storage.

3.6 Limiting Dilution

The process of limiting dilution was conducted to ensure single cell cloning of hybridomas. This was performed by resuspending cells in CSL-MDM supplemented with 20% BDS, to a cell density of 20 cells/ml. A 96 well tissue culture plate (Nunc, Denmark) was divided into 4 groups containing 3 columns each. To the first group 200 μ l of this solution was added, 100 μ l to the second, and 50 μ l and 25 μ l to the final 2 groups. Each group was then filled to 200 μ l total with growth medium containing 20% BDS. This process ensured that 4, 2, 1 and $\frac{1}{2}$ cells were present in the respective groups 1 through 4. The cells were allowed to grow for 10 days before being screened by enzyme-linked immunosorbent assay (ELISA). Wells testing positive for specific antibody were examined via a microscope, and those thought to contain a single colony were re-cloned, following the above procedure. Cloning was considered complete when 100% of the wells containing cells tested positive in ELISA.

3.7 Formalin Fixation of *Streptococcus iniae*

All stocks of *S. iniae* were stored at -80 C in multiple aliquots. Unless otherwise stated the isolate #28 was used through the entire study, grown at 27 C on brain heart infusion agar (Oxoid, Basingstoke UK). A 1 L flask, containing 500 ml of sterile brain heart infusion broth (BHI; Oxoid, Basingstoke UK) was inoculated with *S. iniae* and incubated at 27^oC for 12 h on an orbital shaker. The resulting solution was centrifuged (10,000 g X 20 minutes) to obtain a bacterial pellet, before being resuspended in 100 ml of phosphate buffered saline (PBS; Appendix 1.2). To this solution 2 ml of formaldehyde (Sigma, St Louis, MO) was added to achieve a final concentration of 3% formalin and the solution was gently agitated for another 6 h. The killed cells were harvested, washed three times and resuspended in PBS at 1 X 10⁹ cell/ml supplemented with 0.03% formalin to maintain sterility.

CHAPTER 4

THE ISOLATION AND CHARACTERISATION OF BARRAMUNDI IMMUNOGLOBULIN

4.1 Introduction

The basic structure of teleost immunoglobulin has been well characterised through numerous published reports (Hall *et al.* 1973; Lobb and Clem 1983; Lobb *et al.* 1984; Lobb 1986; Lobb and Olson 1988; Kaattari *et al.* 1999). Teleost Ig is composed of 8 heavy and 8 light chains that are assembled intracellularly to form a tetramer that can be expressed either on the surface of B lymphocytes or secreted into the serum from plasma cells (Kaattari and Yui 1987). The form and function of teleost Ig has been compared to that of mammalian IgM, however, notable differences do exist.

The most obvious difference is that of the quaternary structure of the Ig molecule, tetrameric versus pentameric for teleost and mammalian Ig. A second and very unique difference occurs in the way in which the Ig is assembled. The pentameric Ig from mammals is completely cross-linked through the use of disulfide bonds, while teleost Ig displays a varied assembly pattern that can involve complete, partial, or no disulphide linkages. This is despite the fact that predominantly all fish Ig is tetrameric *in vivo* (Lobb and Clem 1983). The significance of this form of assembly is still to be fully elucidated, but it has recently been suggested that this may represent a unique approach to antibody maturation in fish (Kaattari *et al.* 1999). Generating antibodies with various redox forms would allow considerable flexibility in the antibody molecule, something that is unparalleled in the mammalian immune system. This flexibility may allow an antibody molecule to bend to adhere to many antigen binding sites, thus dramatically increasing the molecule's affinity.

This chapter will examine the structure of barramundi immunoglobulin and compare it to other teleost species.

4.2 Methods

4.2.1 Collection of blood

Whole blood was collected from the caudal vein of adult fish (1-3 years old) using a 5 ml syringe with a 22 gauge needle. Blood processing occurred as described in Chapter 3.2. A rainbow trout serum was supplied from Virginia Institute of Marine Science.

4.2.2 Purification of Ig using Protein A

The purification of Ig was achieved through the modification of a method employed by Estevez and co-workers (1993). First the serum was prepared by adding a 1/10 volume of 1 M Tris (pH 8.0, Sigma, St. Louis, MO). The resulting solution was filtered through a 0.22 μm filter (Millipore, Australia) to remove particulate matter. The antibody solution was passed through a protein A column (BioRad, Australia) at a flow rate of 0.25 ml / min, with the flow through being monitored via spectrophotometry at 280 nm. When all the protein was passed through, the beads were washed with 10 column volumes of 100 mM Tris (pH 8.0) followed by 10 column volumes of 10 mM Tris (pH 8.0). The bound IgM was eluted from the column using 50mM glycine (pH 3.0, Sigma, St. Louis, MO) at a flow rate of 0.35 ml / min. The resulting IgM sample was collected; pH neutralised by adding 1 M Tris (pH 8.0) to the sample, and concentrated. The column was re-equilibrated with 1 M Tris (pH 8.0) and the initial flow through placed back onto the column and the process repeated again. The eluted Ig samples were concentrated and buffer exchanged to phosphate buffered saline (PBS) using a Centriprep 30 (Amicon, Beverly, USA). Protein concentrations of the eluted samples

were determined using BCA (Pierce Chemicals, USA) and purity confirmed by SDS-PAGE (Chapter 3.1).

4.2.3 Characterisation and molecular weight determination

A column 100 cm in length and 26 mm in width was packed with Sephacryl-S300 (Pharmacia-Biotech, Castle Hill, Australia) following manufacturer's instructions. Bed height was 91cm with a bed volume of 230 ml. The column was equilibrated with a protein buffer (0.5 M sodium phosphate pH 7.0 + 0.15 M NaCl) running at 1 ml/min. Molecular weight standards (Sigma, St Louis, MO), blue dextrose 2000 kDa, thyroglobulin 669 kDa, beta-amylase 200 kDa, bovine serum albumin 66 kDa and cytochrome C 12 kDa, were added to the column and monitored via ultraviolet (UV) absorbance at 280 nm and plotted on a chart recorder (BioRad, Australia). One milligram of purified barramundi Ig was added to the column, and the flow through collected in 1 ml samples. Each sample was tested for the presence of Ig by SDS-PAGE and ELISA. The elution profile was plotted against the standards, and molecular size estimated for the eluted fractions.

4.2.4 Ig detection in samples

Monoclonal antibodies produced against barramundi Ig were used in a capture ELISA (Chapter 5) to determine whether protein A completely absorbs all Ig from serum and to test the fractions eluted from the S300 column. The absorbance was read while the rate of the reaction was still linear. The amount of Ig captured was determined by comparison to a serially diluted control of purified Ig that was added in duplicate to each plate.

4.2.5 Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis

Purified barramundi Ig was electrophoresed on SDS-PAGE under reducing conditions (Chapter 3.1). The gels were silver stained (Sigma, St Louis, MO) and molecular weights of the proteins were determined by comparison against broad range markers (BioRad, Australia) and computed using the Syngene system (Syngene Inc. USA).

4.2.6 Redox profile

One µg of purified Ig was added to 2 x SDS-PAGE loading buffer minus 2-beta-mercaptoethanol (Appendix 1.3) and boiled for 5 minutes (denaturing but not reducing). A 3.1% composite agarose - acrylamide gel (CAGE gel) was made according to the methods of Jackson and co-workers (Jackson *et al.* 1980). Immunoglobulin samples were added to individual lanes with affinity purified rainbow trout Ig, human IgM, IgA, and IgG (Sigma, St Louis, MO) as standards. Samples were run for 2 hours at 100 V or until the dye front reached the bottom of the gel. The contents of the gel were transferred to PVDF as per Chapter 3.3. The membrane was washed thoroughly with ddH₂O and stained with colloidal gold (BioRad, Australia) to visualise the proteins.

4.3 Results

4.3.1 Purification of Immunoglobulin

Protein A was effective in purifying Ig from barramundi sera and *Oreochromis mossambicus*. However it failed to extract Ig from rainbow trout. The first purification of barramundi Ig resulted in 1.2 mg of purified Ig from 1 ml of whole serum as determined by BCA and ELISA. When the initial flow through was passed through the column again, 0.9 mg of Ig was obtained, followed by 0.3, 0.2 and 0.05 mg, resulting in a total of 2.65 mg of Ig from 1 ml of sera. The tilapia followed a similar profile (Table 4.1) to that of barramundi, while no Ig could be recovered from rainbow trout sera.

Table 4.1. The amount of Ig recovered using protein A from 1 ml of whole serum from several different fish species as determine via BCA and ELISA.

Species	Wash					Total	Residual
	1 st	2 nd	3 rd	4 th	5 th		
Barramundi	1.2	0.9	0.3	0.2	0.05	2.65	0.05
Tilapia	1.4	0.8	0.5	0.4	0.1	3.4	N/A
Rainbow trout	0	0	0	0	0	0	3.25

4.3.2 Characterisation

The purified Ig samples obtained from the Protein A purification process showed that reduced Ig consisted of a single heavy and light chain with approximate molecular weights of 72 and 28 kDa for barramundi and 74 and 30 kDa for tilapia (Figure 4.1).

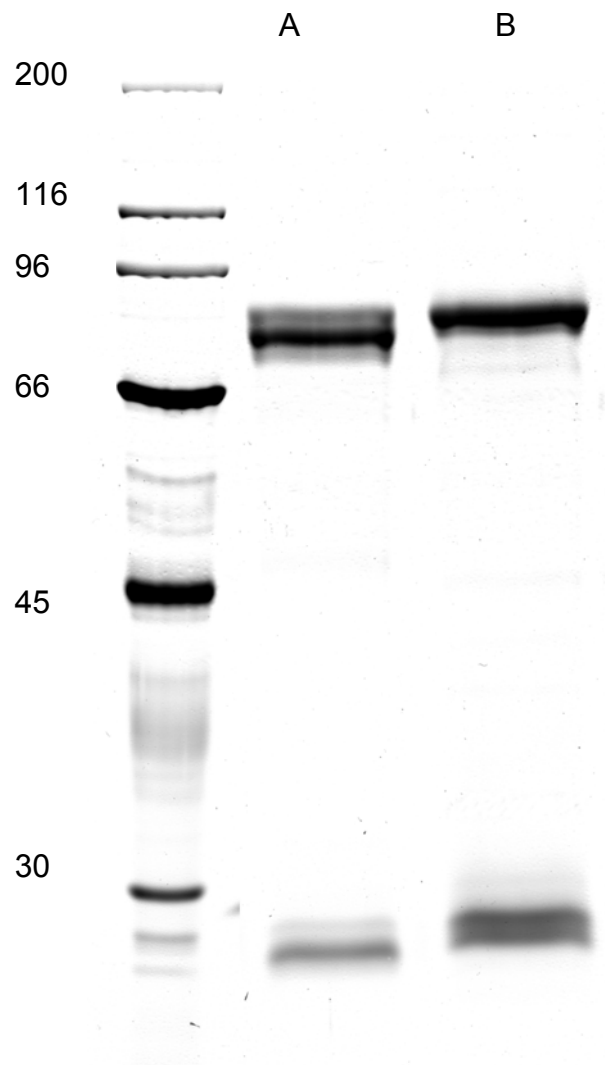


Figure 4.1 The SDS-PAGE profiles of immunoglobulin isolated from the serum of (A) *Lates calcarifer* and (B) *Oreochromis mossambicus* using protein A.

4.3.3 Molecular weight determination

The void volume of the column, as indicated by elution of blue dextran was determined to be 74.5 ml. Native Ig chromatography of the purified barramundi Ig estimated its molecular weight to be 788 kDa (Figure 4.2) from the single well-defined peak observed (Figure 4.3). The ELISA confirmed that protein peak was barramundi Ig. Rainbow trout Ig was estimated to 802 kDa and tilapia 807kDa (data not shown).

When whole barramundi sera was applied to the column and fractionated (Figure 4.4), Ig could only be detected via ELISA in the high molecular weight fractions corresponding with the same Ig positive fractions in the purified sample.

4.3.4 Redox forms

Under denaturing but non-reducing conditions the various barramundi Ig displayed unique redox profile when compared to the other species tested (Figure 4.5). Barramundi Ig redox profile consisted of dominant bands estimated at 790, 580, 400 and 200, representing tetramer, trimer, dimer and monomer. There is also the presence of $\frac{1}{2}$ mer stepwise progression of Ig. The Ig purified from tilapia displayed a similar profile to that of the rainbow trout.

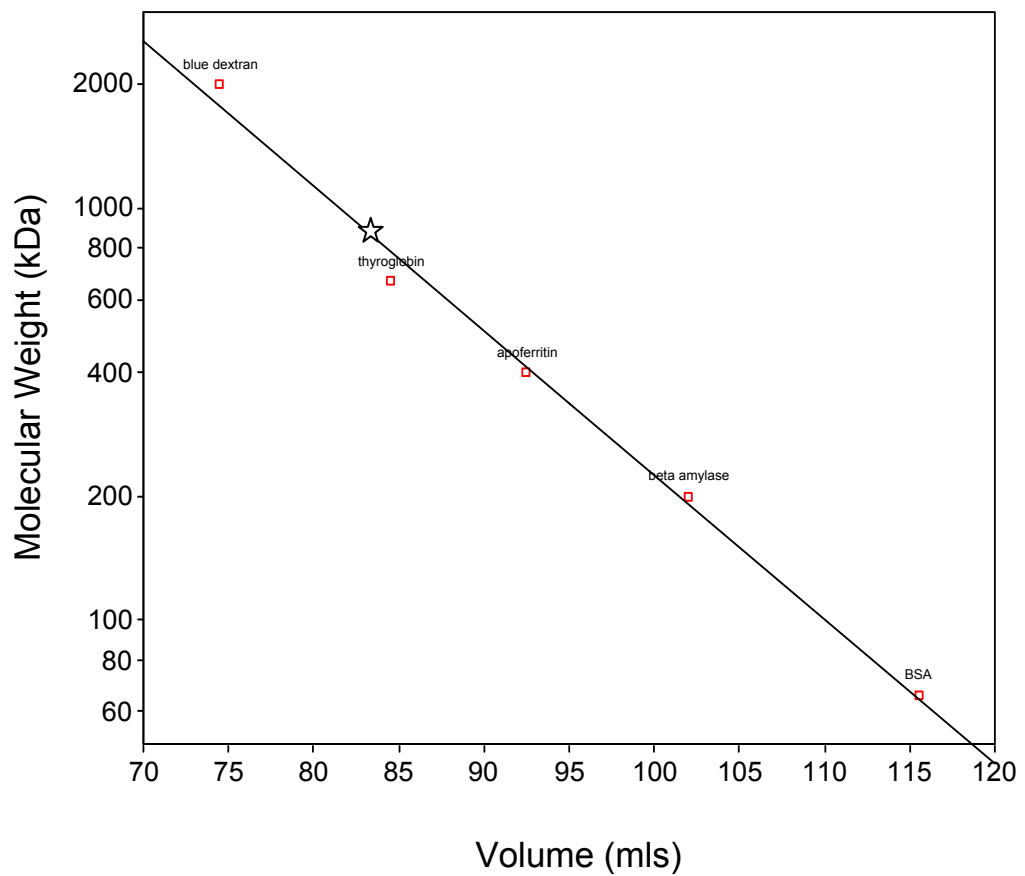


Figure 4.2 The elution profile of various molecular weight standards on a Sephracryl S300 column and the size estimation of barramundi Ig (Star) using these standards ($r^2=99.6$).

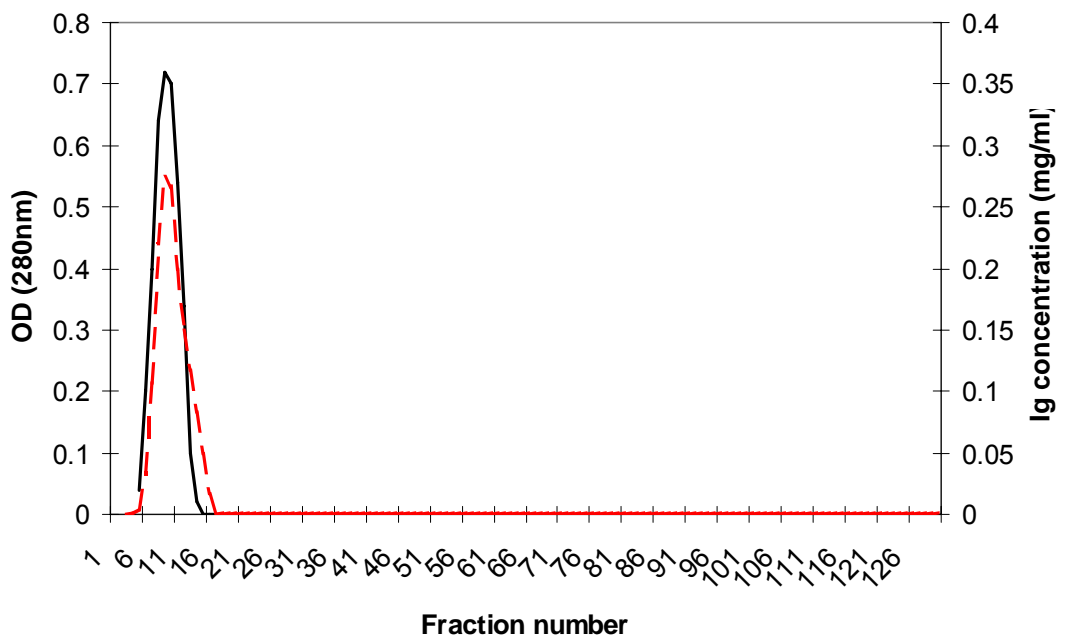


Figure 4.3 Elution profiles of protein A purified barramundi Ig using a Sephracryl S300 column. The secondary y axis indicates the amount Ig detected using a capture ELISA (red dashed line).

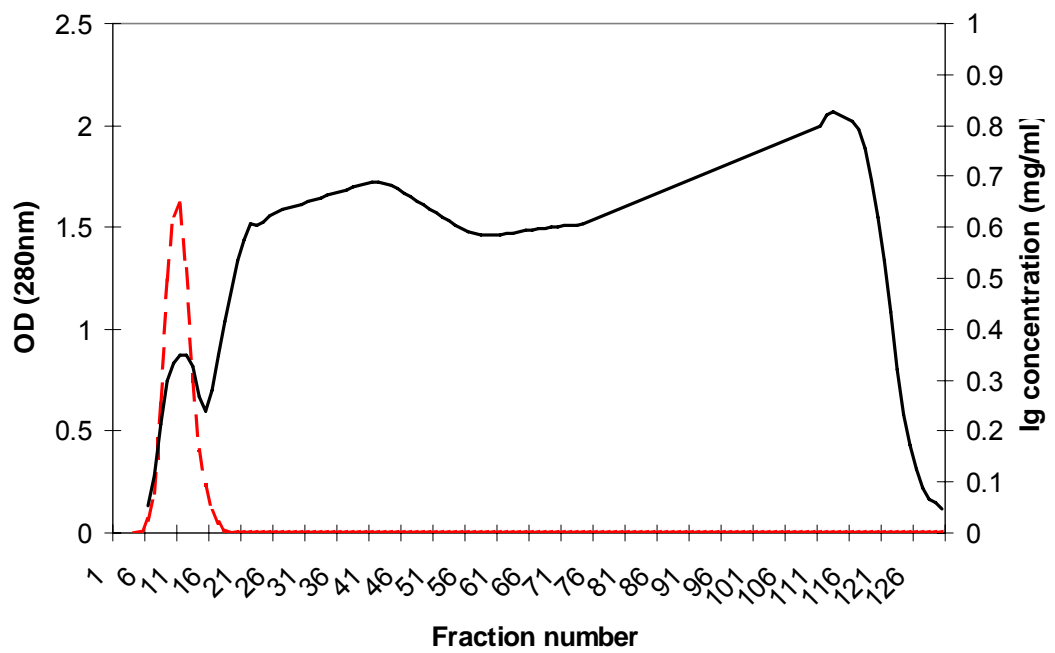


Figure 4.4 Elution profiles whole barramundi sera using a Sephracryl S300 column. The secondary y axis indicates the amount Ig detected using a capture ELISA (red dashed line).

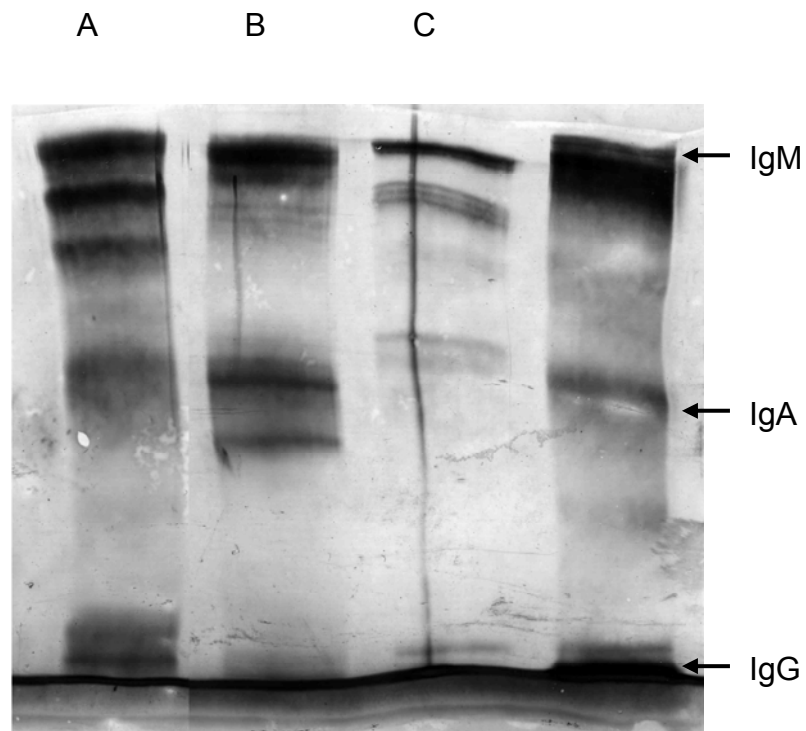


Figure 4.5 The structural diversity of teleost Ig when subject to denaturing but not reducing conditions within a composite agarose acrylamide gel (SDS-CAGE). Lane (A) barramundi (B) tilapia (C) rainbow trout. Last lane is human Ig markers.

4.4 Discussion

The use of protein A for the purification of barramundi immunoglobulin resulted in excellent purification, with minimal contamination from other serum proteins. Furthermore, Protein A was able to remove all Ig present in serum as determined by ELISA and SDS-PAGE.

Despite the reported specificity of Protein A for IgG (Protein A Manual, BioRad, Australia), it has been successfully used for the purification of IgM from various fish species such as the southern blue fin tuna (*Thunnus maccoyii*) and turbot (*Scophthalmus maximus*) (Estevez *et al.* 1994; Watts *et al.* 2001). However, it has never been demonstrated whether Protein A is able to extract all Ig from serum. This set of experiments determined that Protein A was able to successfully purify virtually all Ig from barramundi serum. This was only possible through the successive addition of the filtrate back through the Protein A column, whereas increased incubation time or the use of MAPS buffers failed to increase the yield of Ig (data not shown).

Under reducing conditions, barramundi immunoglobulin resolved into a single heavy and light chain of approximately molecular weight 72 and 28 kDa, respectively. This is in good agreement with the findings of (Bryant *et al.* 1999), but in contrast to those of (Crosbie and Nowak 2002), who determined the heavy and light chains of barramundi to be 86 and 24 kDa respectively. The discrepancy between the 2 results is difficult to explain. Glycosylation of teleost heavy chain can contribute up to 17% its molecular weight (Sanchez and Dominguez 1991). Partial deglycosylation via degradation of the Ig can result in different MW heavy chains becoming apparent, however there is no published data of L chain glycosylation in fish. Therefore, the discrepancies must be due to technique differences, or molecular weight estimation methodology.

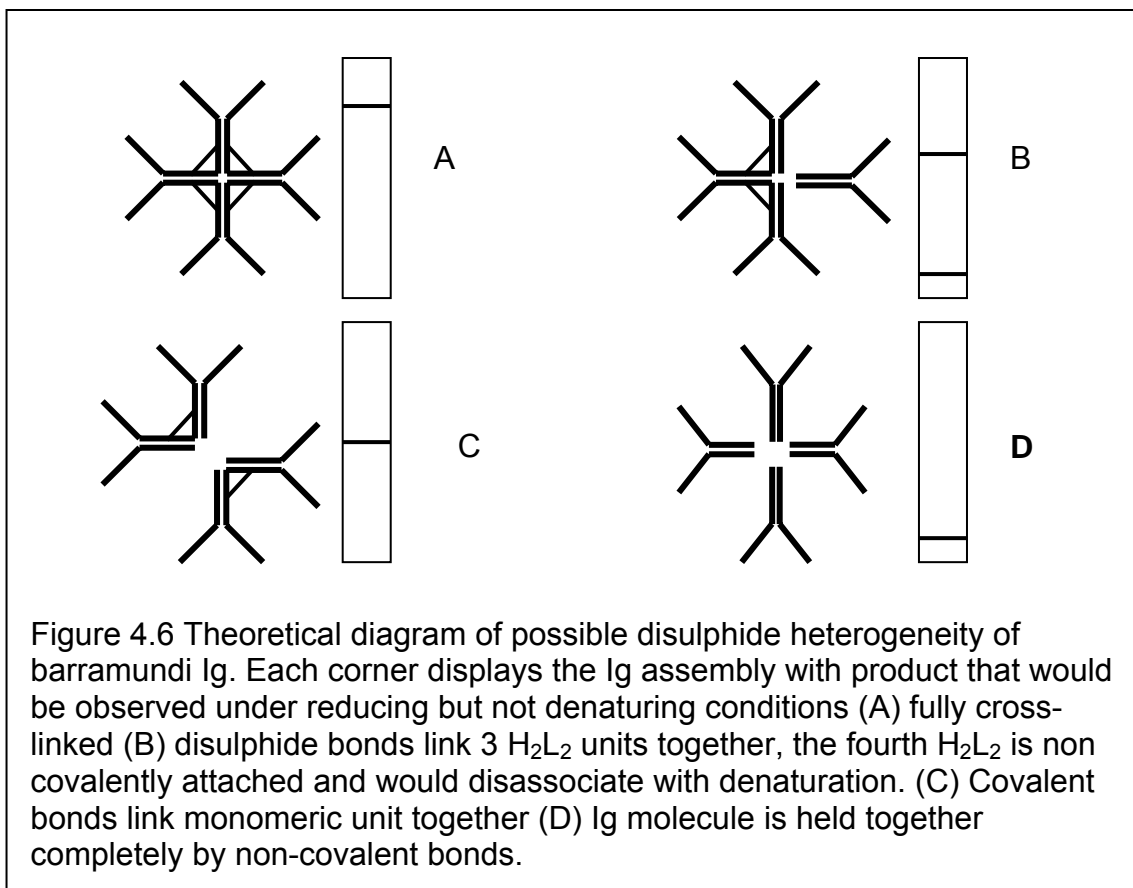
When protein A purified barramundi Ig was processed through an S300 column, the Ig eluted as a single peak with an estimated molecular weight of 788 kDa. The peak was confirmed as barramundi Ig by the positive ELISA

reaction as well as by the presence of pure heavy and light chains in SDS-PAGE. No protein peak was observed in the range of a monomeric subunit (200 kDa) which is contrast to the findings of Bryant and co-workers (1999), who suggested that a unique population of monomeric Ig was present in barramundi serum. The ELISA results from the whole sera processed through the column also indicate a single molecular weight population of barramundi Ig, however it must be noted that the mAb (17D8) used in this study has not been tested to ensure that it recognised 100% of serum Ig.

While the detection of monomeric Ig in serum has been reported by primary studies (eg. Bryant *et al.* 1999), this potential Ig isotype has not been subsequently verified in the same species by other researchers. Indeed in a study of 6 species of teleosts in diverse families, it was determined that all expressed serum Ig is tetrameric (Bromage *et al.* 2004, Appendix 6). However, even though a single tetrameric form of Ig is consistent among teleost species, at least two different transcribed μ genes in salmon (Hordvik *et al.* 1997, Hordvik *et al.* 2002) and a δ gene in salmon (Hordvik *et al.* 1999; Hordvik, 2002), catfish (Wilson *et al.* 1997), cod (Stenvik and Jorgensen, 2000; Stenvik *et al.* 2001), and Japanese flounder (Hirono *et al.* 2003) have been identified. Despite these recent findings of possible Ig isotypy in teleosts, relative expression of these forms has yet to be forthcoming. However the possibility remains that they may be expressed in small quantities in serum or are only expressed under certain conditions.

This study clearly demonstrates that all barramundi Ig is tetrameric *in vivo*. However under denaturing but not reducing conditions (i.e. the presence of heat and SDS) the tetrameric barramundi Ig was shown to possess considerable structural heterogeneity. The Ig disassociated into 4 major redox forms representing monomeric (H_2L_2) steps from the completely cross-linked tetramer. Figure 4.6 diagrammatically displays 4 possible combinations available for differential Ig polymerization. Barramundi Ig displayed a similar profile to that of the rainbow trout standard which is in agreement with the published results for this species (Kaattari *et al.* 1999).

The presence of the various Ig redox forms observed in barramundi and in other teleosts may provide very unique functional advantages over that of mammalian Ig (Appendix 6). Mammalian IgM, IgA and IgG are completely polymerized (Figure 4.5), while teleost antibody is differentially cross-linked, leading to a dramatic increase in the molecule's flexibility. This flexibility may allow the Ig to bend allowing the Fab region to come in contact with many more antigenic sites on the surface of a bacteria or virus, thus increasing the Ig's overall avidity. This in turn may have a dramatic effect on the effector function of the antibody molecule. Clearly, much research needs to be performed to address this evolutionary strategy evolved by teleosts, which may be the functional equivalent to that of mammalian Ig isotypy.



CHAPTER 5

PRODUCTION OF POLYCLONAL AND MONOCLONAL ANTIBODIES AGAINST BARRAMUNDI IMMUNOGLOBULIN AND THE DEVELOPMENT OF IMMUNOASSAYS

5.1 Introduction

The production of antibodies against invading pathogens forms an integral part of vertebrate's immune response (Janeway *et al.* 2001). The antibodies produced are directed at specific antigenic determinants on the surface of the invader. Any one invader can carry a multitude of different epitopes to which antibodies can respond. Thus, at any time there is a pool of heterogenous antibodies directed at the same antigen but different epitopes (Harlow and Lane 1988).

This is the basis of polyclonal antibody production used for diagnostic research. By immunising an animal with the target antigen, the hope is that the animal will mount an adaptive immune response, resulting in the production of circulating antibodies. These in turn can be collected, purified and utilised for diagnostic assays such as the ELISA. Polyclonal antibodies are a powerful research tool and have been utilised widely in all fields of medical research. They are, however, limited in clinical use because of their lack of specificity and the increased chance of cross-reactivity with similar antigens resulting in high background responses (Harlow and Lane 1999).

Kohler and co-workers (1976) developed the method of isolating and immortalising specific antibody producing B-lymphocytes by fusing them with an 'immortal' myeloma cell. This resulted in the production of identical daughter cells secreting an unlimited amount of epitope specific antibodies. The benefit of this technique is that you have a homologous reagent, with

defined specificity and affinity for the antigen that can be produced in unlimited amounts (Harlow and Lane 1988).

The widespread use of polyclonal and monoclonal antibodies for the investigation of humoral immunity in teleosts has only occurred in the last 15 years (Coll and Dominguez-Juncal 1995). The driving force behind the research is the significant increase in fin-fish aquaculture production and the associated drive for knowledge of the animal physiology to increase production further.

Polyclonal antibodies are relatively cheap to produce and can be done with very limited equipment, while the production of monoclonal antibodies requires a special suite of knowledge and equipment. To date there have been only 2 studies conducted on the immune response of barramundi to challenge by an antigen (Chao and Chong 1986; Bryant *et al.* 1999), both conducted with polyclonal antibodies.

This chapter aims to develop these immunological tools, characterise their specificities and develop assays for use in the following chapters.

5.2 Methods

5.2.1 Immunisation protocols

5.2.2.1 Immunisation of rabbits

Two New Zealand white rabbits (2 years old) were immunised over a period of 36 weeks, initially with an oil-in-water emulsion of Freund's Complete Adjuvant (FCA; Sigma-Aldrich, St. Louis USA) and 100 µg of purified barramundi Ig (bIgM; Chapter 4), with boosters every 4 weeks using Freund's Incomplete Adjuvant and 10 µg bIgM (FIA; Sigma-Aldrich, St. Louis USA). When an acceptable titre developed, as measure by ELISA (described below) rabbits were anaesthetised and blood collected from the marginal ear vein (performed by Prof Phil Summers) and processed as outlined in Chapter 3.2. The serum was aliquoted and stored at -20°C until required.

5.2.2.2 Immunisation of mice

Six week old female Balb/c mice were utilised for the three immunisation techniques used for the production of monoclones against barramundi Ig. The first group (6 mice) used an oil-in-water emulsion of whole IgM (50 µg) and FCA, followed by booster inoculations at week 3 and week 5 of bIgM (5 µg) in FIA. The second and third groups of six mice each were immunised with the heavy and light chain of bIgM respectively. This was achieved by running a purified sample (200 µg) of bIgM on a 10% SDS reducing slab-gel. A small sample of each gel was cut off and stained in Commassie blue (BioRad, Australia). The stained portion of the gel was aligned with the original gel and the heavy and light chain bands were individually excised, mixed in 3 ml of sterile PBS, and emulsified to a stage where the solution would pass through a 25 gauge needle. At weeks 0, 5, and 7, a 400 µl dose of the acrylamide protein mix, which contained approximately 20 µg protein,

was injected intraperitoneally (IP) into the respective mice. At regular intervals, 50 µl of blood was collected from the tail vein of immunised mice so that titres could be monitored during the immunisation process, and specificity monitored via Western blotting. Four days before the fusion the mouse with the highest titre was injected intravenously (IV) with 10 µg of bIgM and 50 µg of bIgM IP.

5.2.3 Determining serum properties

Serum properties of the experimentally injected animals were determined using ELISA and Western blotting. Unless otherwise stated, all steps were performed using 100 µl of each reagent, reacting over a period of 1 h at room temperature. Before the next step, all fluid was removed from the plates and washed 5 times with washing buffer (TropBio, Townsville, Australia).

5.2.3.1 Optimisation of indirect ELISA for detection of barramundi Ig.

Purified barramundi Ig starting at 100 µg/ml diluted in carbonate coating buffer (CCB, TropBio, Townsville, Australia) was added to column 1 of an 96-well round bottom ELISA plate (Sarstedt, Pooraka, Australia). Two-fold dilutions in CCB were performed across the plate and allowed to bind to the plate for 1 h at room temperature or overnight at 4⁰C. The plates were subsequently blocked with post-coating buffer (TropBio, Townsville, Australia). Doubling dilutions of the antiserum (rabbit or mouse) were applied down the plate (Figure 5.1) starting at an initial dilution of 1:100 in row A. Following incubation and washing, the secondary labelled antibody consisting of either goat anti-mouse horseradish peroxidase (HRPO; BioRad, Australia) or sheep anti-rabbit HRPO (Jackson Immuno Research, USA) was applied to the plate at a constant dilution of 1:5000 and allowed to incubate. The ELISA was completed through the addition of 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; KPL, Gaithersburg, USA) and the colourimetric reaction read on a 96 well plate reader (Labsystems,

Multiskan EX) at a wavelength of 414 nm. Controls included on the plate were, no primary antibody added, no secondary, or both.

5.2.3.3 Western blotting

In order to check the specificity of polyclonal antiserum, the technique of Western blotting was performed (Chapter 3.3). In brief whole sera was loaded onto a 10% SDS-PAGE gel and run until the loading dye reached the bottom of the gel. The gel was placed in the Western blotting apparatus (BioRad, Australia) as per manufacturer's instructions. Transfer onto PVDF (Millipore, Bedford USA) was performed at 4⁰C, 30 volts for 3 h. The membrane was then allowed to dry before further processing.

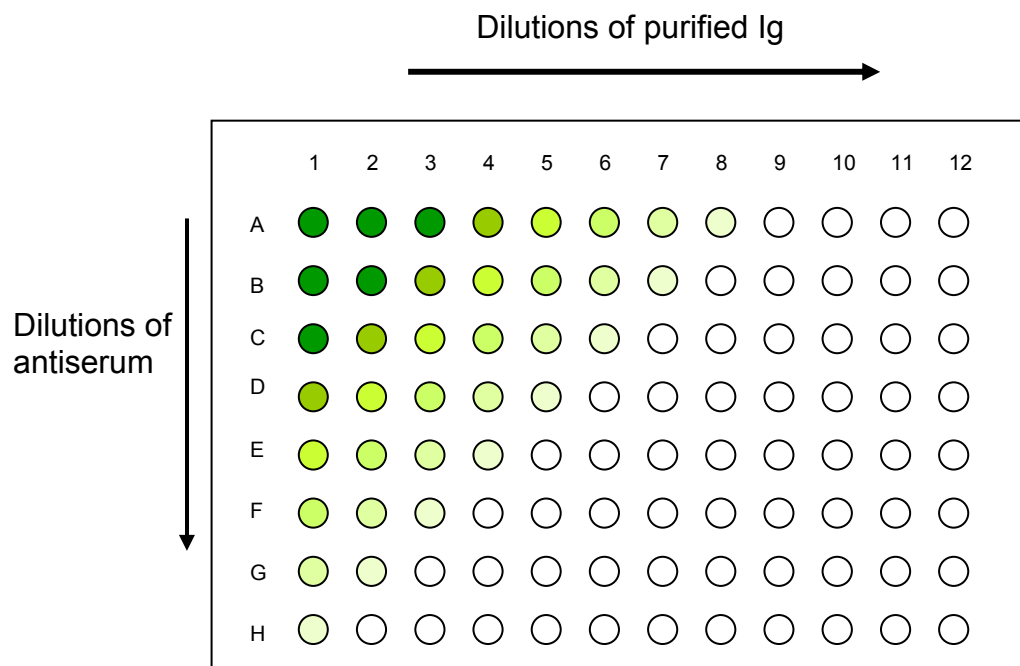


Figure 5.1 The checkerboard system used to determine the optimum coating concentration for barramundi Ig, with consideration for antiserum concentration

Following the drying step, various dilutions of the mouse or rabbit antiserum was applied to the PVDF and gently agitated on an orbital shaker at room temperature for 1 h. The PVDF was washed with 3 changes of PBS prior to the appropriate secondary antibody being added (goat-anti-mouse or goat-anti-rabbit, 1:3000). The chromogens 3, 3' diaminobenzidine tetrahydrochloride (DAB) or amino-ethyl-carbazole (AEC) (Sigma-Aldrich, St. Louis USA) was applied to the PVDF and the reaction was closely monitored. When the desired colour intensity was achieved, the reaction was halted by washing the membrane in distilled water.

5.2.4 Monoclonal antibody production

As per Chapter 3.4

5.2.5 Ascitic fluid production

Balb/C mice were primed with 200 μ l of pristane (Sigma-Aldrich, St. Louis USA), 2 to 4 weeks prior to the injection of hybridoma cells. Each mouse was then given approximately 10^8 hybridoma cells IP, and observed over a period of 4 weeks. Upon the observation of abdominal swelling (usually 7-12 days), mice were euthanased with CO₂ and the ascitic fluid was collected. The fluid was spun at 1000 g for 15 minutes and the supernatant collected and placed at 4⁰C overnight. The next morning the clot was removed from the ascites and the remainder was aliquoted into smaller units and stored frozen at -20⁰C.

5.2.6 Monoclonal antibody characterisation

5.2.6.1 Titre

Using the optimum coating Ig concentration (0.5 μ g/ml; Chapter 5.2.3.1), ELISA plates were coated, blocked and stored at -20⁰C until required. The

titres of cell-culture supernatants and ascites derived from the hybridomas were determined as the dilution at which the optical density (OD) is 50% of the maximum OD rate achieved for that sample (Arkoosh and Kaattari 1990). Each antibody solution was titred across the plate in duplicate starting at an initial concentration of 1:100 for ascites or neat for cell culture supernatant. A HRPO labelled goat-anti-mouse secondary was added to each well (1:2000), incubated for 1 h, washed thrice, and ABTS added.

5.2.6.2 Antibody cross-reactivity

Each monoclonal antibody was tested for cross-reactivity with Ig from other local fish species. Fish were obtained via hook and line from local creeks and estuaries and bled immediately from the caudal vein. Where multiple fish of the same species were obtained, the derived serum was pooled before being used in the following assay. In total, sera from 11 species of fresh and saltwater fishes were tested for cross-reactivity with the mAbs. Specificity of the mAbs were tested via indirect ELISA. Whole sera, diluted 1:5000 in CCB, was applied to 96-well round-bottom ELISA plates and allowed to bind overnight at 4°C. Following blocking, cell culture supernatant from each hybridoma line was titred against the sera. The ELISA was completed through the subsequent addition of goat anti-mouse HRPO and ABTS. The reactions rates were compared to that of the barramundi control on the same plate. For each species the various monoclonal antibodies were ranked into 4 groups according to the comparison of their OD rate / min to the barramundi control, the groups were negative < 25% of the control OD rate / min, low 25% < 50%, moderate 50% < 75%, and positive > 75%.

5.2.6.3 Antibody specificity

Western blots were performed to assess the specificity of the monoclonal antibodies for barramundi Ig. Ascites from each monoclonal antibody was tested against the protein products derived from protein A purification and resolved on SDS-PAGE and

SDS-CAGE (Chapter 4). The proteins were transferred to PVDF (Chapter 3.3) and the membrane allowed to dry. The PVDF was placed into a 20-chamber slot blot apparatus (BioRad Laboratory USA) and the ascites diluted to 1:500 applied. The slot blot was placed on a bench top rocker with gentle agitation. After 1 hr incubation the solution was removed and the apparatus flushed 5 times with PBS before the membrane was removed and washed a further 3 times. The secondary antibody (goat anti-mouse HRPO) diluted 1:1000 was applied to the membrane and allowed to incubated with gentle agitation for 1 hr. Five, 1 minute washes of the PVDF removed any unbound secondary reagent, and the reaction was visualized with either DAB (Sigma, St Louis, MO) or AEC (TropBio, Townsville Australia) and stopped through the addition of excess dH₂O.

5.2.6.4 Antibody isotyping

Isotyping of the monoclones was performed using the Sigma isotyping kit (Sigma-Aldrich, St. Louis USA), following the manufacturer's instructions.

5.2.7 Assay development

5.2.7.1 Capture enzyme-linked immunosorbent assay

Each monoclonal antibody produced was tested for its ability to capture barramundi Ig and present it in a way that could be detected by the polyclonal antibody for an Ig quantification assay. Each protein A purified monoclonal antibody (Chapter 4) was serially diluted in CCB starting at an initial concentration of 100 µg/ml and applied to two ELISA plates and allowed to bind overnight. Plates were washed, subsequently blocked with 3% casein, before the addition of 0.1 µg of purified barramundi Ig to each well on plate or tilapia Ig as the negative control. Following this step, serial dilutions of the rabbit polyclonal antibody were added to each plate, and allowed to incubate for 1 h before goat-anti-rabbit HRPO (Jackson Immuno Research, USA) (1:5000) was added. ABTS was

added and the reaction was read while linear. Each monoclonal was analysed for the optimum signal to background ratio, by dividing the reading from each well of the barramundi Ig plate by the equivalent reading on the control tilapia Ig plate. Monoclonals that possessed wells with high ratios (>15:1) were considered for further evaluation.

5.2.7.2 Indirect enzyme-linked immunosorbent assay for the determination of specific antibody titre

To determine specific antibody titres present in barramundi sera, an optimised indirect ELISA was developed. Antisera from a barramundi previously challenged with formalin killed *S. iniae* (#28) was obtained from a previous study (Bromage 1997), and was used throughout this development process and in all further ELISAs. In order to determine the optimum binding concentration of bacteria the following procedure was used. Formalin killed bacterial cells (Chapter 3.5) were diluted to a concentration of 2×10^9 cells ml in CCB. To the first row of Nunc flat bottom ELISA plates (Immunosorb), 100 μ l of the bacterial suspension was added, doubling dilution of this solution were performed down the plate. Plates were placed in a 37°C drying room until the wells were completely dry (approximately 12 h). The plates were washed once with washing solution (TropBio, Townsville Australia) then blocked with post-coating buffer (TropBio, Townsville Australia) for 2 h at room temperature. The blocking solution was then flicked off and the plates were allowed to dry again at 37°C. Each plate had barramundi sera titred down the plates, starting at a 1:100 dilution. Following incubation and washing, the various monoclonal antibodies produced were tested by diluting them across one plate. The ELISA was completed through the subsequent addition of goat-anti-mouse HRPO and ABTS. The reaction was read while linear and expressed as an OD rate / min. Naive barramundi serum and immune tilapia serum was used as the negative control.

5.3 Results

5.3.1 Indirect enzyme-linked immunosorbent assay optimisation

The optimum coating concentration of protein A purified barramundi Ig was determined to be the minimum barramundi Ig concentration which gave the maximum OD rate (0.5 µg/ml) (Figure 5.2). No variation in the result was observed when the plates were coated for 1hr or overnight (data not shown).

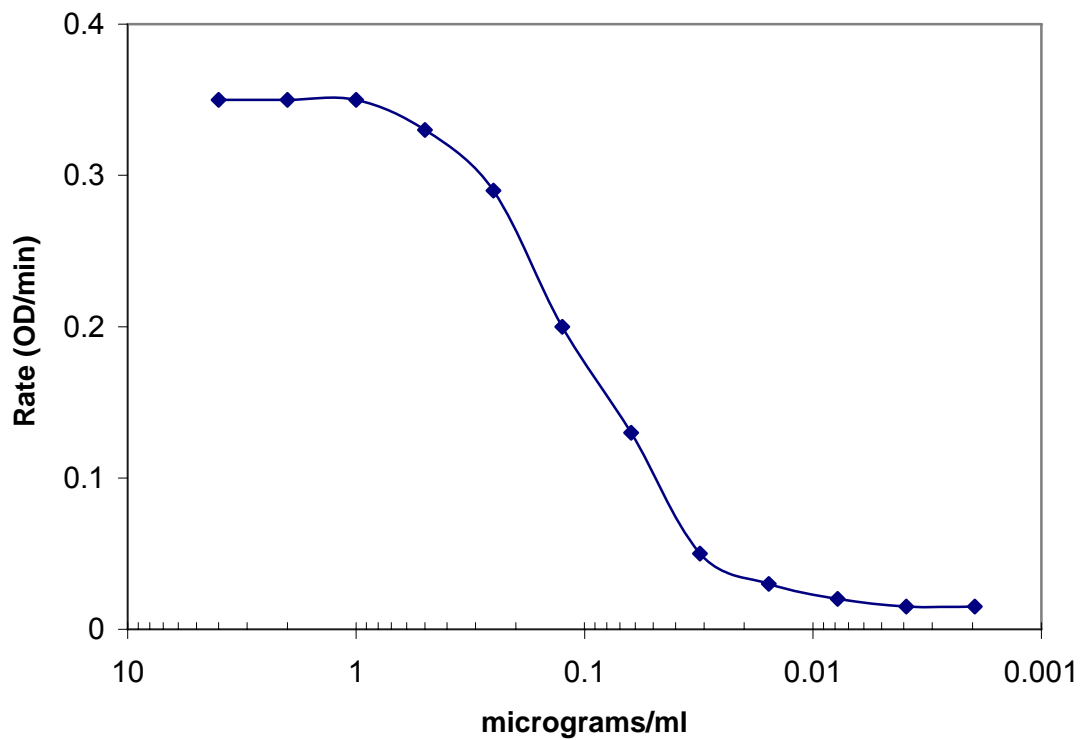


Figure 5.2. The plot of coating concentration of barramundi Ig versus OD rate/minute utilising rabbit-anti-barramundi Ig antiserum.

5.3.2 Polyclonal antiserum

5.3.2.1 Titre

At the optimum coating concentration (described above), the titre for the rabbit-anti-barramundi Ig was determined to be 1,700,000 units of activity / ml and 6,000,000 units of activity / ml (Figure 5.3) for the two rabbits vaccinated. Mice displayed variable antibody titres depending on the method of immunization. Mice immunised with whole barramundi Ig developed a strong antibody response with titres ranging from approximately 450,000 to 700,000 units of activity / ml (data not shown). Mice immunised with either barramundi Ig heavy or light chain displayed a diminished immune response with titres reaching only 2000 units of activity / ml (data not shown). Pre-bleed from all animals was less than 200 units of activity / ml.

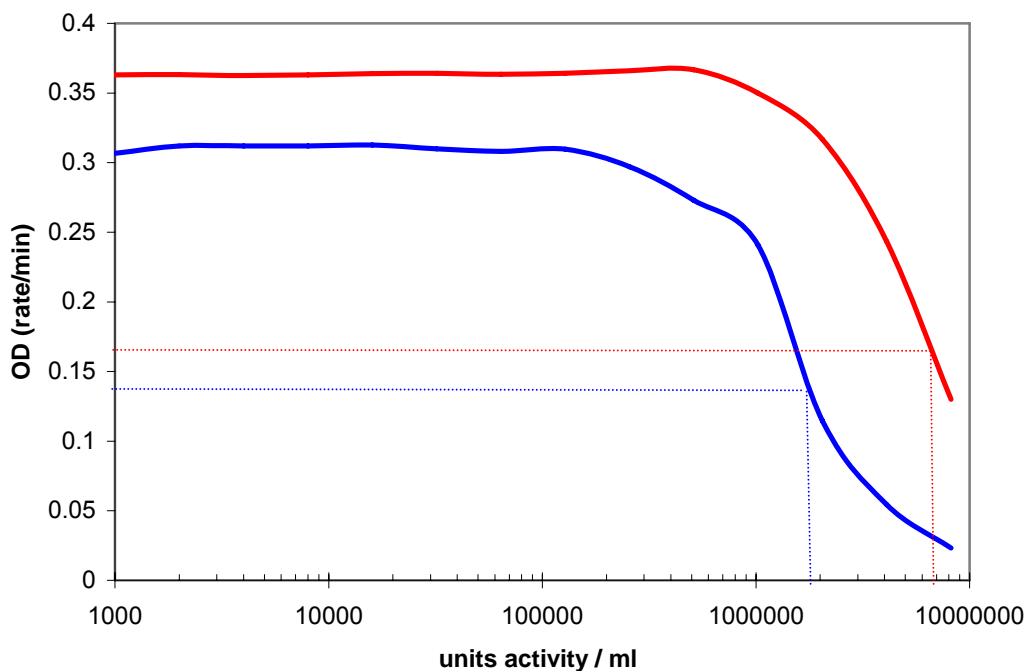


Figure 5.3 The serum titre profile of 2 rabbits (solid red and blue lines) immunised with purified barramundi immunoglobulin detected via indirect ELISA. The dashed lines indicate the 50% point of the maximum rate of OD observed for each rabbit expressed as units activity / ml.

5.3.2.2 Specificity

Antiserum from both the rabbit and mouse recognised the heavy and light chain with little cross-reactivity with other serum proteins (Figure 5.4). There is little cross-reaction of the antiserum with the tilapia sera control (data not shown). Mice vaccinated with either heavy or light chain recognized only the component they were vaccinated with. The antisera generated from these mice worked well at a dilution of 1:1000.

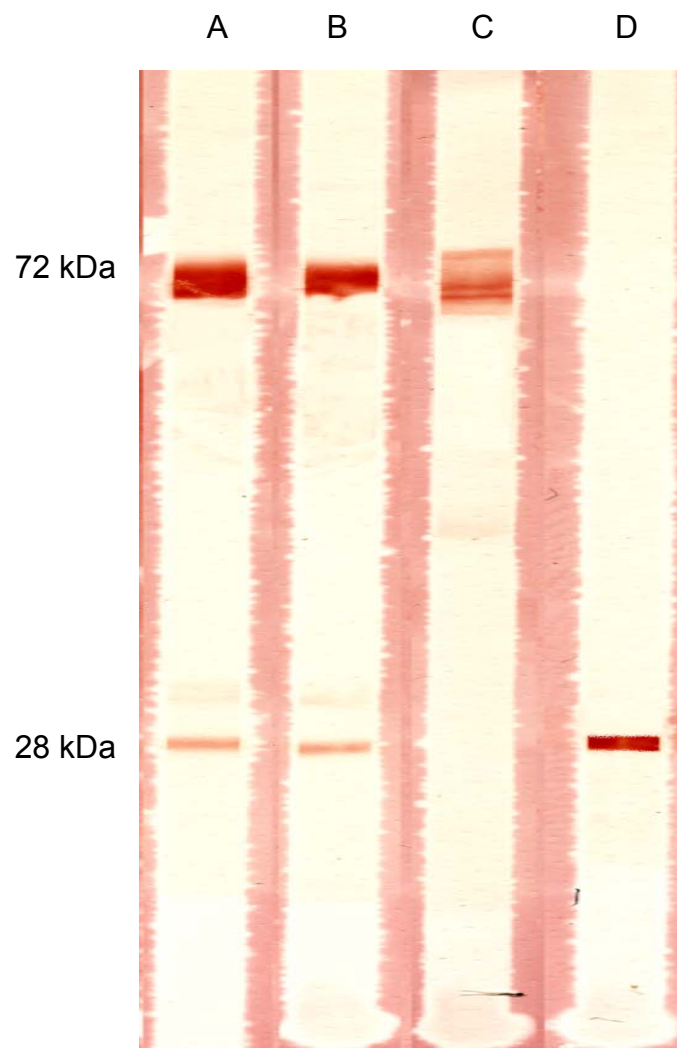


Figure 5.4 Western blotting of antiserum obtained from rabbit and mice vaccinated with barramundi Ig. Rabbits (A) and mice (B) were vaccinated with protein A purified Ig and antisera Western blotted against SDS-PAGE separated barramundi serum. Western blotting of antiserum from mice vaccinated with either the heavy chain (C) or light chain (D). Molecular weights of the heavy and light chain are shown.

5.3.3 Monoclonal antibodies

5.3.3.1 Production

In all seven fusions were performed; five for whole barramundi Ig, and single fusions each for mice vaccinated with heavy or light chain. The fusions resulted in an average of 40% of the wells having at least one viable colony present by day ten.

The screening of the whole barramundi fusions resulted in the production of 257 positive wells that were expanded into 24 well plates. Screening of the 24 well plates via ELISA five days after transfer reduced the number to 120. Each of these were transferred to 25 cm² flasks, grown to 70% confluence and the cells frozen at -80⁰C (Chapter 3.5), while the supernatant were stored at -20⁰C for further testing. Following screening the 15 best hybridomas were single cell cloned three times via limiting dilution (Chapter 3.6). This resulted in seven stable hybridoma colonies that were expanded and used throughout the experiment (Table 5.1).

The two fusions performed with mice injected with either barramundi Ig heavy or light chain resulted in the production 26 and 53 positive wells respectively. Cloning and re-screening resulted in the production of two heavy chain specific and 5 light chain specific hybridomas (Table 5.1).

5.3.3.2. Titre

The cell culture supernatant derived from the whole barramundi Ig fusion possessed titres ranging from 32 to 4000 units of activity / ml (Figure 5.5), while those derived from heavy or light chain extracts had a much lower titre (Figure 5.6) to those derived from the whole Ig.

Table 5.1 The characteristics of the hybridomas produced against barramundi IgM. Fourteen monoclonal antibodies were produced against whole, or components of barramundi Ig. Each monoclonal was isotyped, as well as being screened on a reducing SDS-PAGE to determine whether the monoclonal recognised an linear or conformational epitope.

Hybridoma ID	Isotype	Linear epitope
17D8.1 B*	IgG ₁	No
17E11.2 B	IgG ₁	No
19C10 B	IgG ₁	No
16F4 B	IgG ₁	No
23F8 B	IgG ₁	No
17F8 B	IgG ₁	No
17E10.1 B	IgG ₁	No
6F3.3.1 BH*	IgM	Yes – heavy chain
2F2.2.2 BH	IgM	Yes – heavy chain
4F10.1 BL*	IgM	Yes – light chain
8D1.1 BL	IgM	Yes – light chain
4A8.1 BL	IgM	Yes – light chain
7D8 BL	IgM	Yes – light chain
9B12.1 BL	IgM	Yes – light chain

* denotes the protein utilised for vaccination and boosting of mice (B – whole barramundi Ig, BH – heavy chain only, BL – light chain only).

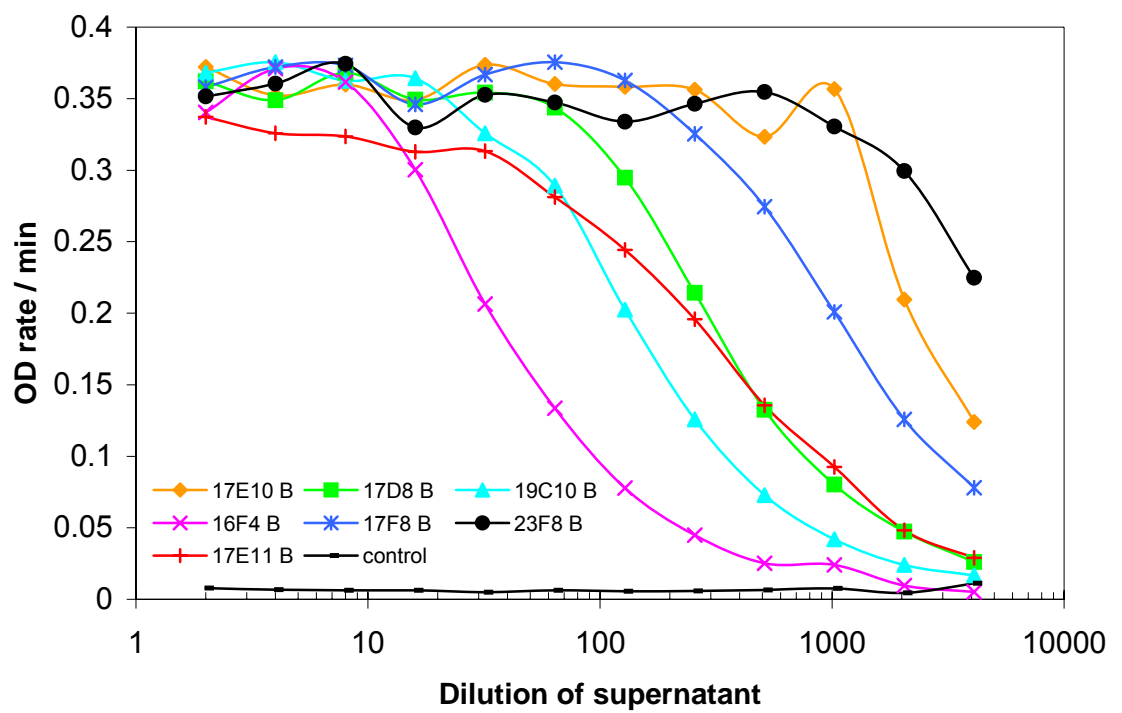


Figure 5.5. The titre profile of cell culture supernatants containing monoclonal antibodies produced against whole barramundi Immunoglobulin.

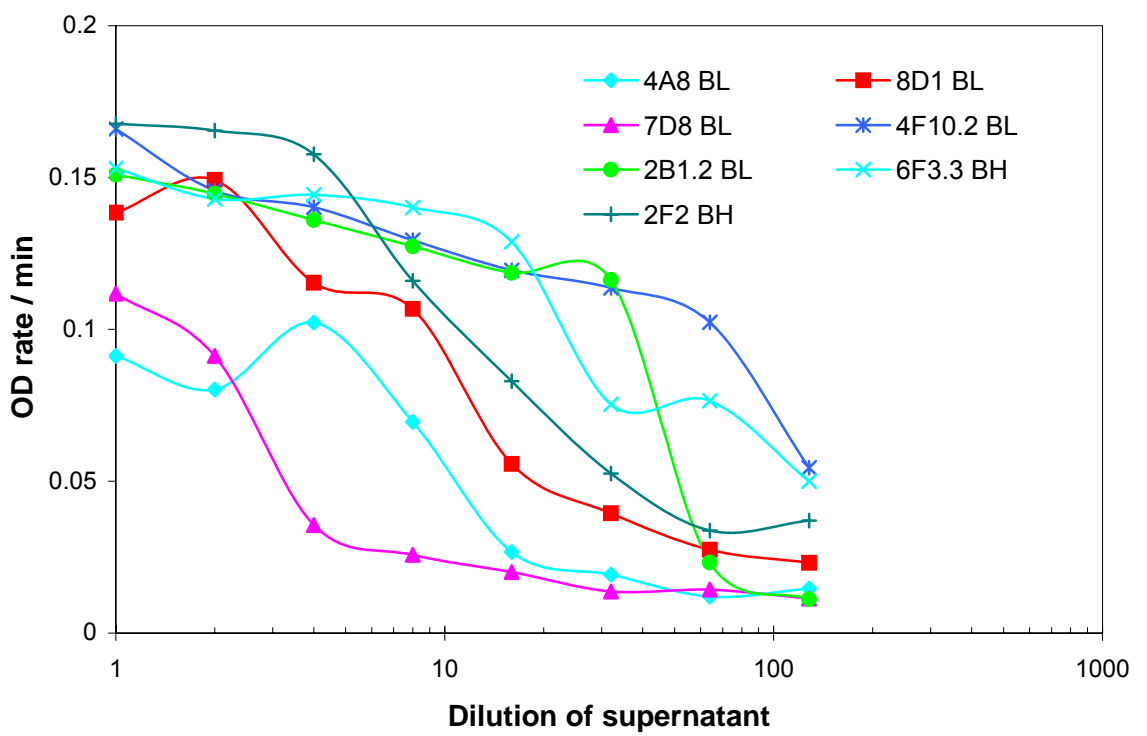


Figure 5.6 The titre profile of cell culture supernatants containing monoclonal antibodies produced against heavy or light chain of barramundi Immunoglobulin.

5.3.3.3 Cross reactivity

When the monoclonal antibodies were screened against Ig derived from other species very different profiles were observed (Table 5.2). The monoclonal antibodies specific for heavy and light chain were species specific. The hybridoma 17E10.1 B was the least specific monoclonal antibody produced reacting positively to 9 out of the 12 species tested. With regard to the species of fish tested, 17D8.1 B was the only monoclonal antibody produced from the whole Ig fusions that was specific for barramundi.

Table 5.2. The cross-reactivity of monoclonal antibodies generated against barramundi immunoglobulin for other teleost Ig (- negative, + low, ++ moderate, +++positive).

Species	17D8.1 B	17E11.2 B	19C10 B	16F4 B	23F8 B	17F8 B	17E10.1 B	6F3.3.1 BH	2F2.2.2 BH	4F10.1 BL	8D1.1 BL	4A8.1 BL	7D8 BL	9B12.1 BL
<i>Late calcarifer</i>	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +	++ +
<i>Oreochromis mossambicus</i>	-	+	-	-	-	-	+	-	-	-	-	-	-	-
<i>Lutjanus russelli</i>	-	+	-	-	-	-	+	-	-	-	-	-	-	-
<i>Letherinus nebulosus</i>	-	-	-	-	-	-	+	-	-	-	-	-	-	-
<i>Epinephelus coioides</i>	-	-	+	+	+	-	+	-	-	-	-	-	-	-
<i>E. corallicola</i>	-	-	+	+	+	-	+	-	-	-	-	-	-	-
<i>E. quoyanus</i>	-	-	+	-	-	-	+	-	-	-	-	-	-	-
<i>Platycephalus</i> sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Acanthopagrus</i> sp	-	-	-	-	-	-	+	-	-	-	-	-	-	-
<i>Pseudorhombus</i> sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Megalops cyprinoides</i>	-	-	-	-	-	-	+	-	-	-	-	-	-	-
<i>Sillago</i> sp	-	-	-	-	-	-	+	-	-	-	-	-	-	-

5.3.3.4 Antibody specificity

None of the 120 monoclones generated in the whole barramundi Ig fusion were chain specific when tested via Western blotting. Six of the seven chosen for further evaluation also failed to detect the redox products of the SDS-CAGE. One single monoclonal antibody, 17D8.1 B, recognized all barramundi Ig redox forms when tested via Western blotting (Figure 5.7).

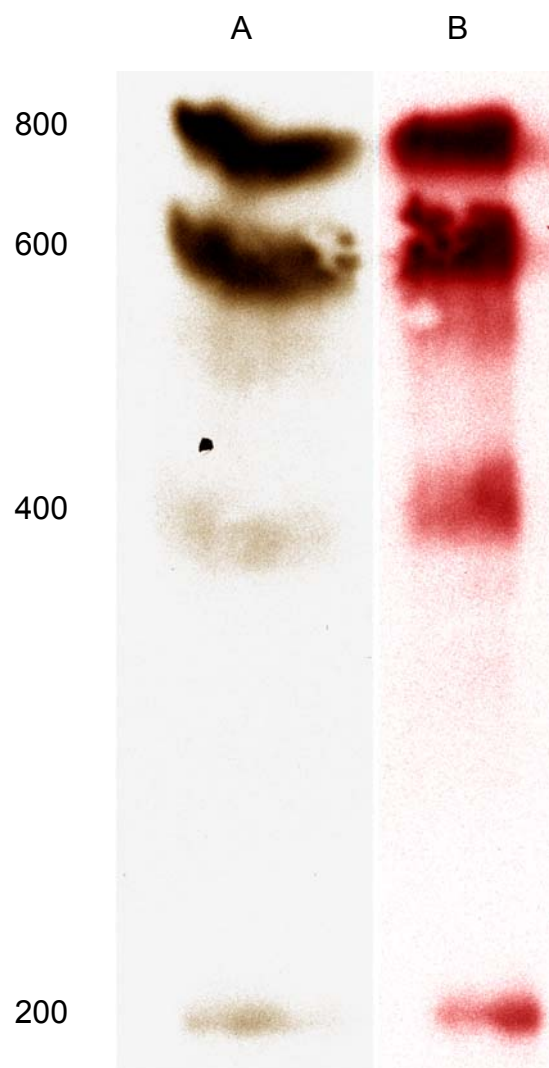


Figure 5.7. Western blot of selected monoclonal antibodies generated against whole barramundi immunoglobulin. Redox forms of barramundi Ig as observed in SDS-CAGE (A), Western blotting of monoclonal antibody 17D8.1 B (B). Approximate molecular weights (kDa) shown on left.

The hybridomas generated from the heavy chain fusion were specific for the heavy chain (Figure 5.8) under reducing conditions and recognised all redox forms (Figure 5.9). The light chain hybridomas were specific for light chain (Figure 5.8) and also recognised all redox forms (Figure 5.9).

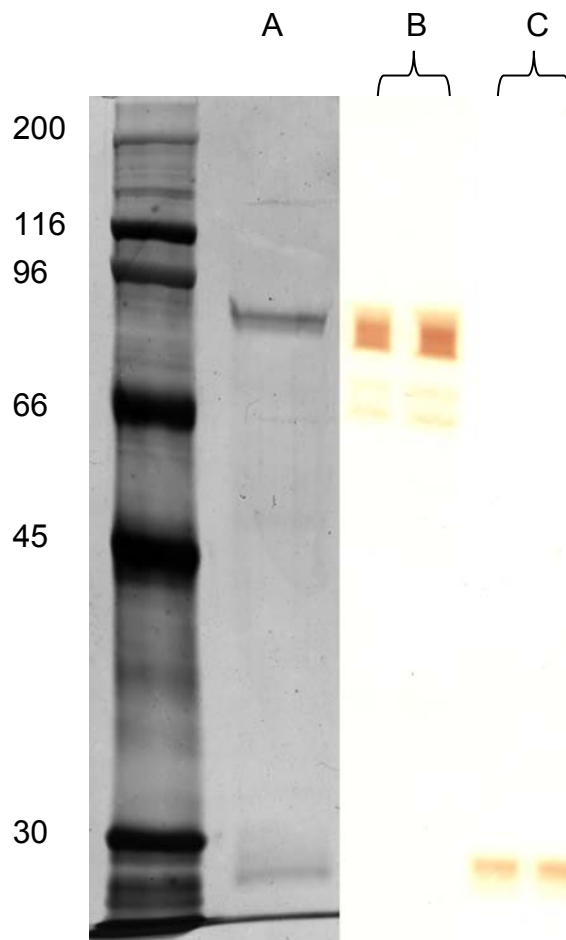


Figure 5.8. Western blotting of selected monoclonal antibodies generated against the heavy or light chain of barramundi immunoglobulin. SDS-PAGE profile of barramundi Ig (A), Western blotting of monoclonal antibodies 2F2.1 BH (B) 4F10.1 BL (C) against SDS-PAGE separated barramundi Ig. Left lane is molecular weight markers

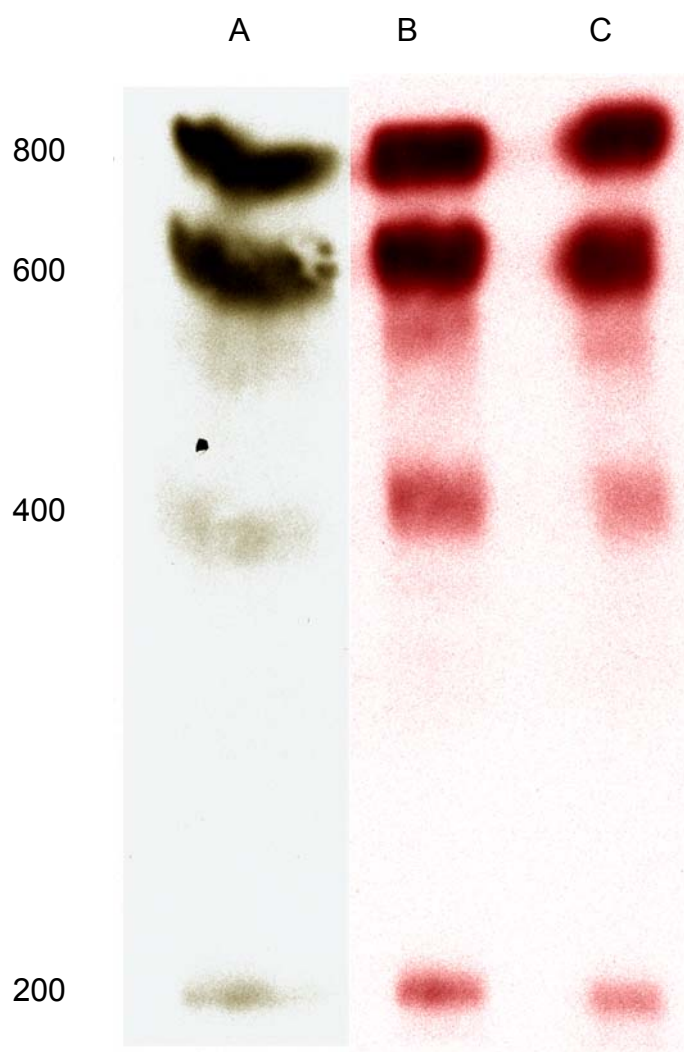


Figure 5.9. Western blotting of selected monoclonal antibodies generated against the heavy or light chain of barramundi immunoglobulin. SDS-CAGE profile of barramundi Ig (A), Western blotting of monoclones 2F2.1 BH (B) 4F10.1 BL (C) against SDS-CAGE separated barramundi Ig. Approximate molecular weights (kDa) shown on left.

5.3.3.5 Isotyping

All monoclones produced from the whole Ig fusions were of the IgG₁ isotype (Table 5.1). While those from the heavy and light chain fusions were all of the IgM isotype.

5.3.4 Assay development

5.3.4.1 Capture enzyme-linked immunosorbent assay

The development process of the capture ELISA resulted in one monoclonal (17D8.1 B) being superior to others tested. This monoclonal produced the lowest background and could be used at a high dilution. The optimum coating concentration was determined to be a 1:16,000 dilution of unpurified ascitic fluid or 0.2 µg/ml of purified antibody. To detect the captured barramundi Ig the optimum concentration of the rabbit polyclone was 1:10,000. At these concentrations the range of 0.1 to 0.6 µg of barramundi Ig could be accurately estimated.

5.3.4.2 Antigen specific enzyme-linked immunosorbent assay

The cell coating concentration of *S. iniae* found to provide the optimum ELISA response was determined to be 100 µl of a 1×10^5 cells/ml solution applied to a flat bottom tissue culture plate (Nunc, Denmark). The plate was left at 37°C until all fluid had evaporated (approximately 12 h) before being blocked. This procedure resulted in the optimum signal to background of all the variations tested. All the anti barramundi Ig monoclonal antibodies tested using this configuration performed well. The IgG monoclonals were able to be used at a much higher dilution than the IgM monoclonals and once again 17D8.1 B had the lowest background.

5.4 Discussion

In order to monitor the humoral immune response of any animal species, the production of appropriate tools is vital. One method employed is the use of ELISA technology arising through the development of monoclonal antibodies against the target species Ig. These antibodies can be used to non-lethally monitor the immune response after vaccination or exposure to an antigen. There have been several monoclonal antibodies produced and effectively used to monitor the immune response of aquatic species (Estevez *et al.* 1994; dos Santos *et al.* 1997; Nakayasu *et al.* 1998). The most widely used is the 1-14 monoclonal (DeLuca *et al.* 1983) which is directed at the heavy chain of rainbow trout (*O. mykiss*) Ig. The production of this monoclonal has allowed researchers to study in great detail many of the aspects of the development and maturation of the immune system of this highly prized species.

This is the first report of monoclonal antibodies produced against barramundi Ig and one of the first against a species of fish cultured in the tropics. The fusion process was successful in generating many positive clones, but there was difficulty in generating clones specific to either the heavy or light chain under reducing conditions. The benefit of a heavy or light chain specific monoclonal is that it can be used in the widest array of immunoassays, such as the ELISA and Western blotting. Polyclonal mouse serum obtained from mice vaccinated with whole barramundi Ig developed an excellent immune response, as measured by ELISA, and did recognise both heavy and light chains under reducing conditions. However, following the fusion process none of the 120 clones screened were chain specific under reducing conditions. This is an unexpected result as fusions following identical protocols utilising rainbow trout Ig as the antigen resulted in a ratio of 2:7:3 for conformational, heavy and light chain epitopes respectively (unpublished results), with a similar result obtained from a fusion with tilapia Ig (unpublished results).

The desire to produce a heavy or light chain specific monoclonals led to the process of excising the already reduced heavy and light chains from SDS-PAGE gels and vaccinating the mice with the homogenised protein-acrylamide mix. Once again the mice responded, albeit with a diminished titre. The subsequent fusion of these mice did produce heavy and light chain specific hybridomas, with the caveat that they were of the IgM isotype. This vaccination process, while producing clones of the desired specificity, appeared to fail to initiate a secondary response or class switching in the mice, resulting in the lack of monoclonals of the IgG isotype. This has been previously reported by Jones (1989) and therefore may have not been the optimum method for vaccination. Eluting the proteins from the gel into solution using a product such as the BioRad electro-eluter or possibly transferring the protein onto nitrocellulose (Harlow and Lane 1998) may have provided a better result. The monoclonals were functional in all assays tested but the IgM isotype is not regarded as an optimal monoclonal due to its lower affinity (Harlow and Lane 1988)

In spite of these drawbacks the monoclonals produced performed extremely well in the immunoassays developed. One monoclonal (17D8.1 B) provided an excellent signal, with low background, and was chosen for use in all assays. The clone recognised all redox forms in equimolar ratios to those observed in the CAGE gel, while not responding to reduced Ig. It is hypothesised that this clone recognises a linear epitope located on, or near, an intra-monomer disulphide bond and would be worthy of further characterization in the future.

CHAPTER 6

ONTOGENY OF IMMUNOGLOBULIN, IMMUNOGLOBULIN BEARING CELLS AND THE HUMORAL IMMUNE RESPONSE IN BARRAMUNDI

6.1 Introduction

The development of barramundi fry is rapid, hatching within 14-17 hours of release and actively feeding within 1-2 days (Barlow 1997). In comparison, the development of rainbow trout fry occurs over a number of months; at 14⁰C, 28 days are required for the fertilized eggs to hatch (Vernier 1969), and another 2 months before the larvae feed. Complete utilization of the yolk in rainbow trout takes up to 12 weeks, while barramundi only 2-3 days (Vernier 1969; Barlow 1997). The yolk not only provides the nutritional support for the developing fry, but it also provides various protective factors (Alexander and Ingram 1992; Breuil *et al.* 1997; Brown *et al.* 1997; Takemura and Takano 1997). The majority of these components are non-specific defensive factors such as lectins (Alexander and Ingram 1992). In addition to these non-specific factors it has also been shown that there is the passive transfer of maternal antibodies (Castillo *et al.* 1993; Yousif *et al.* 1995; Takemura and Takano 1997). The elimination of the maternal antibody is directly related to the absorption of the yolk (Takemura and Takano 1997). Female barramundi invest very little energy into individual fish, preferring to produce a large quantity of eggs. Therefore, it is hypothesized that larvae need to develop immuno-competence very quickly after hatch.

Recent research with catfish, *Ictalurus punctatus*, have shown that it is able to mount a weak antibody response to bacterial challenge by week 4 post-hatch (PH) (Petrie-Hanson and Ainsworth 1999). Rainbow trout can respond to B-cell antigens such as *Renibacterium salmonarium* by week 8, while T-dependent antigens like human gamma globulin are not recognised until week 12 post-hatch (Tatner 1986). There has been no comparable research performed with barramundi fry, and due to the physiological differences in

time of development and the temperature of culture, it is very difficult to relate these findings to the development of the barramundi immune system.

Therefore, this chapter has been designed to examine whether there is maternal antibody transfer, how long it lasts and at what age barramundi fry develop B-cell competence.

6.2 Methods

6.2.1 Animals

Fish from 3 separate spawnings were utilised in these experiments (Bluewater Barramundi Pty Ltd, Mourilyan). Larvae were maintained at either the hatchery or at the Aquaculture Department at JCU. Fish were transferred from the hatchery at 14 d post-hatch to the Microbiology and Immunology aquatic facilities where they were held in 0.125 m³ (50 x 50 x 50 cm) floating cage with 1 mm² netting. The cage was housed in a 2000 L freshwater recirculation facility held at a constant 27⁰ C. They were fed initially with a 1-2 mm crumble at 2 % of their body weight, four times a day.

6.2.2 Sample collection and preparation - Ontogeny

Following egg release and fertilization (spawning performed by Bluewater Barramundi), samples of eggs / developing fry were collected and placed in universal vials and immediately frozen (-80 C). Samples were collected over a period of 38 days and for three separate spawnings. When all samples were collected, specimens were thawed and washed three times in PBS. Excess water was removed and 1 g (wet weight) of each was added to 1 ml of PBS and homogenised using a tissue homogeniser (Pyrex, USA). Samples were centrifuged at 12,000 g, before the supernatant was collected and filtered through a 0.22 µm filter (Millipore, Australia) and used immediately in the capture ELISA (Chapter 5). Each sample was titrated in

duplicate and compared to protein A purified barramundi Ig standards covering the range of 0.1 to 0.6 µg / ml on the same plate.

6.2.3 Sample collection – B cell response

At week two post-hatch, 30 fish (approx. 20mm) were injected IP with 25 µl of formalin killed *S. iniae* (1×10^9 cells / ml) (without adjuvant) (Isolate #28). Two weeks post-inoculation, and weekly thereafter, five fish were sacrificed for collection of blood via tail amputation. Blood samples were processed as per Chapter 3.2 and stored at –20 C until required. This was repeated with six groups of 30 fish from week three through seven. Titre was monitored using an indirect ELISA (Chapter 5) comparing the samples to high titre sera.

6.2.4 Immunohistology

Every third day post-hatch, five fish were collected and euthanased with 2-phenoxyethanol (Sigma, St Louis, MO). Whole fish were immediately placed into histology cassettes and overlaid with imbedding medium (TBS, Durham, USA). The cassettes were placed just above the surface of liquid nitrogen and snap frozen. The samples were then stored at –80°C until processed. Frozen samples were sectioned (5 µm) on a cryostat (Shandon 620/E Cambridge England) at a temperature of –11 C and placed on silanised slides (Sigma, St Louis, MO). Slides were placed at 20°C for two hours enhance binding, then fixed in ice cold acetone for ten minutes and allowed to air dry.

Endogenous peroxidase was eliminated by placing the sections in a solution of 0.03% hydrogen peroxide (Sigma, St Louis, MO). The slides were incubated for 3 h in a 1:200 dilution of monoclonal 17D8.1 B, before being gently washed three times with PBS. Goat anti-mouse HRPO (1:500) was applied to the slides before washing and detection with AEC (TropBio, Townsville Australia). The reaction was stopped after 15 minutes through the

addition of excess PBS. Slides were counterstained with haemotoxylin and cover slips attached with aqueous mounting medium.

6.3 Results

6.3.1 Ontogeny

Using the capture ELISA, trace amounts of Ig were detected in eggs and day old fry. Following this period Ig could not be detected again until day 7. There was a steady increase in Ig from day 7 until day 38 when the experiment concluded (Figure 6.1).

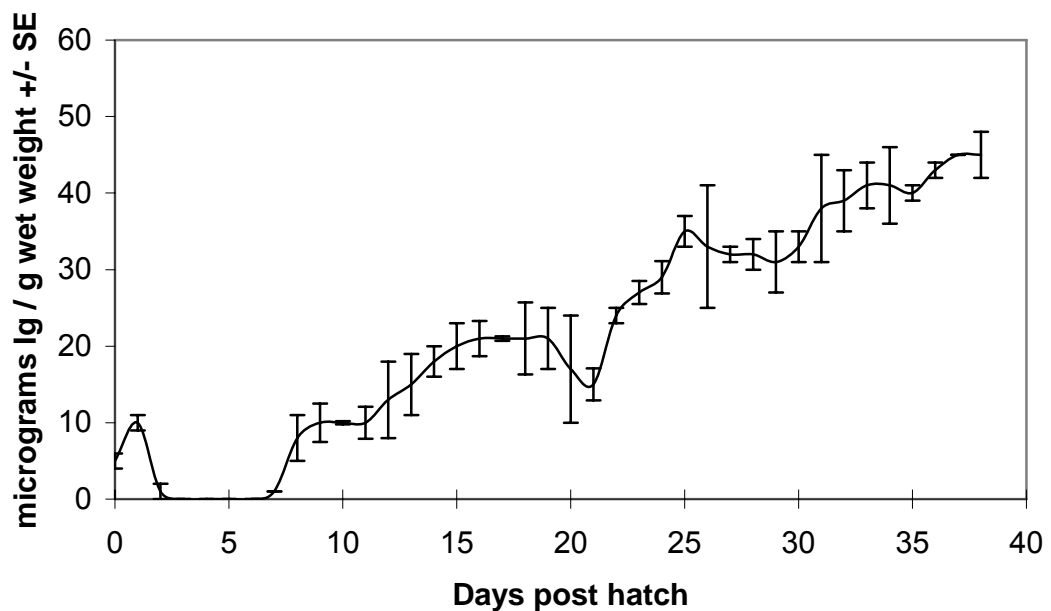


Figure 6.1. The relationship between age and level of Ig detected by a capture ELISA in barramundi homogenates

6.3.2 Specific antibody response

Barramundi vaccinated at four weeks age (average 30 mm total length) were the first to display an antibody response to *S. iniae* (Figure 6.2 A-F). Three weeks post-vaccination a detectable titre developed in this group (Figure 6.2b), diminishing rapidly in the following weeks. No response was observed in the group of fish vaccinated before this. Fish vaccinated at seven weeks PH were the first to display an extended immune response (Figure 6.2 E).

6.3.3 Immunohistology

At day four post hatch the monoclonal 17D8.1 B positively detected cells located in the anterior kidney. Two types of cells were observed simultaneously; one lymphoid cell containing cytoplasmic Ig, and a smaller cell bearing surface Ig. Both cell types were located in what appeared to be developing germinal centres (Figures 6.3 and 6.4). As the fish aged the relative amount of Ig⁺ cells increased in the anterior kidney (Figure 6.5). In the spleen, Ig⁺ cells were not detected until day 16. Ig⁺ cells were concentrated in germinal centres but were also dispersed throughout the splenic tissue (Figure 6.6). Little differentiation of cell types were observed in the spleen. No cells stained in the control slides (Figure 6.7).

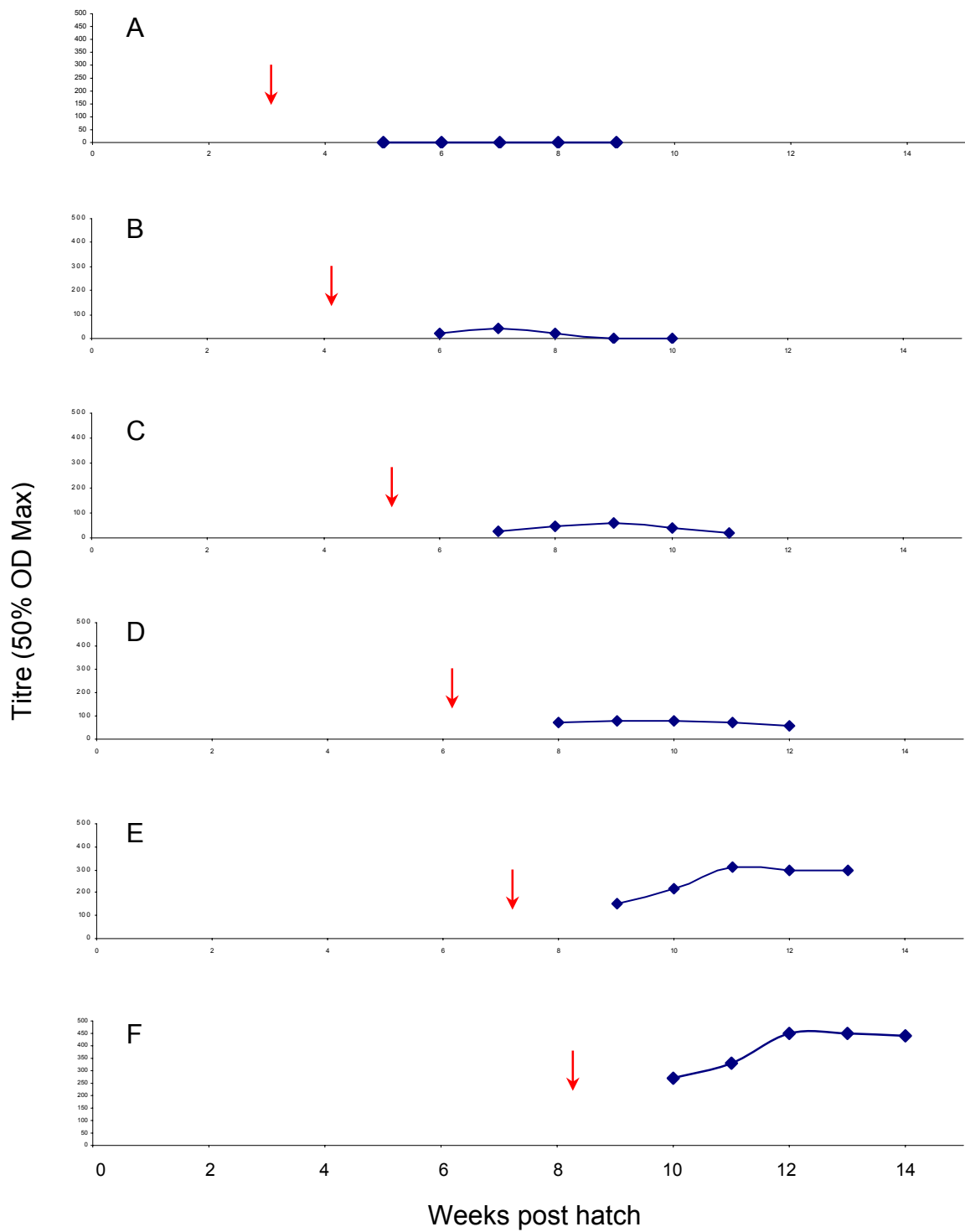


Figure 6.2 A-F. The specific antibody titre (50% OD Max) of barramundi fry vaccinated with *Streptococcus iniae*. Fish vaccinated week 3 (a) week 4 (b) week 5 (c) week 6 (d) week 7 (e) week 8 (f). Arrow denotes when vaccine was administered.

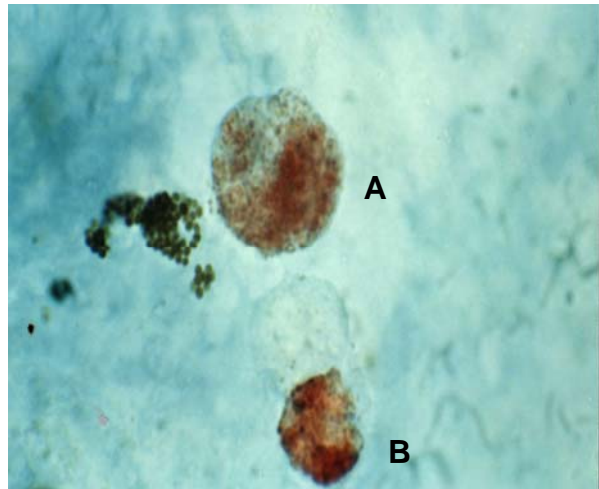


Figure 6.3 Immunostaining of Ig⁺ cells located in anterior kidney of 6 day old barramundi. (A) pre-B cell (B) mature B cell. (990x) AEC staining of the monoclonal 17D8.1 B with haematoxylin counterstain

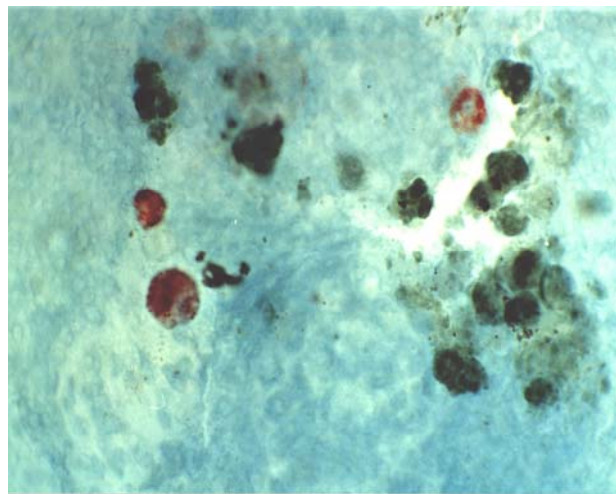


Figure 6.4 Immunostaining of Ig⁺ cells located in developing germinal centres of the anterior kidney of 6 day old barramundi. (540x) AEC staining of the monoclonal 17D8.1 B with haematoxylin counterstain

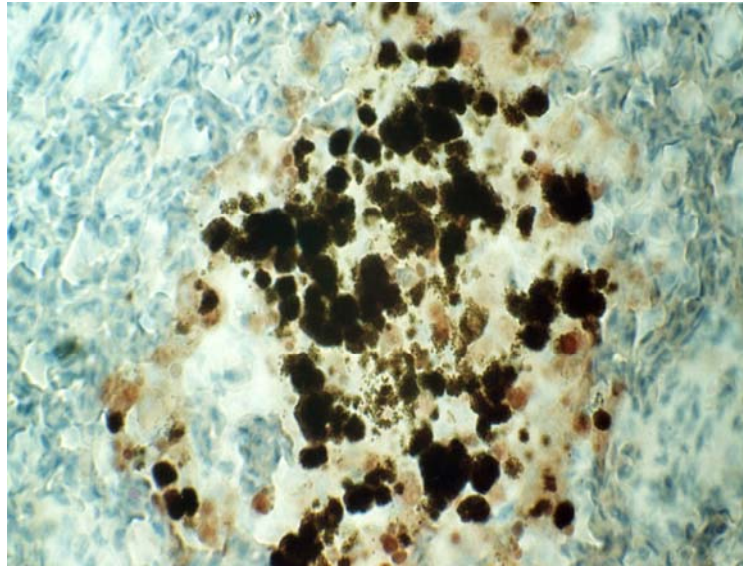


Figure 6.5 Immunostaining of Ig+ cells located in apparent germinal centres of the anterior kidney of 15 day old barramundi. (400x) AEC staining of the monoclonal 17D8.1 B with haematoxylin counterstain

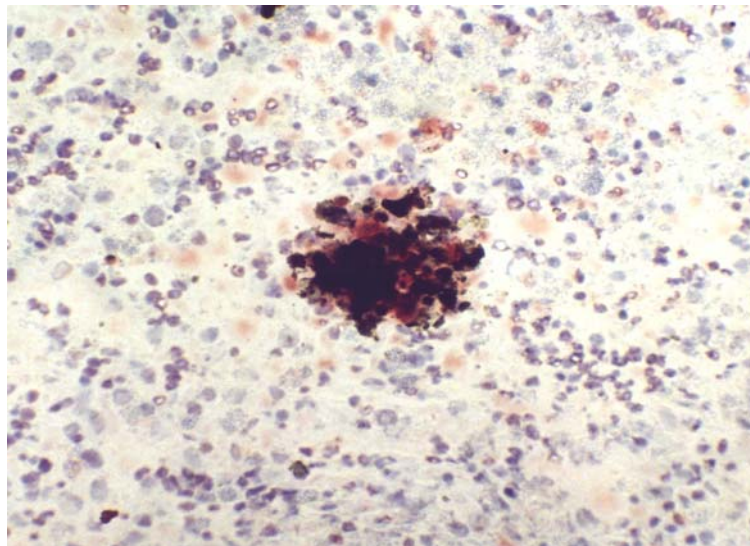


Figure 6.6 Expression of Ig positive cells located in spleen of 22 day old barramundi. (340x) AEC staining of the monoclonal 17D8.1 B with haematoxylin counterstain

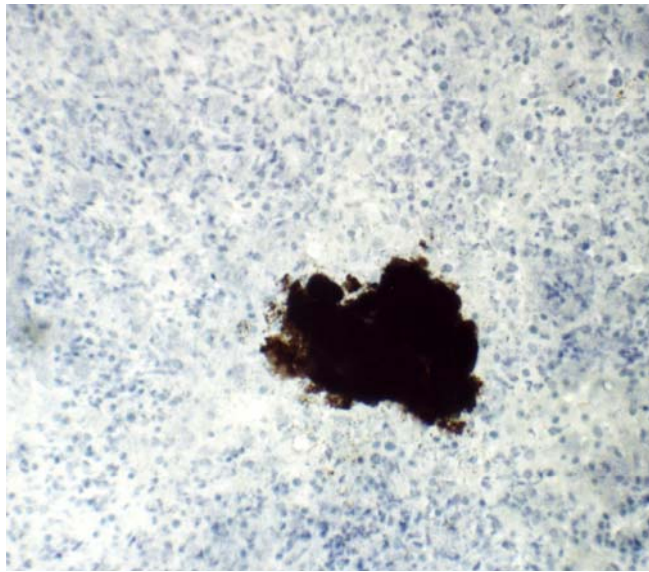


Figure 6.7 Control spleen of 22 day old barramundi. (300x) AEC staining of the monoclonal 17D8.1 B with haemotoxylin counterstain

6.4 Discussion

There have been a number of studies that have investigated the ontogeny of the teleost immune system (Tatner 1996). The development of the immune system follows a similar path irrespective of the species. During the production of the eggs, the female not only supplies the required nutrients for initial growth and development, but she also provides a suite of protective factors, such as antibodies, that may offer the larvae a survival advantage. The transfer of maternal antibodies into eggs has been reported from numerous species: coho salmon, *Oncorhynchus kisutch* (Yousif *et al.* 1995, Brown *et al.* 1997), tilapia, *Oreochromis mossambicus* (Takemura and Takano 1997), and the seabass, *Dicentrarchus labrax* (Breuil *et al.* 1997). It was demonstrated via capture ELISA that Ig was present in barramundi eggs, which is likely to be of maternal origin. The elimination of the Ig in developing fry appeared to coincide with yolk absorption. The rate of loss of maternal antibody appears to be species dependent. In sea bass (*D. labrax*), maternal antibodies are lost five days post hatch (Breuil *et al.* 1997), while tilapia (*O. mossambicus*) have detectable maternal antibodies for up to 13 days after egg release (Takemura 1993). In both cases, the loss of maternally derived antibody is related to the absorption of the egg.

Barramundi invest minimal energy into individual larvae, preferring to produce vast quantities of eggs, up to 5 million, with a limited nutritional supply (Sivaloganathan *et al.* 1998). In comparison, rainbow trout produce relatively few eggs (5000), with a large nutritional supply (Vernier 1969). There has been considerable interest in vaccinating broodstock against pathogens that affect larvae. The hope is that the specific antibody developed by the female will be transferred to the egg and provide effective protection for the larvae until they are able to mount their own immune response. This specific protection has been demonstrated in tilapia (*O. aureus*) against ichthyophthiriasis (Sin *et al.* 1994). However, the rapid utilization of the yolk and consequent loss of maternal antibodies would preclude barramundi broodstock from vaccination for this purpose.

Ig positive cells were first detected in anterior kidney of developing fry at four days post hatch and in the spleen by day 12. This is similar, albeit faster than the development of Ig+ cells in channel catfish (Petrie-Hanson and Ainsworth 2001). The cytoplasmic stained cells are likely to be immature or pre-B cells while the surface stained lymphocytes indicate mature B-cells. The simultaneous appearance of these cell types is in agreement with other published reports (Grossi *et al.* 1977; Lassila 1981) of teleost ontogeny. It is important to realize that the presence of specific types of immune cell does not imply functional humoral immunity. This was demonstrated by the lack of detectable circulating antibody until day seven and the lack of a specific antibody response in vaccinated fry until four weeks post-hatch. It is thought that the development of well defined lymphoid structures in the kidney and spleen is required before a specific immune response can be mounted (Petrie-Hanson and Ainsworth 2001). Although this was not examined directly in this study, the complete development of barramundi germinal centres did appear to coincide with the production of a specific immune response and would warrant further investigation.

The more pronounced immune response did not occur until week seven. It is not known whether *S. iniae* is a T-dependent or a T-independent antigen. The lack of a prolonged immune response in fish before week seven may indicate that a T-cell development is important in the immune response of barramundi against *S. iniae*. To date, antibodies against barramundi T-cells are not available, and their development and use would be invaluable in completing the picture of the ontogeny of adaptive immunity in barramundi. It may also be the case that barramundi vaccinated before week seven may not have developed other immune components needed to enhance or maintain a specific antibody response, such as complement. Clearly there is the need to develop assays to investigate further the ontogeny of immunity in barramundi.

CHAPTER 7

PHYSICAL PARAMETERS AFFECTING THE SUSCEPTIBILITY OF BARRAMUNDI TO *STREPTOCOCCUS INIAE*

7.1 Introduction

Seasonal variations in the prevalence of diseases in cultured fish have been reported in a number of species. For example furunculosis outbreaks in Atlantic salmon (*Salmo salar*) occur most frequently during the summer and early autumn, while the onset of cooler temperatures vibriosis becomes a significant problem (Lillehaug *et al.* 2003). Columnaris disease, caused by *Flavobacterium columnare*, generally occurs when water conditions are favourable for the bacteria and stressful the fish, most commonly when the water temperature is between 20-30°C (Wakabayahi 1991).

Previously I examined the effect of cultural conditions on the growth of *S. iniae in-vivo* (Bromage 1997). It was determined that the optimum growth conditions were 27°C, pH 7 and salinity of 0.5 ppt. It was also observed that there was considerable seasonal variation in the mortality observed at sea cage facilities. The peak mortality occurring during the summer months, when there is considerable fluctuation in the physical parameters of the water.

There have been a number of studies examining the effects of various water quality parameters on streptococcal infections. Bunch and Bejerano (1997) examined the effect of oxygen and nitrite concentration on the infection of tilapia (*Oreochromis niloticus* x *O. aureus*) with *Streptococcus* sp. They found the stress associated with low oxygen levels and increased nitrite resulted in significant increases in mortality. Crowding stress has also been linked to increased mortality in hybrid striped bass (*Morone saxatilis* x *M. chrysops*) in culture facilities (Stoffregen *et al.* 1996). Many early reports of streptococcal outbreaks indicated that the disease was most prevalent during the warmer

months (Munday *et al.* 1993; Al-Harbi 1994; Young-Gill and Lee 1994) but little has been done to examine this phenomenon. This chapter examines long term environmental and mortality data at an estuarine cage facility and the effect of three water quality parameters (temperature, salinity and pH) on the susceptibility of barramundi to *S. iniae*.

7.2 Materials and Methods

7.2.1 Long-term environmental data

Environmental data was recorded over an eight-year period from estuarine cage facility suffering from ongoing mortalities due to streptococcosis. Daily recordings of mortalities, salinity, pH and temperature, were analysed from the initial disease outbreak in 1992 up to March 2000. Salinity, pH, and temperature readings were recorded at a depth of 1 m. The data was averaged per week and plotted to determine any long-term patterns in the outbreak of disease.

7.2.2 Confirmation of etiology

Confirmation of etiology of the mortalities was performed initially by the Queensland Department of Primary Industries, Oonoonba Veterinary Laboratory (1992-1995), and subsequently by the author. During periods of increased mortality at the farm, all morbid or moribund fish were collected and a sample of fish (>20) were randomly selected and either bacterial isolation was performed on site by the author or shipped on ice to JCU. Bacterial isolation was performed from the brain, kidney, and spleen, while characterisation of the recovered bacterial isolates was performed as previously described (Bromage *et al.* 1999)

7.2.3 Challenge experiments

A series of challenge experiments were conducted at varying levels of pH, salinity and temperature. Challenge trials were conducted in triplicate with groups of 20 fish (100 g) per treatment. Barramundi were challenged with an isolate of *S. iniae* (#28) that was previously recovered from the farm in which these fish were sourced (Bromage 1997). Challenge occurred via a ten minute bath exposure to an LD₅₀ (3.2×10^4 cfu / ml) previously determined for this isolate (Bromage 1997). Prior to challenge, groups of fish were held in 100 L glass aquaria initially containing seawater (27°C, 30 ppt, pH 7.8). The water parameters were gradually changed to the required conditions via water exchange over a period of three weeks. Fish were held for another week at the desired conditions. The parameters tested were pH (4.5, 6.0, 7.5, 8.5, 9.5) at a constant 27°C and 30 ppt, temperature (18, 21, 24, 27, 31, 34 °C) at a constant pH of 7.8 and 30 ppt, and salinity (0, 10, 20, 30, 40 ppt) at a constant 27°C and pH of 7.8. Water conditions were monitored and adjusted daily if required. One tank of control fish (20) were held at each of the environmental conditions and subjected to bath challenge with formalin killed *S. iniae* cells. Mortalities were recorded and animals subject to bacterial isolation and characterisation (Bromage *et al.* 1999).

7.2.4 Statistical analysis

Statistical analysis of the physical variables and mortality at the seacage facility were performed by recoding the variables into groups based on the highest and lowest reading of each physical parameter. Temperature was recoded into 10 groups, salinity into 8 and pH in 5. The data was analysed using a general linear model, with a post-hoc stepwise comparison using least significant difference (SPSS).

For the experimental challenge data a pair-wise comparison of survival using the Wilcoxon (Gehan) statistic for barramundi challenged with *Streptococcus iniae* at different physical variables was used.

7.3 Results

7.3.1 Environmental data

There was considerable seasonal fluctuation in the environmental factors monitored at the farm over the eight-year period (Figure 7.1). The fish were subject to salinity ranging from a high of 46 ppt and dropping as low as 2 ppt during periods of heavy rain, with the average salinity being 34 ppt. The water temperature had a recorded high of 33⁰C dropping to a low of 18⁰C in the winter. The mean growing temperature was 26.5⁰C. The pH of the water was relatively constant with the average pH being 7.9. Dramatic drops in pH were observed infrequently, which coincided with the anthropogenic disturbances of the surrounding mangrove forests.

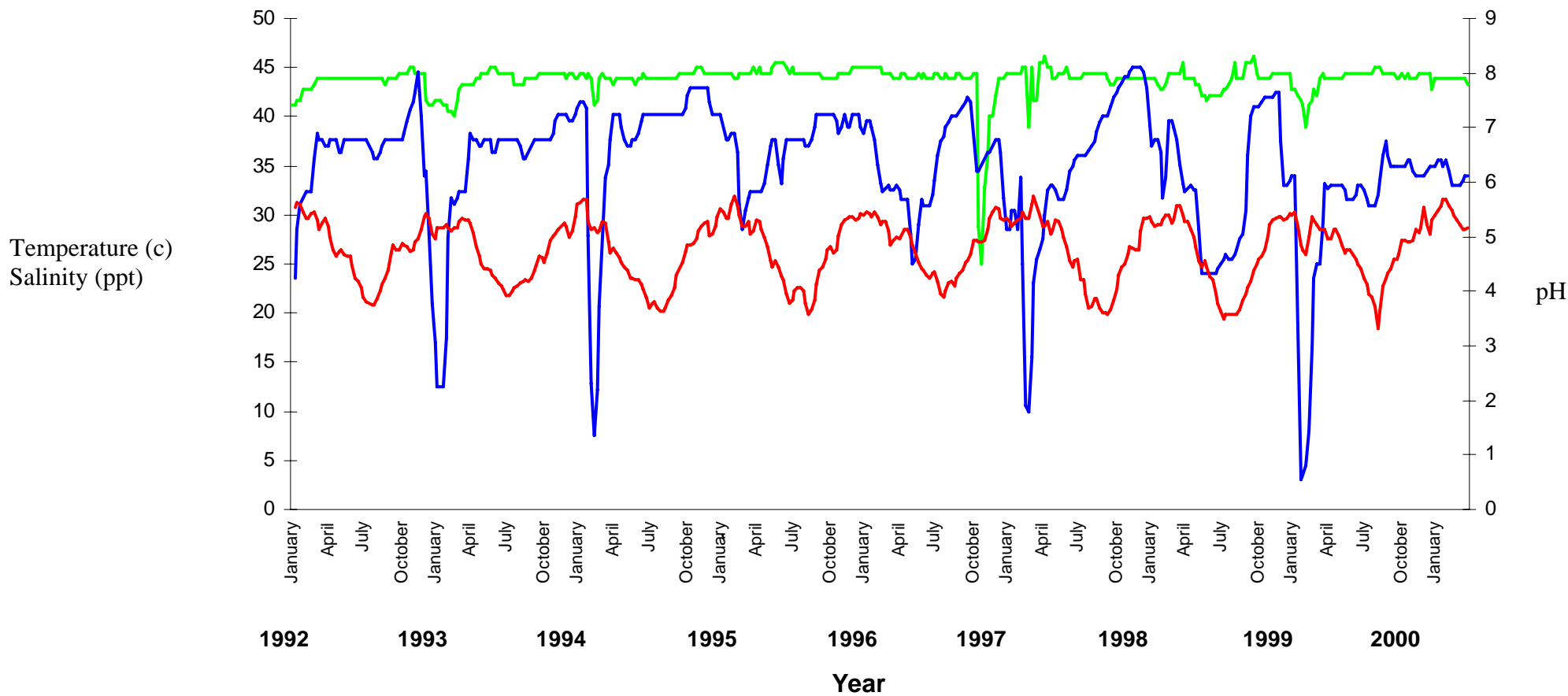


Figure 7.1 Environmental data from a barramundi sea cage facility monitored over the period of 1992 to 2000. Temperature (—) and salinity (—) are plotted on the left axis, while pH (—) on the right.

7.3.2 Mortality data from cage facility

During the period 1996 to 2000, there were 140,000 mortalities at the farm (Figure 7.2). Four thousand and seventy of these fish were subject to bacterial isolation, and where appropriate, bacterial characterisation. The mortality was attributed to *S. iniae* when greater than 90% of the fish sampled at any one time gave pure cultures of the bacterium from the brain. This method indicated that 102,550 (73.3%) of the mortalities observed could be attributed to *S. iniae*.

The peak mortality was observed primarily during the warmer months of September through March, when the water temperature was greater than 25°C. When the temperature data was recoded into ten equal groups, no significant difference was observed ($F = 1.845$, $df = 9$, $p = 0.07$), however post-hoc analysis revealed that group 4 and 5, representing temperatures 25 to 28°C, had significantly higher mortality than the other groups (Appendix 3.1). No relationship was observed between mortalities and pH ($F = 1.32$, $d.f. = 3$, $p = 0.272$) or salinity ($F = 0.736$, $d.f. = 7$, $p = 0.642$) and post-hoc comparison yielded no within group effects with either variable. There was a significant reduction in mortality in 1998 resulting from a coordinated vaccine and management trial at the farm which was removed from analysis, and is reported in detail in Chapter 9. No significance was found between the interaction of factors (Table 7.1).

7.3.3 Challenge data

Barramundi adapted easily to changes in salinity and temperature in the ranges tested. While trying to lower the pH of water, fish displayed considerable signs of stress at or below pH 6 (respiratory distress and erratic swimming behaviour), and mortalities were observed below pH 5. Therefore, challenge trials were aborted for pHs below 6.

When fish were subject to bath challenge with *S. iniae*, relationships were evident with the various factors tested. There were highly significant profiles associated with temperature (Appendix 4a). The highest mortality occurred when the temperature was 27⁰C. At all other temperatures tested, with the exception of 24⁰C, there was a significant difference in the level and schedule of mortality observed (Figure 7.3; Appendix 4a). Increased mortality was observed when the pH of the water was lowered to 6 units when compared to fish challenged at pH 7 or pH 8. At the other levels tested no differences were observed between the groups (Figure 7.4; Appendix 4b). Changes in salinity had little effect on mortality (Figure 7.5; Appendix 4c). No mortality was observed in any of the control fish.

Table 7.1. The general linear model to assess the impact of physical environmental variables on the level of mortality due to *S. iniae* observed at a sea cage facility over a period of 8 years.

Source	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	511702185	86	5950025	.944	.607
Intercept	289835696	1	289835696	45.975	.000
pH	24969089	3	8323029	1.320	.272
Salinity	32470330	7	4638618	.736	.642
Water Temperature	104670088	9	11630009	1.845	.070
pH x Salinity	65257587	7	9322512	1.479	.184
pH x Water temperature	164673612	15	10978240	1.741	.055
Salinity x water temperature	117183174	29	4040799	.641	.914
pH x Salinity x Water temp	50856707	3	16952235	2.689	.051
Error	605201390	96	6304181		
Total	1406739272	183			
Corrected Total	1116903575	182			

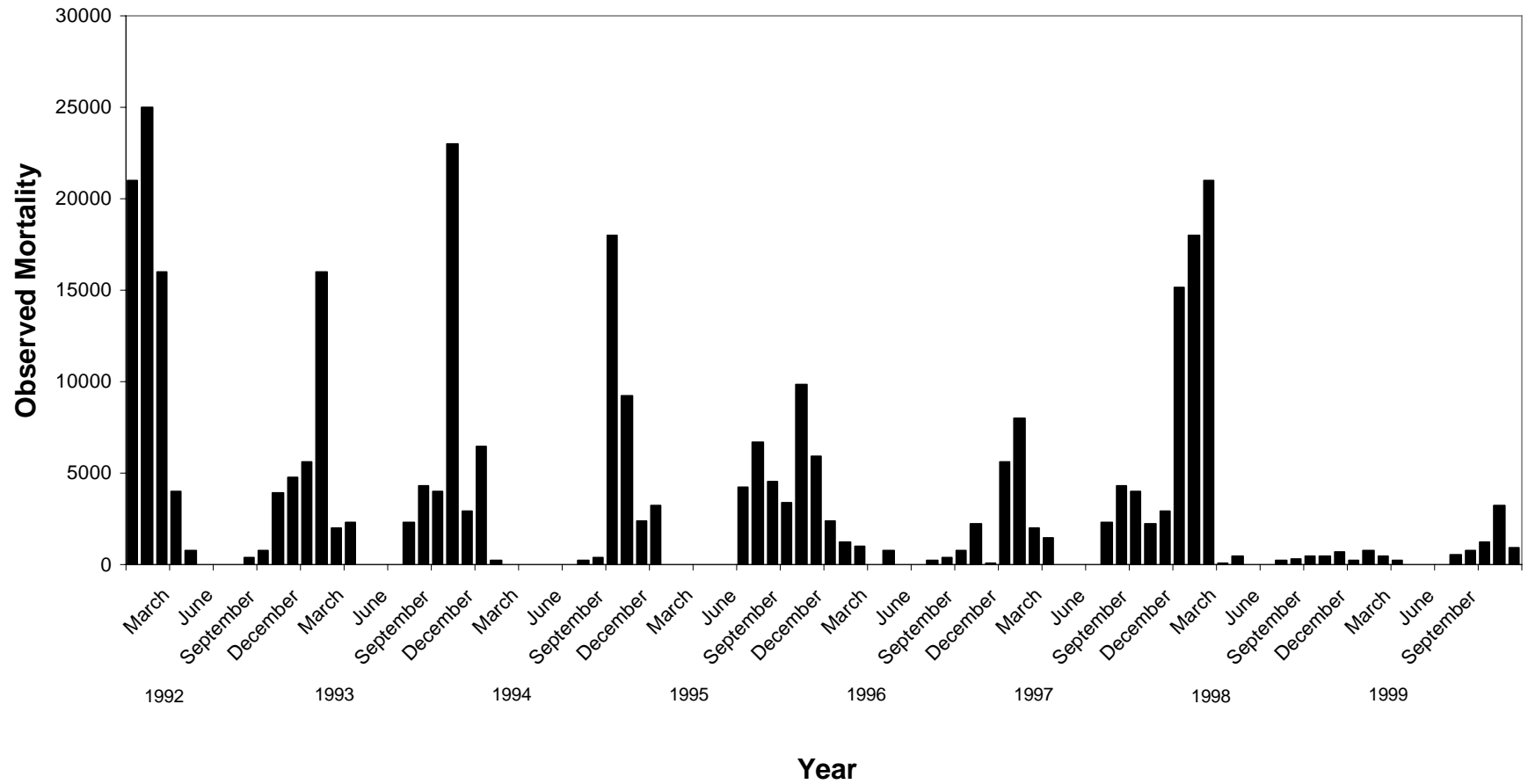


Figure 7.2 The mortality of barramundi attributed to *S. iniae* at a sea cage facility over the period 1992 to 2000

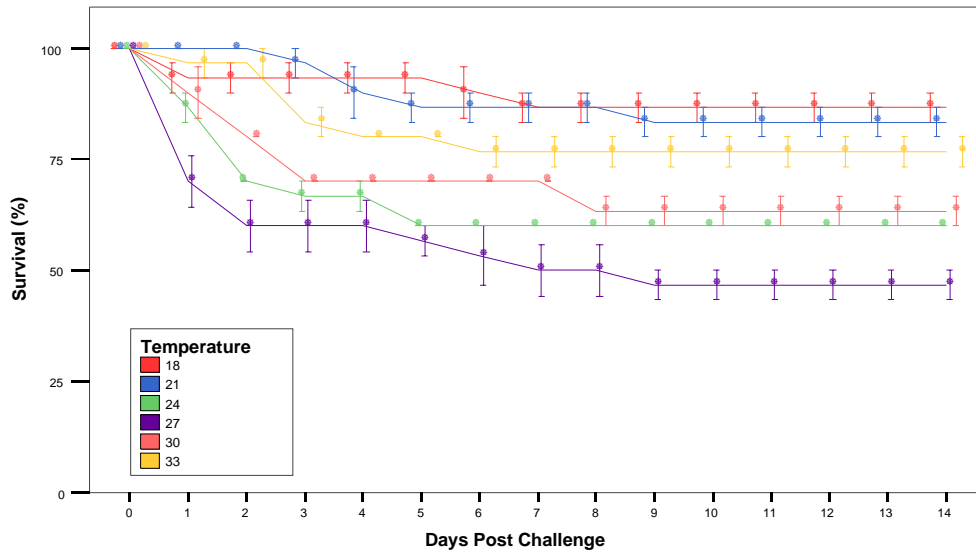


Figure 7.3 The relationship between temperature and mortality observed during challenge trials of barramundi with *S. iniae*.

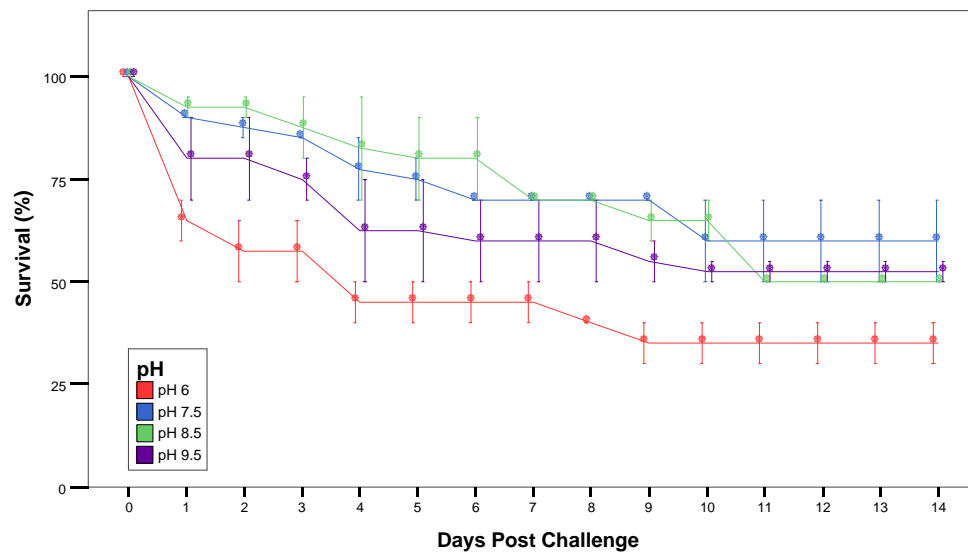


Figure 7.4 The relationship between pH and mortality observed during challenge trials of barramundi with *S. iniae*.

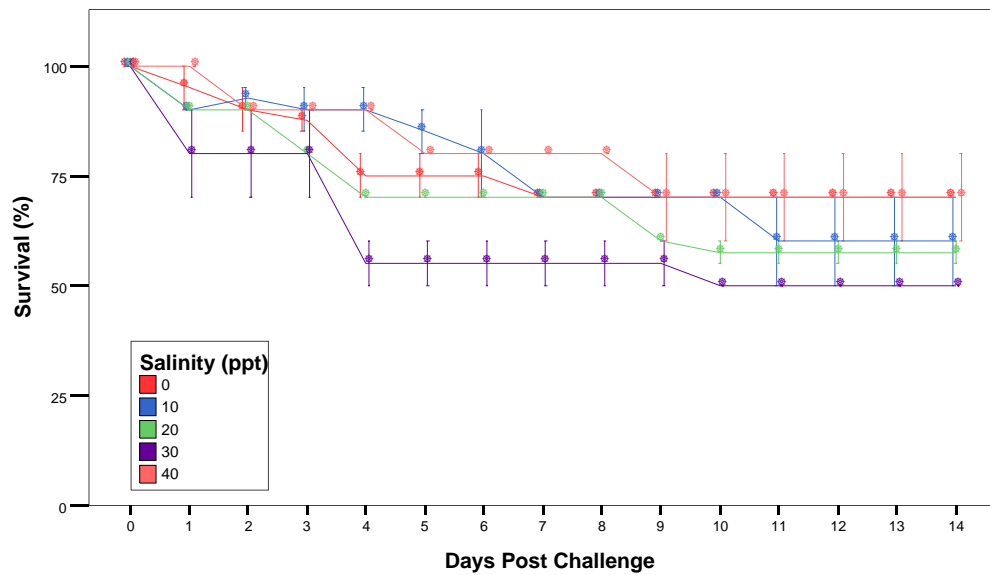


Figure 7.5 The relationship between salinity and mortality observed during challenge trials of barramundi with *S. iniae*.

7.4 Discussion

During the monitoring period, the estuarine environment in which these fish were cultured was subject to seasonal fluctuations in temperature, pH and salinity. No link could be found between salinity or pH and infection with *S. iniae*, but a strong association was determined with temperature. In laboratory challenge experiments, the link between temperature and mortality was also established, while no relationship was determined with either salinity or pH.

The estuarine environment in which the fish were cultured was subject to dramatic fluctuations in salinity, where shifts were recorded from 45 to 5 ppt within 24 h. This corresponded primarily with the end of the dry season and the beginning of monsoon rains. Despite the dramatic changes, surprisingly there was no statistical link between salinity and mortality due to *S. iniae* at the farm.

The pH was much more static throughout the entire year, with only minor fluctuations observed. The sole exception occurred in 1997 when the pH dropped dramatically in the estuary to a low of 4.6 units. This pH shift coincided with heavy losses of barramundi at the farm. The mortality data from this period was not included in the current analysis, despite the fact that more than 70% of the moribund fish observed during this epizootic were positive for *S. iniae* (data not shown). While seawater has a high buffering capacity (Millero 1996), the estuary was subject to severe anthropogenic disturbance to the acid sulphate soils of the surrounding mangrove systems during this period (personal observation). This disturbance was able to overcome the pH buffering capacity of the surrounding water dropping the pH to the level observed. It is therefore likely that the low pH had a deleterious effect on the fish, possibly through heavy metal toxicity occurring at this low pH (Hall 1987) leading to the mortalities observed.

The relationship between temperature and infection with *S. iniae* was pronounced. Mortality peaks during the months when the water temperature is greater than 25°C, typically September through March, with nominal mortality due to *S. iniae* occurring below this temperature. Many previous reports infer a connection with temperature and infection with streptococcal pathogens. For example MacMillan and Santucci (1990) were only able to isolate *Streptococcus* sp. from the intestine of farm raised channel catfish (*I. punctatus*) during the warmer months, though no mortality was reported. While Munday *et al.* (1993) indicated that there was a relationship with temperature and streptococcal infections in rainbow trout (*O. mykiss*), they did not examine this phenomenon further. In a review of streptococcal infections in yellowtail monthly incidence of streptococcosis were provided (Kitao 1993). Infections were observed year round, however, the relative frequency increases dramatically during the warmer months of August through November. The findings of this current study demonstrate conclusively that the streptococcal infections observed in sea cultured barramundi are linked to temperature.

The laboratory challenge experiments supported the relationship between temperature and mortality. The highest prevalence of disease occurred when the temperature was between 24 and 30°C, with the peak mortality observed at 27°C. At the highest and lower temperatures tested (33, 21, 18°C) there was significantly reduced mortality when compared to the group challenged at 27°C. It has been previously determined that the optimum growth rate of *S. iniae* occurs at 27°C (Bromage 1997), above and below this temperature growth of the bacteria was reduced. This temperature may represent the point at which the bacteria are able to overwhelm the host's immune system due to rapid growth. Above this temperature, when the specific immune system is responding more rapidly to bacterial challenge, the barramundi are better able to neutralise the infection, while concurrently the growth of the bacterium is reduced. *Streptococcus iniae* infection at the lower temperatures may be able to be effectively cleared because of slower replication of the bacteria.

Challenge experiments conducted at varying salinity verified previous reports of salinity having little effect on mortality (Bromage 1997; Bromage and Owens 2002). Barramundi have a renowned ability to adapt quickly to salinity fluctuation. Almendras (1996) in his study on osmolality and chloride regulation in barramundi found that after an initial period of crisis, lasting less than 2 days, barramundi plasma returns to pre-crisis levels. This current study utilized a gradual water exchange to achieve to the desired salinity, and then allowed another week for further adaptation, clearly taking the barramundi beyond the crisis phase. Challenge after a dramatic change in salinity (35 to 5 ppt) was not examined in this study, even though salinity shifts of this magnitude were recorded at the farm. This is mainly because analysis of the data from the farm did not indicate that this change led to an increased incidence of disease.

The effect of different pH values on susceptibility of barramundi to *S. iniae* only had an effect only when it was lowered to 6.0 or below. No mortality was observed in the control fish held at pH 6.0; however it was visibly obvious that the fish were stressed under these conditions. Fish that were attempted to be held at lower pH's died during the pH adjustment process. This demonstrates that barramundi are unable to tolerate seawater of low pH, which makes them more susceptible to infection with *S. iniae*. There are many reports of the effects of low pH on the health of freshwater fishes (for review see Sayer and co-workers 1993), but only one report with estuarine fishes (Sammut 2001). In this case, low pH caused considerable effects on fish's gills and integument making them more susceptible to infection by epizootic ulcerative syndrome, a fungal infection.

It was determined that during the period monitored directly by the author (1996-2000), a total of 102,550 barramundi were lost to infection with *S. iniae*. The unrealized market value of the infected fish was estimated to be \$492,000. This is the first comprehensive study on the physical factors that can affect the susceptibility of fish to *S. iniae*, with the caveat to this work that physical parameters were studied individually, while they obviously act in unison. However it has been shown conclusively that infection with *S. iniae* is

dependant on elevated water temperature, while environmental stressors such as estuarine acidification can render barramundi more susceptible to disease.

CHAPTER 8

TEMPERATURE MEDIATED HUMORAL IMMUNITY – A KINETIC STUDY

8.1 Introduction

It has long been known that temperature has a dramatic effect on the immune response of poikilothermic vertebrates (Bisset 1948). Typically, when a fish is challenged at low environmental temperatures there is minimal specific response to the invasion. Temperatures where fish can mount an adaptive immune response have been termed 'permissive', while temperatures that are unfavourable for specific immune response have been termed 'non-permissive' (Bly *et al.* 1986; Bly *et al.* 1990).

It has been suggested that the primary effect of temperature is in the helper T cell functions (Bly and Clem 1991), while others have found that interactions between B and T cells to be the most critical step affected (Avtalion 1981). At non-permissive temperatures there is no response seen in either B or T cells. At temperatures on the margin of being non-permissive the immune response (B-cell) is slower to develop, but the magnitude of the response is thought to be unaffected (Stolen *et al.* 1984; Lillehaug *et al.* 1993; Hrubec *et al.* 1996; Eggset *et al.* 1997). When these reports are more closely examined, it is clear that the mean titre of the population appears to be unaffected by the lower temperature of challenge, but there is clearly increased variability in the response when compared to animals challenged at the higher temperatures.

This study examined the specific antibody response of adult barramundi vaccinated and maintained at varying temperatures. I further wished to explore the specific antibody variability observed in vaccinated animals at these temperatures and sought to identify a link between antibody production and protection against *S. iniae* in these animals.

8.2 Materials and Methods

8.2.1 Experimental design

The barramundi used in the experiment were obtained from Bluewater Barramundi and were of an average total length of 30 cm. All fish were maintained at a salinity of 30 ppt and pH 7.8. Groups of 10 fish (Table 8.1) were held in individual 1000 L Reln bins with aeration and bio-filtration (Figure 8.1). Experimental rooms (2) were held at a constant temperature of either 17⁰C or 27⁰C, and tanks were gradually heated (1 week) to the desired temperature (19, 24, 27, and 33⁰C) using 400 Watt bar immersion heaters. Fish were allowed to acclimatise to the temperature for a further 3 weeks. Two additional groups of fish were held at 19 and 27⁰C. Four weeks after the primary inoculation these fish were given a booster inoculation (details below). One final group was vaccinated and maintained at 19⁰C for 4 weeks, before the temperature was elevated over a one week period to 27⁰C, and the fish maintained at this temperature for a further 8 weeks. All fish were monitored over a period of 14 weeks.

Table 8.1 The temperature and inoculation profile of the seven groups utilised in the study of the temperature effect on humoral immunity.

Group number	Temperature (°C)	Primary inoculation at week 0	Booster inoculation at week 4
1	19	Yes	No
2	24	Yes	No
3	27	Yes	No
4	33	Yes	No
5	19	Yes	Yes
6	27	Yes	Yes
7	19 raised to 27	Yes	No

In each group, seven of the fish were vaccinated via IP inoculation with 10^9 formalin killed *S. iniae* cells (#28) in FIA (200 μ l total), while the remaining three fish were injected with FIA only, and served as controls. The booster inoculation contained the same number of formalin killed cells in FIA (200 μ l total). Each animal was fin clipped to enable identification. One half ml blood was drawn from each animal at week 0, 3, 5, 8, 11, and 14. Blood was processed as per chapter 3.1.

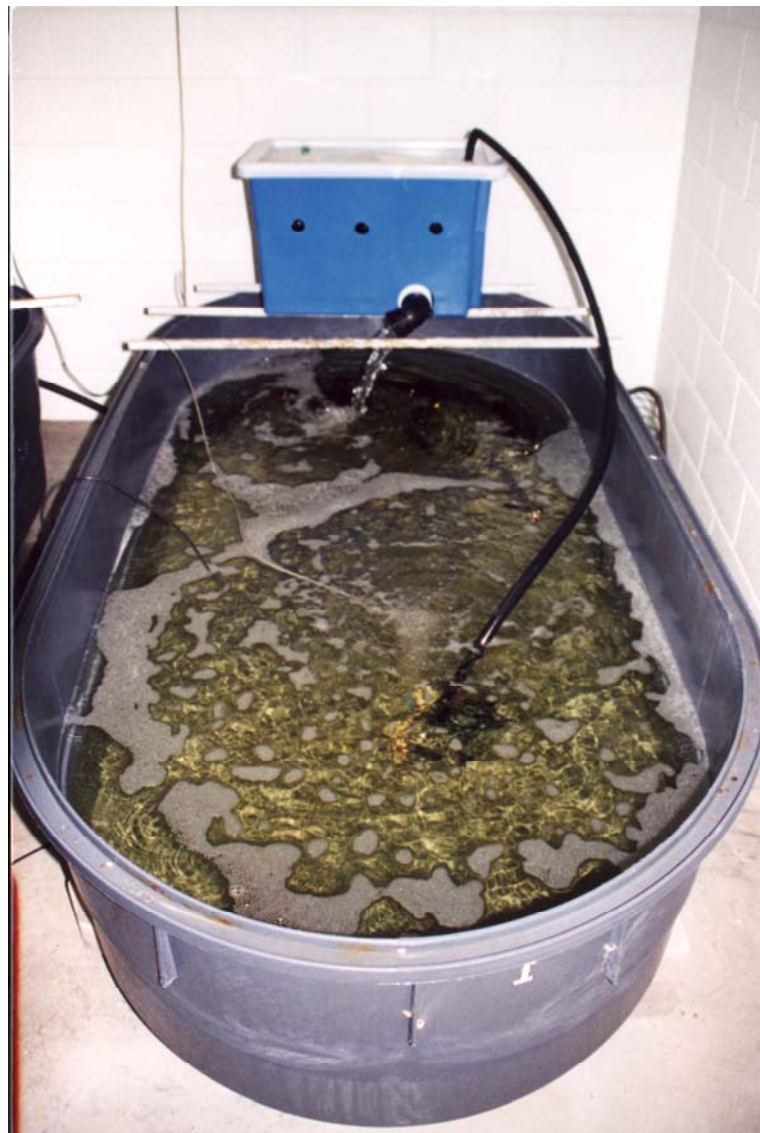


Figure 8.1 The aquarium system used for holding individual experimental groups of barramundi.

8.2.2 Specific antibody response

Serum from each animal was titrated in duplicate on ELISA plates coated with *S. iniae* (#28) as described in Chapter 5. Serum from a hyper-immunised barramundi (obtained from a previous study, Bromage 1997) was used as the control to compare all reactions. The hyper-immune serum was serially diluted (5 fold) in pre-immune sera from the same animal, and added in duplicate to the plate starting at an initial dilution of 1:50. The titration curve produced was divided equally into 8 groups, with 1 being unresponsive and 8 having a response greater or equal to that of the hyper-immune serum (Figure 8.2). All sera to be tested were diluted 1:5000 in PBS, which was approximately equal to 50% of the maximum OD rate per minute achieved from the hyperimmune serum, and added to the plate in duplicate. Thus 40 barramundi serum samples could be screened per plate. The ELISA was performed as per Chapter 5.2.7.2, and each plate read (414nm) while the reaction was still linear. The average OD rate of the samples were compared to the control sera titration curve and ranked (grouped) according to the serogroup they were in.

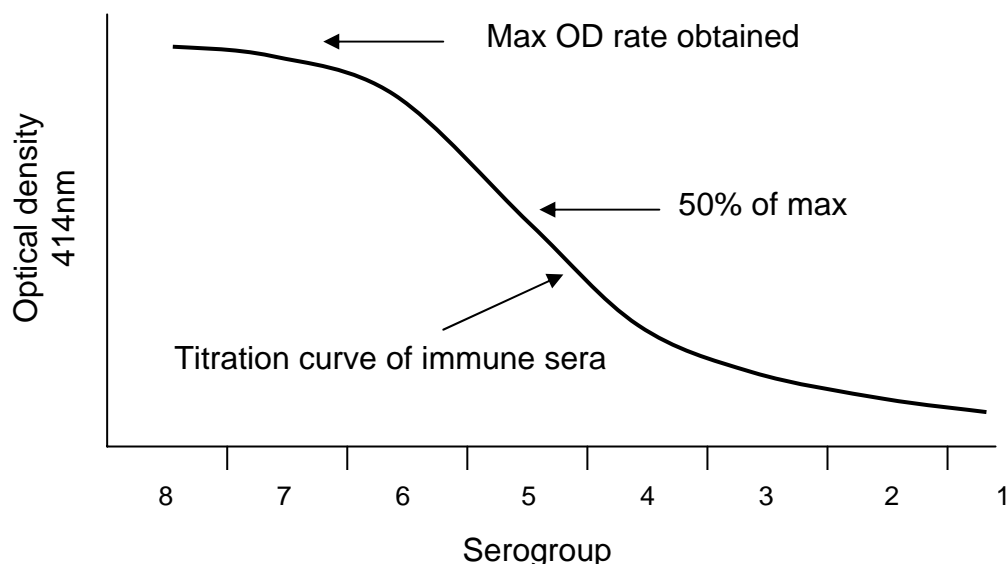


Figure 8.2 The titration curve and serogrouping of anti-*Streptococcus iniae* sera derived from a hyper-immunised barramundi

8.2.3 Total serum antibody

Total antibody was determined using the capture ELISA designed in Chapter 5, and results analysed by ANOVA with a Tukey's post-hoc comparison.

8.3 Results

8.3.1 Temperature effect on specific antibody development

The effect of temperature on the antibody response of barramundi was markedly different only at the lowest temperature examined (19⁰C) (Figure 8.3). Approximately 40% of the fish challenged failed to mount a detectable antibody response before the end of the monitoring period. None of the fish held at 19 and 23⁰C (Figure 8.4) had a detectable response before week 5. Fish held at the higher temperatures, 27⁰C (Figure 8.5) 33⁰C (Figure 8.6), had a higher average response and a lower deviation than those held at lower temperatures.

In all groups examined, the experimental animals displayed a great deal of heterogeneity in the immune response. Within groups, especially at the lower temperatures, some fish failed to respond or responded minimally while others responded rapidly with a high, measurable titre.

The effect of a booster inoculation appeared to be important to the sero-conversion of barramundi maintained at 19⁰C (Figure 8.7), but not at 27⁰C (Figure 8.8). The delivery of a booster to the fish maintained at the lower temperature resulted in all the fish sero-converting, developing an average titre greater than the unboosted group maintained at the same temperature (Figure 8.3). The same result was observed in the fish maintained vaccinated and maintained at 19⁰C before the temperature being elevated to 27⁰C (Figure 8.9).

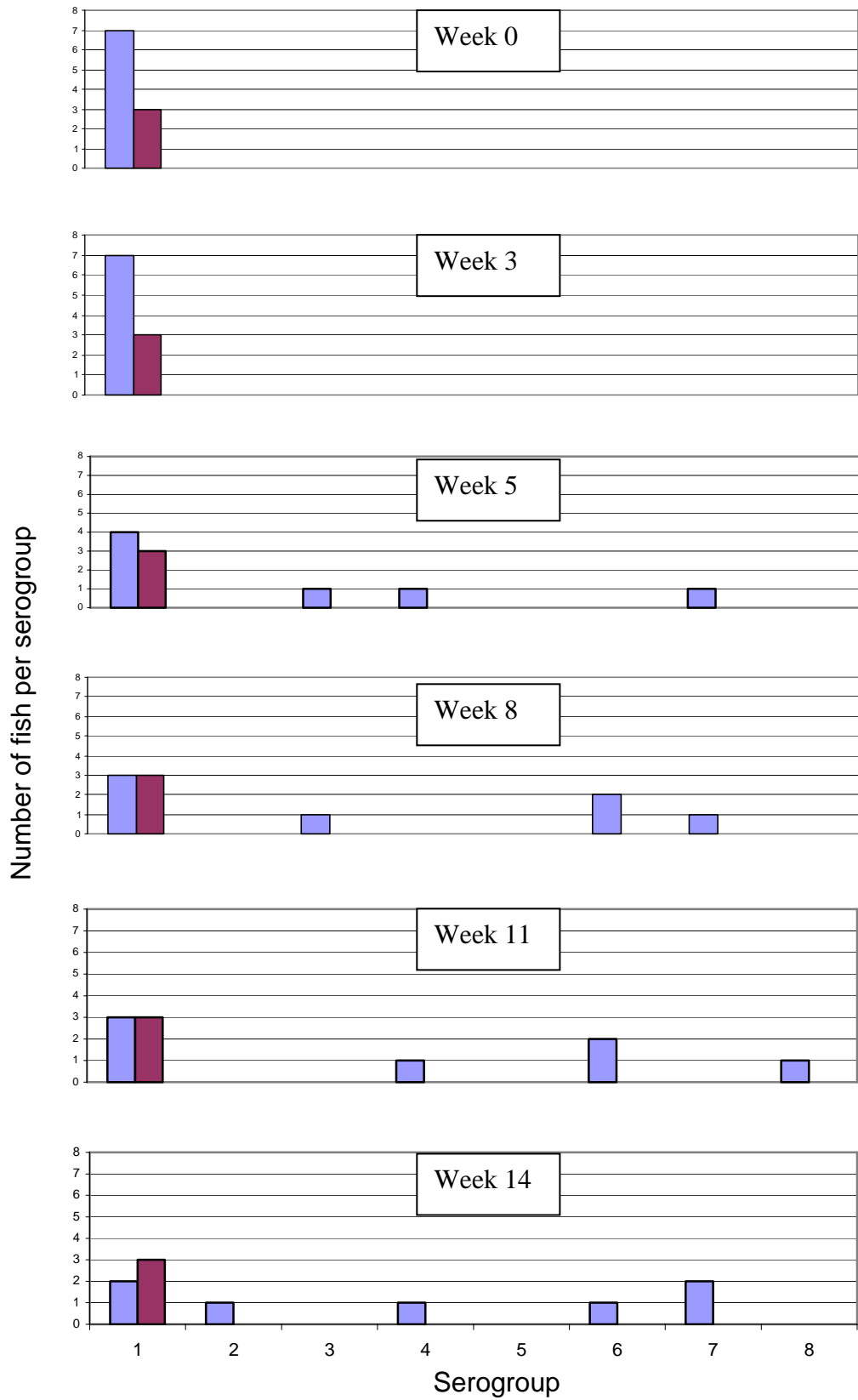


Figure 8.3 The change in the serogroup distribution of barramundi vaccinated with *S. iniae* and maintained at a constant 19°C (blue columns) over a 14 week period, and those of unvaccinated control fish (purple column).

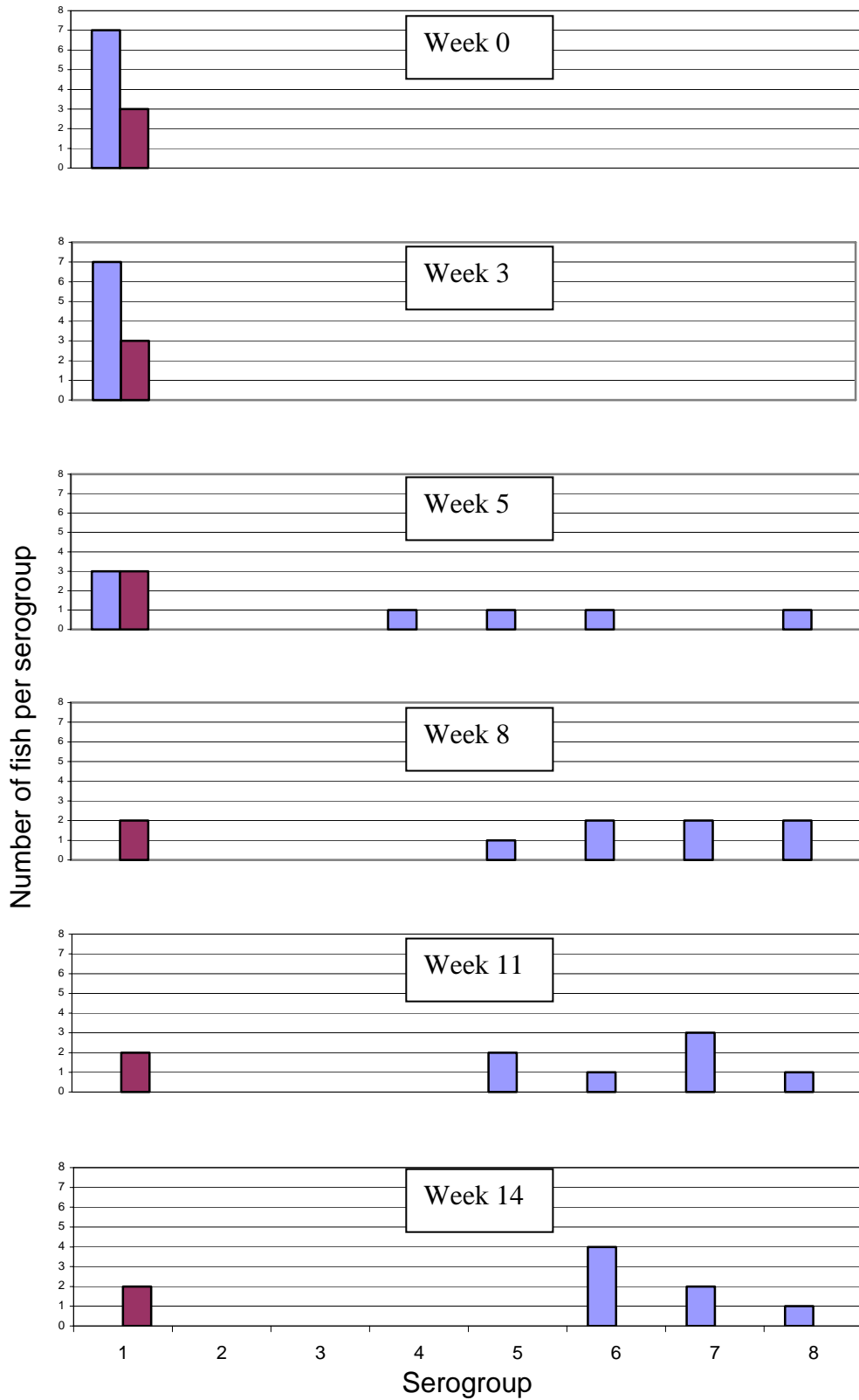


Figure 8.4 The change in the serogroup distribution of barramundi vaccinated with *S. iniae* and maintained at a constant 23°C (blue columns) over a 14 week period, and those of unvaccinated control fish (purple column).

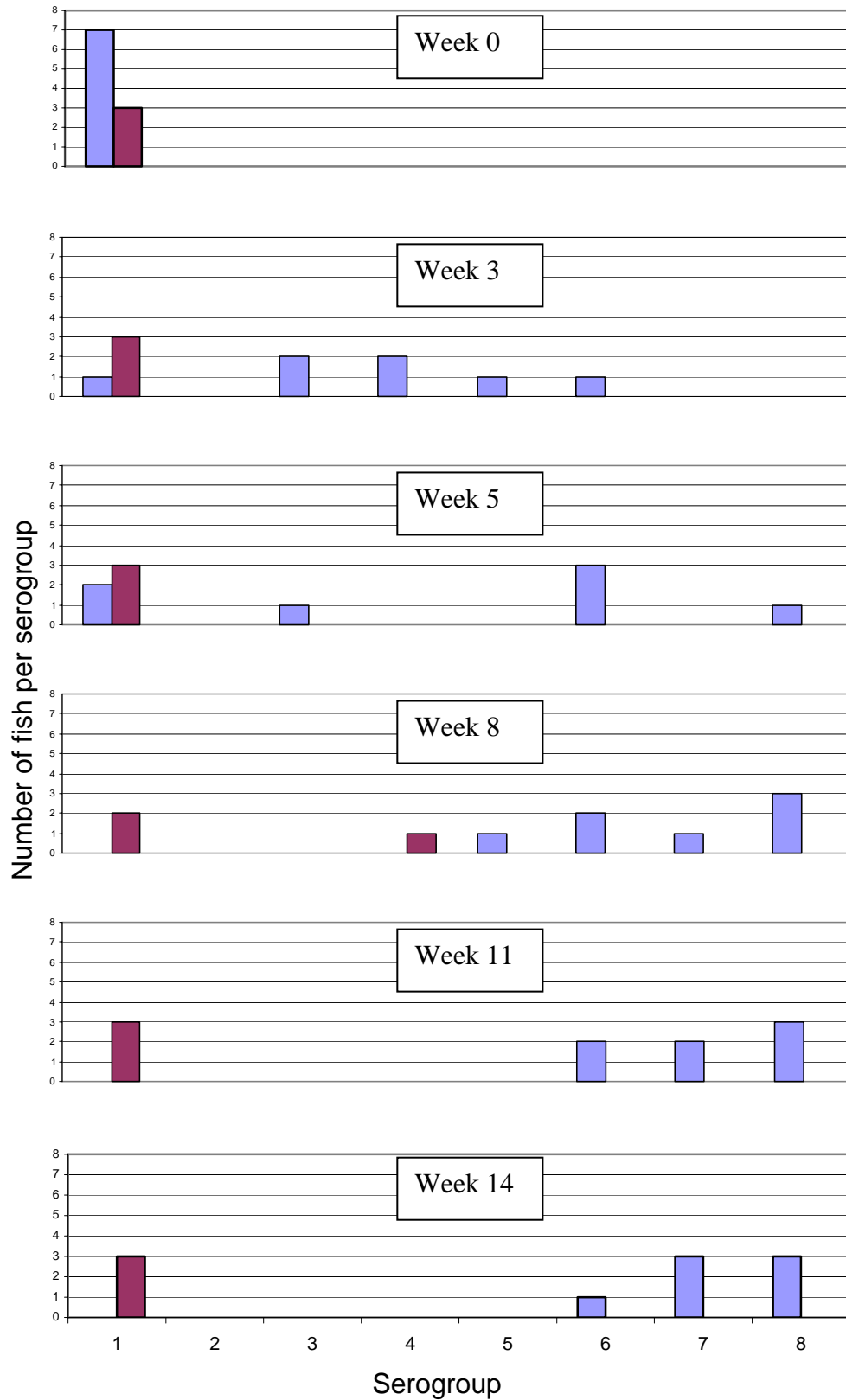


Figure 8.5 The change in the serogroup distribution of barramundi vaccinated with *S. iniae* and maintained at a constant 27°C (blue columns) over a 14 week period, and those of unvaccinated control fish (purple column).

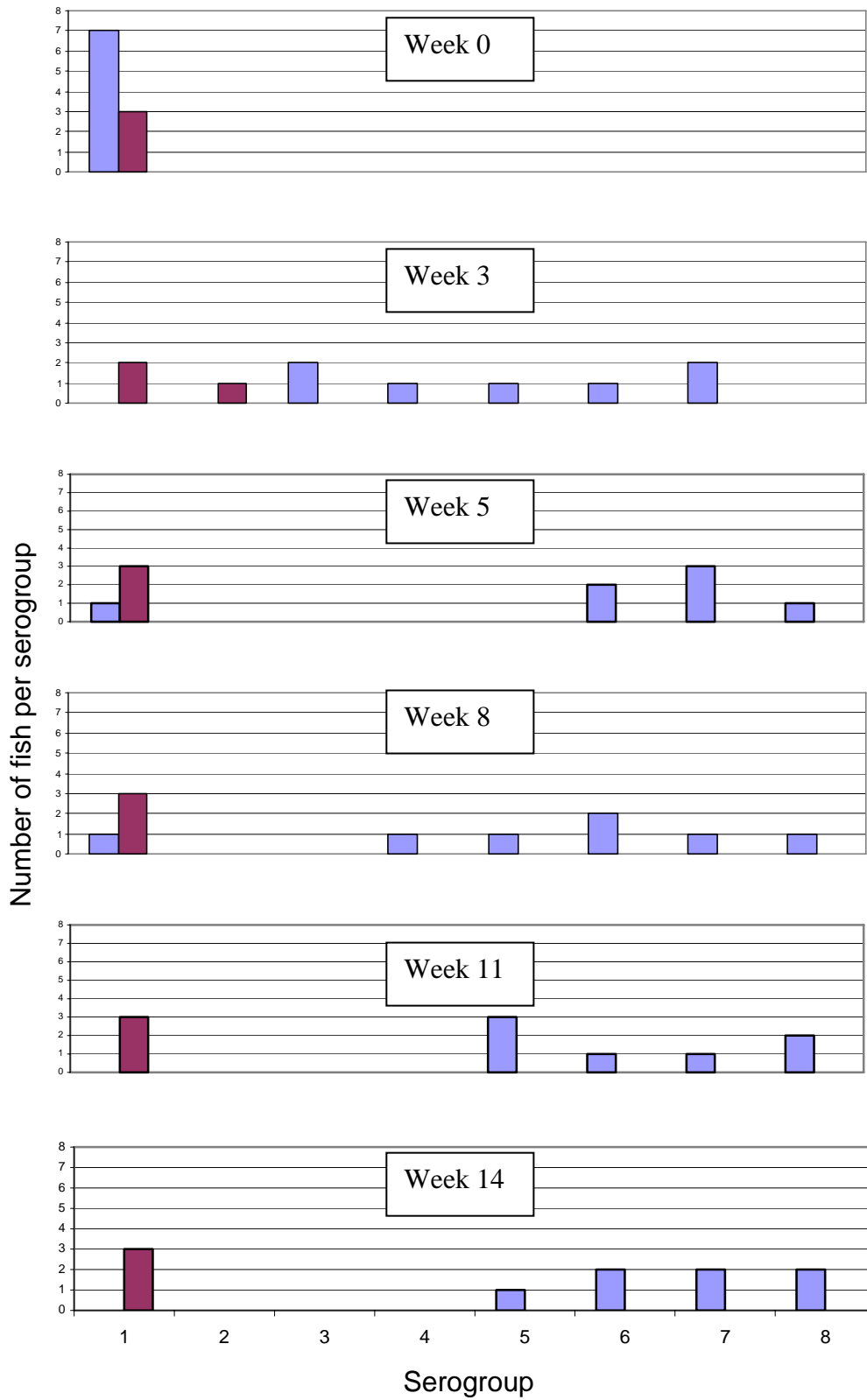


Figure 8.6 The change in the serogroup distribution of barramundi vaccinated with *S. iniae* and maintained at a constant 33°C (blue columns) over a 14 week period, and those of unvaccinated control fish (purple column).

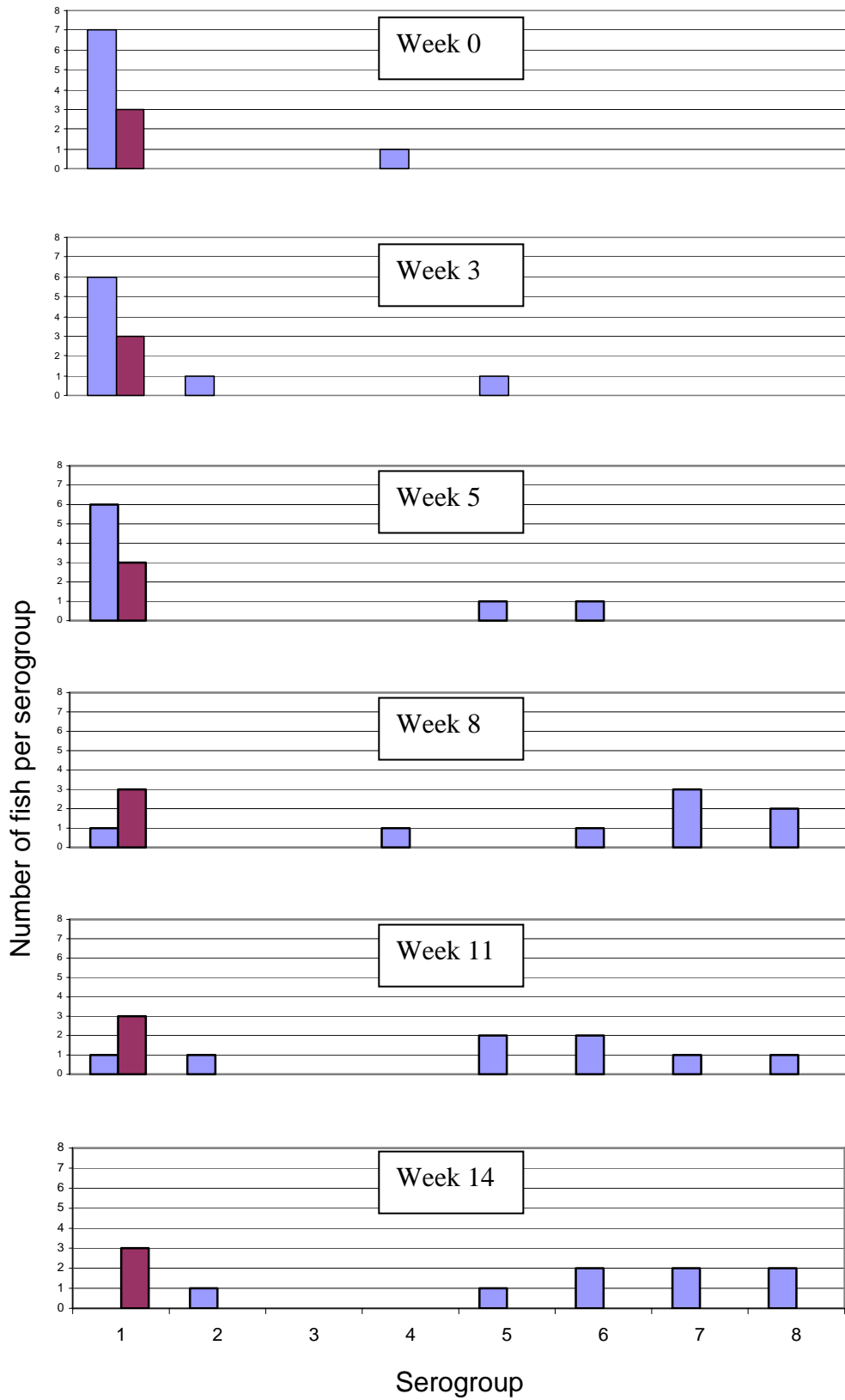


Figure 8.7 The change in the serogroup distribution of barramundi vaccinated (week 0) and boosted (week 4) with *S. iniae* and maintained at a constant

19°C (blue columns) over a 14 week period, and those of unvaccinated control fish (purple column).

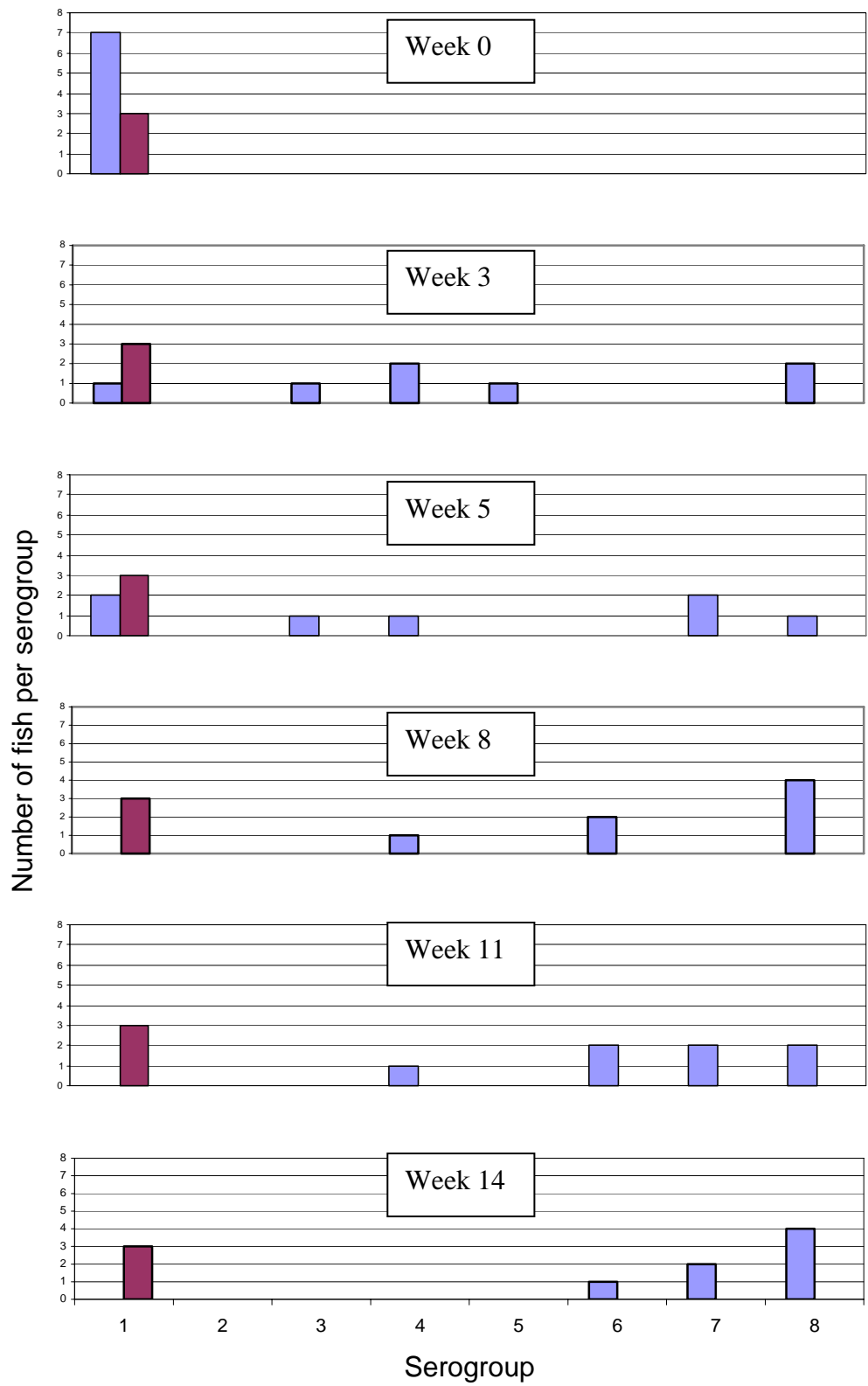


Figure 8.8 The change in the serogroup distribution of barramundi vaccinated (week 0) and boosted (week 4) with *S. iniae* and maintained at a constant

27⁰C (blue columns) over a 14 week period, and those of unvaccinated control fish (purple column).

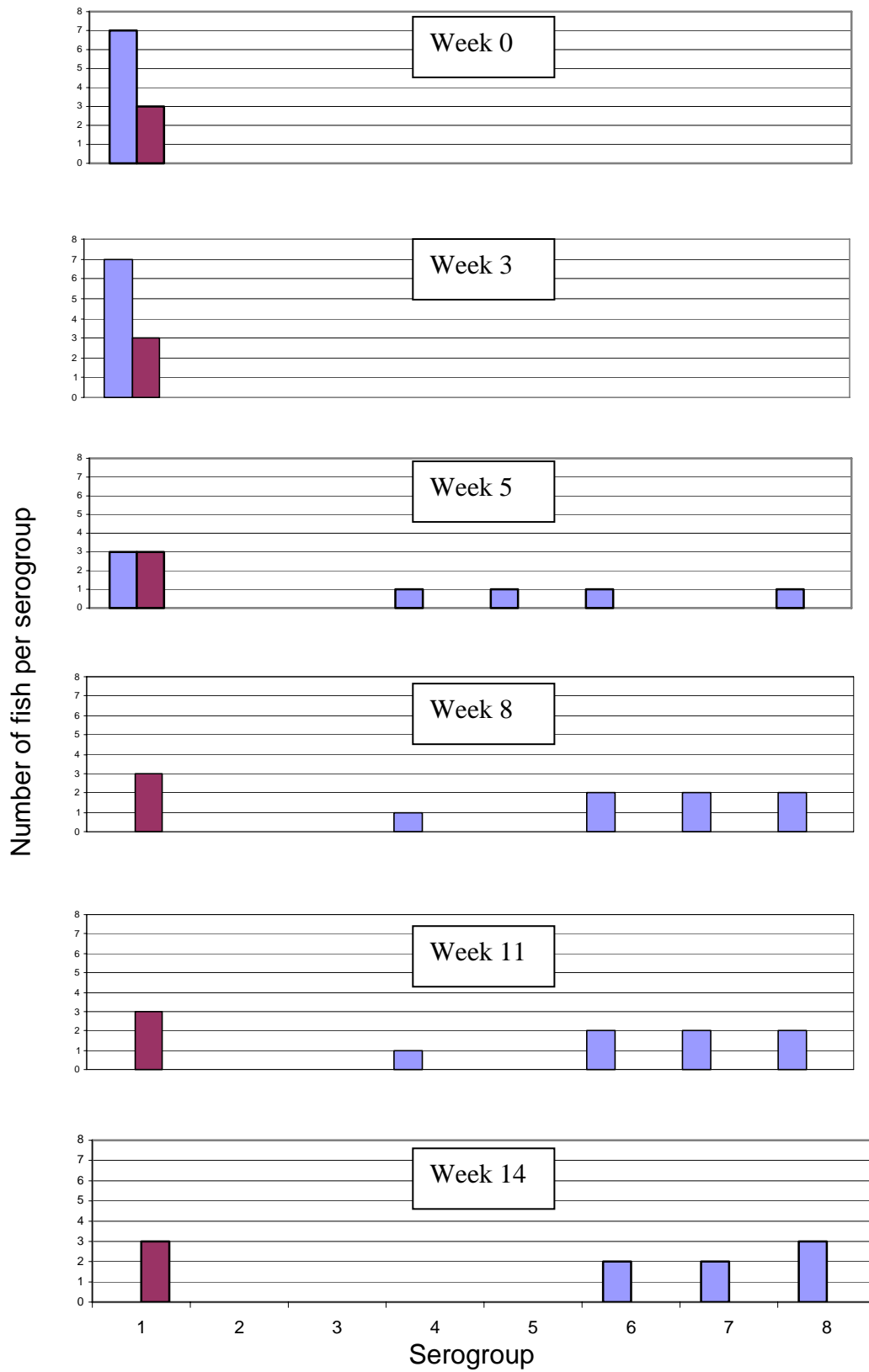


Figure 8.9 The change in the serogroup distribution of barramundi vaccinated with *S. iniae* and maintained at a constant 19⁰C (blue columns) for 4 weeks, before the environmental temperature was elevated to 27⁰C and maintained for a further 8 weeks. Unvaccinated control fish (purple column).

8.3.2 Temperature effect on total serum antibody

The levels of serum Ig varied considerably between experimental groups over the experimental period (Figure 8.10). In particular, fish held at cooler temperatures possessed higher levels of serum antibody than those held at the higher temperatures. However, statistical analysis revealed that these differences were not significant (df=15, F=0.585, p=0.880) at any given time, except during week 8 when the barramundi maintained at 19⁰C were determined to differ (p<0.001) from those held at 27⁰C in a Tukey's post hoc comparison.

The serum Ig levels were also compared for animals that were held at 19⁰C (Figure 8.11) and 27⁰C (Figure 8.12) and subject to an additional booster vaccination at week 4. Barramundi held at 19⁰C displayed a significant decrease in serum antibody one week following the boost when compared to the control (p<0.001) and single injected (p=0.001) fish as determined by post-hoc analysis. The serum Ig levels returned to normal in the subsequent weeks of the experimental period. The fish held at 27⁰C also displayed an initial decrease in serum antibody the week following inoculation; however, the change was determined not to be significant (p>0.05).

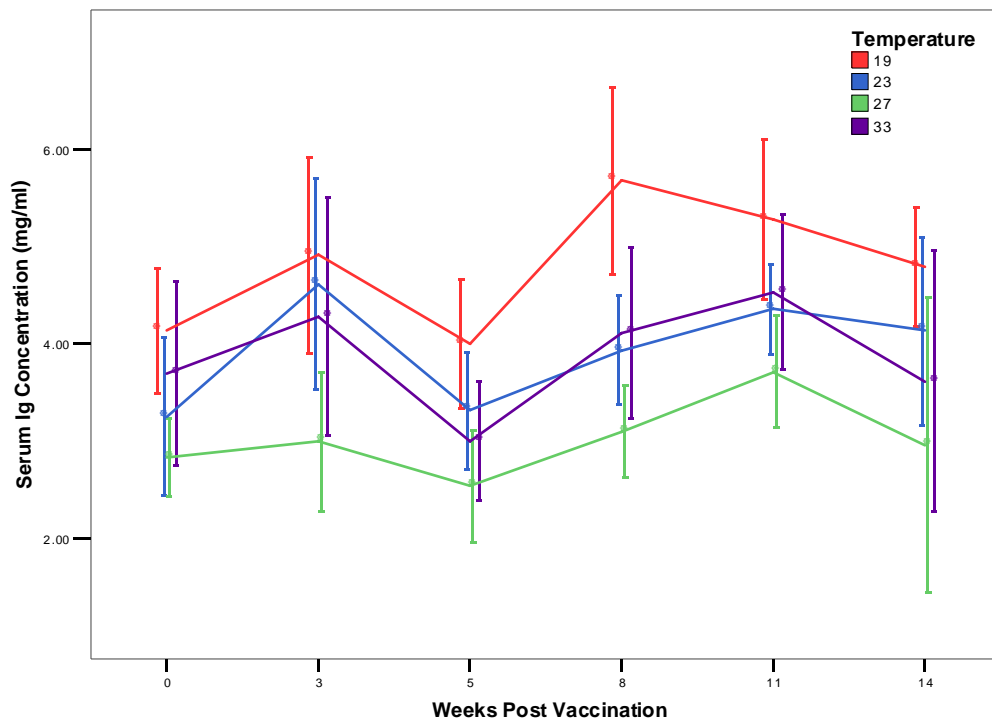


Figure 8.10 Total serum antibody (mg/ml) of barramundi vaccinated with *S. iniae* and maintained at different temperatures over a 14 week period. Bars indicate the standard deviation.

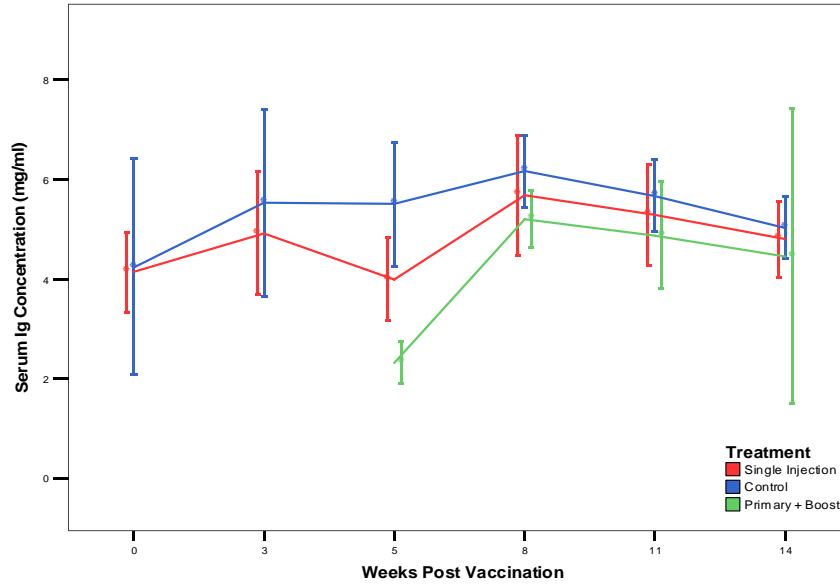


Figure 8.11 Total serum antibody (mg/ml) of barramundi that have been subject to a primary vaccination or primary and a booster inoculation (week 4) with *S. iniae* and maintained at 19⁰C over a 14 week period. Bars indicate the standard deviation.

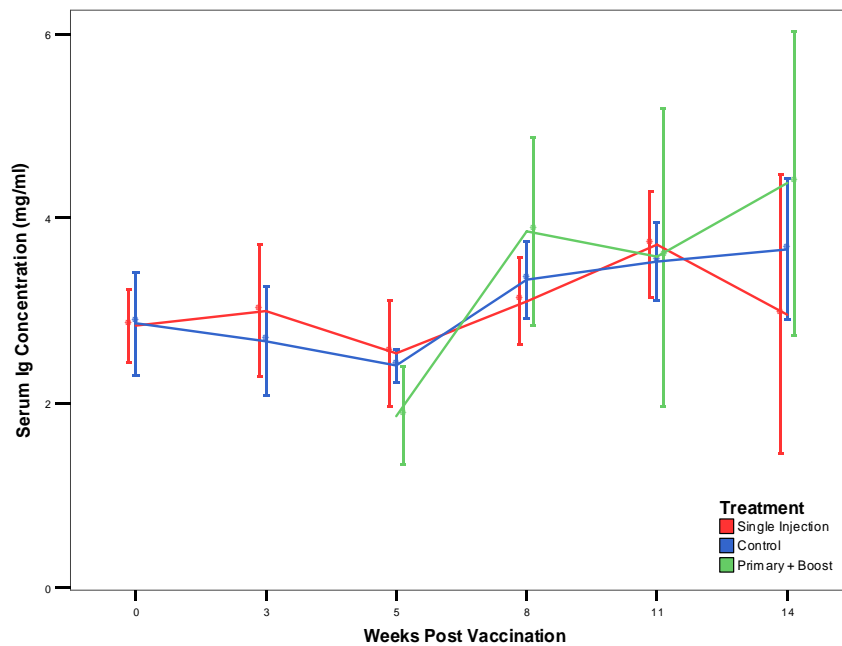


Figure 8.12 Total serum antibody (mg/ml) of barramundi that have been subject to a primary vaccination or primary and a booster inoculation (week 4) with *S. iniae* and maintained at 27°C over a 14 week period. Bars indicate the standard deviation.

8.4 Discussion

The present study documents the effect of temperature on the humoral immune response of barramundi following challenge with killed *S. iniae*. The data clearly demonstrate that at low temperature (19°C), the humoral immune response is diminished, delayed and highly variable between animals. This was in contrast to the groups held at higher temperatures with responses in these animals being higher and more uniform. Environmental temperature also impacted the level of total serum antibody, with animals held at lower temperatures possessing a greater level of circulating Ig. The effect of elevating the temperature (19 to 27°C) following vaccination, or administering a booster vaccination, permitted sero-conversion to occur in all animals compared to the 35% of fish that failed to respond in the control group held at 19°C.

This is the first and only study of teleost humoral immunity that has used this method of ranking the animals into sero-groups. The method has previously been used to examine the humoral immune response in herd animals (Spencer and Burgess 1984; Burgess *et al.* 1985) as this method allows a large number of samples to be analysed rapidly with a substantial reduction in the volume of reagents required when compared to standard titration methods. The technique utilises the rate of reaction of the ELISA measured when the OD is still increasing in a linear fashion, in contrast to endpoint reactions, which then can be proportionally linked to the amount of specific antibody bound to the *S. iniae* (Spencer and Burgess 1984). Using this analytical method and technique of reporting, I was able to highlight the variability in the population, particularly emphasising those animals that failed to respond to challenge.

The effect of low environmental temperature on the immune system of fish has been well examined (for review see Atvalion 1981). However there has been considerable conjecture in the literature whether the low temperature only delays the immune response, with animals still obtaining the same titre as at elevated temperatures (Lillehaug *et al.* 1993), or whether the immune response is diminished (Groberg *et al.* 1983). Proponents of the delayed response suggest that the processing of the antigen occurs at a slower rate at the lower temperature (O'Neill 1980; Lillehaug *et al.* 1993), in effect prolonging the exposure to the antigen allowing a maximal response to develop.

It was clear that the response was considerably delayed at 19⁰C (Figure 8.3), as the barramundi held at this temperature took longer to respond, or failed to respond, when compared to those held at higher temperatures. The data is also strongly weighted towards a diminished immune response, with the caveat being that the experiment was concluded before the barramundi humoral immune response began to decline (as evidenced by a decrease in sero-group). However, only one animal had achieved the maximal sero-group, subsequently dropping to group 7, and 35% of the animals failed to respond.

The administration of a secondary vaccination had considerable effect on barramundi held at 19⁰C. It allowed all animals in this test group to seroconvert as well as enhancing the overall immune response in the group (Figure 8.7). The improved response could be due to an amnestic reaction in these animals, or just from the increased bacterial load delivered to the animals. Either way, this finding highlights that it may be feasible to vaccinate animals during the winter, when typically the workload at farms is diminished, to generate an effective humoral immune response that may protect barramundi during the warmer months when bacterial infection is more prolific (Chapter 7). There was also marginal improvement of the rate seroconversion in the 27⁰C group that had a secondary immunisation, however it did not appear to effect the final sero-group of the animals. This would indicate that a secondary vaccination is of primary importance to animals vaccinated at low temperatures.

Raising the temperature from 19⁰C to 27⁰C four weeks after vaccination also improved the response in animals vaccinated at 19⁰C. The response was similar to that of providing a booster vaccination in that it allowed all animals to seroconvert and to achieve a higher sero-group (Figure 8.9). This is similar to the observations of Pylkko and co-workers (Pylkko *et al.* 2002) who examined the effect of increasing temperature with Arctic charr (*Salvelinus alpinus*) vaccinated at low temperature. This could suggest that barramundi can not process the bacteria as efficiently at the lower temperature, and is worthy of further investigation.

Clearly, the lower temperature used in this study (19⁰C) was not able to completely eliminate the humoral immune response in barramundi. However, the fact that 35% of the fish failed to respond suggests that the temperature was near the margin of being 'non-permissive' for a B-cell response as described by Bly and co-workers (1986). It may have been experimentally possible to hold the fish at a lower temperature, however 19⁰C was chosen as the lower limit for several reasons including: It represents the lower limit at which barramundi are typically cultured (Barlow 1998), it is close to the natural temperature limit of the north-eastern barramundi stock (Keenan 2000), and it

is also not economically feasible to culture barramundi below this temperature due to retarded growth. It may be that this temperature not only affects the growth but also may be the limit at which barramundi can adequately fight the progression of infectious disease (Bly and Clem 1992).

Very few people have examined the effect of temperature of total serum antibody. This study demonstrated that fish held at lower temperatures had a higher antibody level, displayed by both vaccinated and control fish; however there was a considerable amount of variability. The significance of this is yet to be fully understood. However, it is known that the Atlantic cod (*Gadus morhua*), common in Arctic waters, has a very high level of circulating antibodies to compensate for retarded adaptive immune response at the temperatures it is found (Israelsson *et al.* 1991). Indeed, due to the rapid kinetics of the adaptive immune response observed at elevated temperatures, high levels of circulating antibodies may not be required to deal with infection, while at lower temperatures increased Ig levels may confer a protective advantage.

This was a unique way to look at the population dynamics of a group of fish rather than grouped data, and demonstrated that barramundi subject to bacterial exposure at different temperatures demonstrated considerable heterogeneity in immune response. The results could be interpreted in that the temperature used was not low enough to completely eliminate the immune response, but the range monitored was biologically relevant to the culture of the fish.

CHAPTER 9

IMMUNOLOGY OF EXPERIMENTAL AND FIELD VACCINE TRIALS FOR *STREPTOCOCCUS INIAE* IN BARRAMUNDI, *LATES CALCARIFER*

9.1 Introduction

It is well accepted that prevention of infectious diseases is much more desirable than trying to cure a problem once established. Thus the ongoing outbreaks of streptococcosis at barramundi farms throughout Australia have caused considerable concern in the aquaculture industry (Carson and Munday 1990; Bromage *et al.* 1999; Kahn *et al.* 1999; Bromage and Owens 2002). While efficient husbandry can reduce the occurrence of infectious disease (Shoemaker *et al.* 2000), the very nature of aquaculture; with its high stocking densities and single species culture systems makes it even more at risk to microbial attack.

Antibiotic usage has long been established with aquaculture for immediate control of many diseases (Lillehaug *et al.* 2003), but it has fallen out of favour in recent years due to the adverse effects on the environment and the increasing trend towards multi-antibiotic resistant strains of bacteria (Wiklund and Dalsgaard 1998; Gudding *et al.* 1999; Lillehaug *et al.* 2003). This has been the case with *S. iniae* infections at a sea-cage facility monitored during this study. There has been a trend toward antibiotic resistance against tetracyclines, and new drugs have needed to be used to help control outbreaks (personal observations).

The most promising method in successful control of this disease, and many other diseases affecting aquaculture, is through the development and effective delivery of vaccines (Newman 1993). There have been many attempts in the past to produce vaccines against fish pathogenic streptococci, meeting with varying success (Toranzo *et al.* 1995; Eldar *et al.* 1997; Klesius

et al. 2000; Klesius *et al.* 2001). Previously, vaccination of barramundi with a whole cell formulation was attempted and resulted in protection in laboratory trials (Bromage 1997). These results indicated that the trials should be expanded and tested in the field.

Typically the saltwater infections result in an ongoing chronic mortality with peaks of heavy losses when the water temperature is at 27⁰C. More recently, outbreaks of streptococcosis have started to occur in intensive freshwater recirculation systems used in the culture of barramundi (personal observations). Outbreaks of *S. iniae* in freshwater systems are usually rapid, with mortalities doubling every day, and total losses of systems and farms occurring within days. While clinically the disease appears the same in both freshwater and saltwater systems, there is a notable difference in the severity of mortalities observed at the different sites.

This study was devised to examine if there was an immunological basis for the difference in the diseases observed at the barramundi culture facilities, and to examine the effect of vaccination at these facilities. The specific aims of this study were to determine if there is a relationship between specific antibody titre and protection from lethal challenge.

9.2 Materials and Methods

9.2.1. Monitoring of specific serum antibody at various locations

Over a period of one year, monthly serum samples were obtained from barramundi being cultured at 3 different sites. Farm 1 was the sea-cage facility examined in Chapter 7; Farm 2 was a large freshwater recirculation facility that suffers from severe to sporadic epizootics due to *S. iniae*, while farm 3 is a freshwater facility that is regarded as *S. iniae* free. At each facility, fish of approximately the same size were chosen (starting weight 150 g), and this group was monitored for the entire period. At all facilities stocking

densities were maintained between 25 – 40 kg/m³. From each facility blood was obtained from 50 individual fish, allowed to clot, serum collected and stored at –20°C until required.

9.2.2 Relationship of sero-group with protection

One hundred and ten barramundi (200 g) were transported from the sea-cage facility to holding tanks at James Cook University. Fish were individually tagged and maintained 10 per tank at 27°C in 30 ppt seawater. One ml serum samples were collected from each animal and stored –20°C until required. To assess the relationship of serogroup and protection barramundi were bath challenged (as per Chapter 6) with a LD₁₀₀ (4.4 x 10⁶ cells / ml, Bromage *et. al.* 2002) of *S. iniae* (#28). Mortality was monitored over a 10-day period and aetiology confirmed by bacterial isolation and characterisation. A LD₁₀₀ was used to ensure the all fish in the naïve group would succumb to infection and allow a maximal spread in RPS responses dependent on the serogrouping of the animal (0-100%). The RPS was calculated for each sero-group by dividing the mortality observed by the total number of fish challenged for each group (Amend 1981).

9.2.3 Bacterial preparation

Bacteria (#28) were prepared (as per Chapter 3.5) and stored as a concentrate at 1 x 10¹⁰ cells/ml with 0.1% formalin to maintain sterility.

9.2.4 Field trials of vaccine

Experimental field trials were held at two separate facilities, one a freshwater re-circulation facility and the other at a sea-cage farm. Prior to vaccine trials, 50 fish were bled and serum collected to assess native antibody levels to the streptococcal antigen. The vaccine trials consisted of approximately 50,000 fingerlings (20 g) in the vaccinated group and a similar number of

unvaccinated fish as controls at each farm. The formalin-killed vaccine (isolate #28) was administered via bath immersion (10^7 cells/ml) during the process of grading; while the control group was subject to the same handling but was not exposed to the vaccine. Basically, the fish were placed into 300 L oxygenated sorting bins, where they kept for a minimum of 5 minutes. Fish were then scooped out of the bin and sieve sorted, with the smallest fish falling through the mesh back into the bin, thus the largest fish were exposed for 5 minutes while the smallest were exposed for up to 10 minutes. Fish were given a second administration of the killed bacterin approximately 3 weeks later following the same protocol. Mortalities were recorded daily, and bacterial isolation was performed on samples of diseased fish undergoing the trial. Every month for four months, serum samples were obtained from a sample of vaccinated and unvaccinated fish from each facility, and stored at -20°C until required.

9.2.5 Detection of streptococcal antibodies in barramundi serum.

Levels of specific antibodies to *S. iniae* in barramundi serum were measured using an optimised indirect ELISA (as described in Chapter 5). Serum samples were tested in duplicate and compared to control serum on each plate from a hyper-immunised animal. Each result was then placed in a category (1-8) compared to the control sera, where 8 represents high titre serum and 1 represents no specific immune response (as described in Chapter 8).

9.3 Results

9.3.1 Field data




The monthly testing of barramundi serum from the various farms displayed a dramatic difference between the sea-cage facility and both of the freshwater farms (Figure 9.1). However, the ANOVA with post-hoc comparison indicated

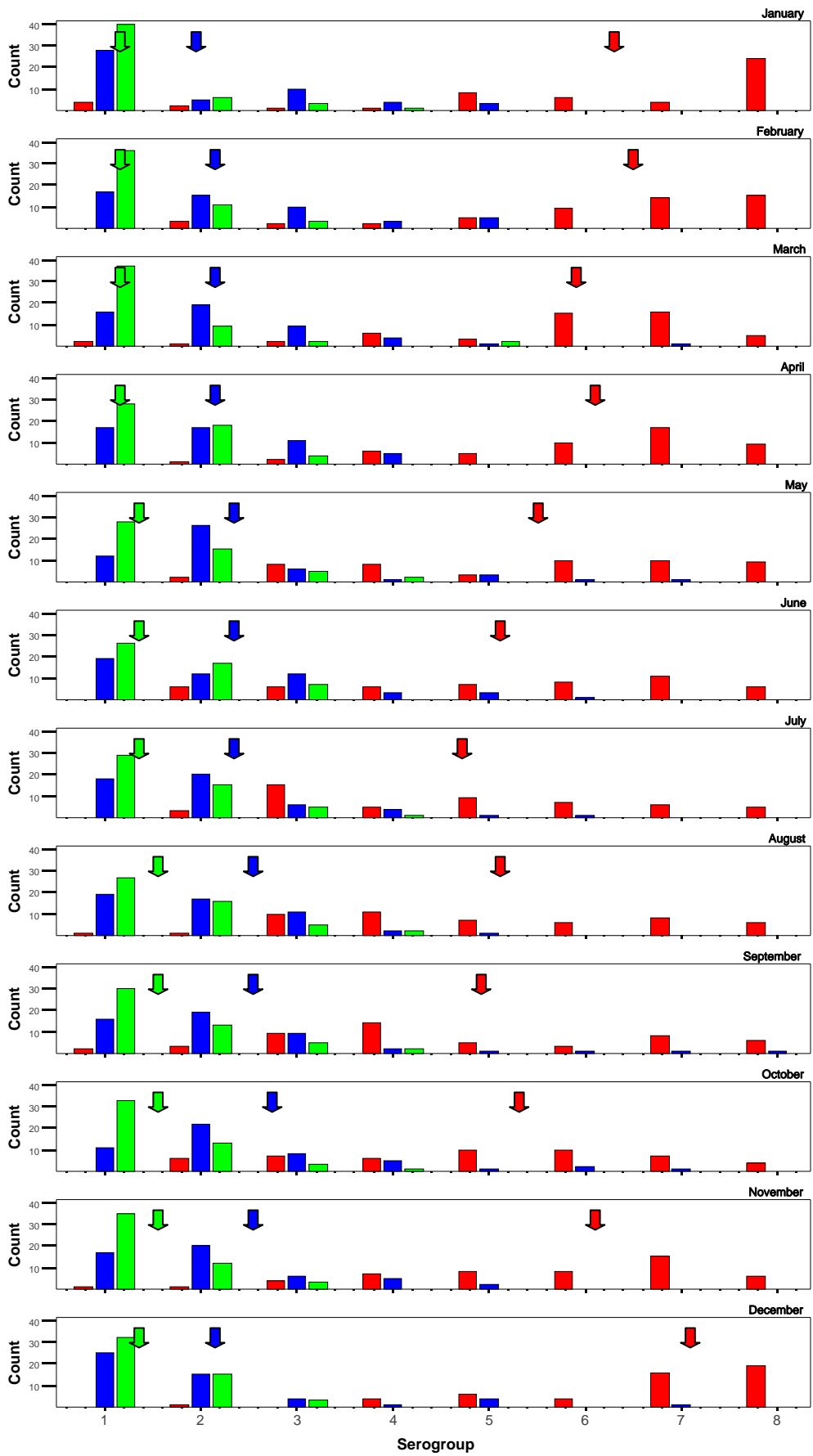
that all the facilities were significantly different from one another ($df=2$, $p<0.001$). There was no observable trend in the data obtained from either of the freshwater facilities. The fish from the freshwater facility possessed a constant low sero-group, with little change over the entire year. The barramundi held at the sea-cage facility displayed a seasonal fluctuation in the sero-group, with an increasing sero-group trend during the summer and a decrease throughout the winter.

When this data was compared to the mortalities observed at the sea-cage facility (Chapter 7), it was evident that upon the onset of the first outbreak of disease at the facility there was a corresponding increase in the sero-group of the fish that was maintained throughout the entire summer. The decrease in average sero-group observed in barramundi starting in May also corresponded with the cessation of mortalities at the farm. This is in contrast to the trend observed at the freshwater facility. The fish did not show any specific immune change over the monitoring year. During this period there were 2 outbreaks of *S. iniae* in the freshwater facility (data not shown), where losses accounted for 22% (April 12th – 19th 2000) and 47% (September 23rd - 30th 2000) of the fish. No mortalities due to *S. iniae* were recorded outside of these two events. No losses to *S. iniae* occurred at the facility regarded as *S. iniae*-free.

There was also a significant dispersal of the sero-groups observed in the barramundi at the sea-cage facility. The grouping was more restricted during the summer months, and greatly dispersed during the winter. The same trend was not observed at the freshwater facility.

Figure 9.1 The temporal sero-group profile of barramundi held at 3 different facilities. The arrows denote the average observed for each group at that particular time point

-  Seawater
-  Freshwater
-  Freshwater - control



9.3.2 Sero-group and protection

There was a relatively good distribution of sero-groups in the unvaccinated fish obtained from the sea-cage facility, encompassing groups 1 through 8; with the caveat that only one fish was determined to be in group 1. This indicated that these barramundi had been naturally exposed to *S. iniae* at the sea-cage facility. To enable further comparison and to serve as a control, an additional 10 fish were added to the experimental challenge from the freshwater control farm, and possessed no detectable serum titre. When barramundi were challenged with a lethal dose of *S. iniae*, the data indicated that the animals in the higher sero-groups were resistant to infection (Table 9.1), whereas animals in the lower sero-groups displayed a higher susceptibility to challenge. Naïve fish were highly susceptible to infection with the group displaying 100% mortality, whereas the single fish in group 1 survived.

Table 9.1 The effect of a lethal challenge (LD₁₀₀) with *S. iniae* to barramundi assigned to various sero-groups depending on their serum titre against *S. iniae* before challenge.

Sero-group	Number of fish challenged	Mortality	Relative Percent Survival
Naive	10	10	0%
1	1	0	100%
2	8	5	37.5%
3	14	10	28.6%
4	14	9	35.7%
5	17	6	64.7%
6	23	7	83.3%
7	18	0	100%
8	14	0	100%

9.3.3 Field Trials

A comparison of the mortality in vaccinated and unvaccinated fingerlings was 0.6% (300) and 7.2% (3600) respectively at the saltwater farm, and 4.4% (2200) and 9.2% (4600) at the freshwater facility over the monitoring period of 4 months (Table 9.2).

Table 9.2. The observed mortality of barramundi maintained at two separate facilities that underwent vaccination and boosting with *Streptococcus iniae*.

Culture conditions	Number of fish in treatment	Cumulative mortality (n)	RPS
Saltwater			
Vaccinated	50,000	300	92 %
Unvaccinated	50,000	3600	
Freshwater			
Vaccinated	50,000	2200	52%
Unvaccinated	50,000	4600	

The naive serum obtained from barramundi at both facilities possessed no detectable titre before vaccination. One month following the initial vaccination, vaccinated fish at the sea cage facility (Figure 9.2) displayed notable sero-conversion with the trend continuing throughout the entire monitoring period. The same was also true for the unvaccinated fish where there was an increasing trend in the sero-group throughout the monitoring period, but the conversion was not as high as in the vaccinated fish. The fish maintained at the freshwater facility (Figure 9.3) also displayed sero-conversion following vaccination and subsequent boosting. However the average sero-conversion group achieved was not as high as observed at the saltwater facility (2.9 vs 3.4). The unvaccinated fish ended the experiment with an average sero-group of 1.5.

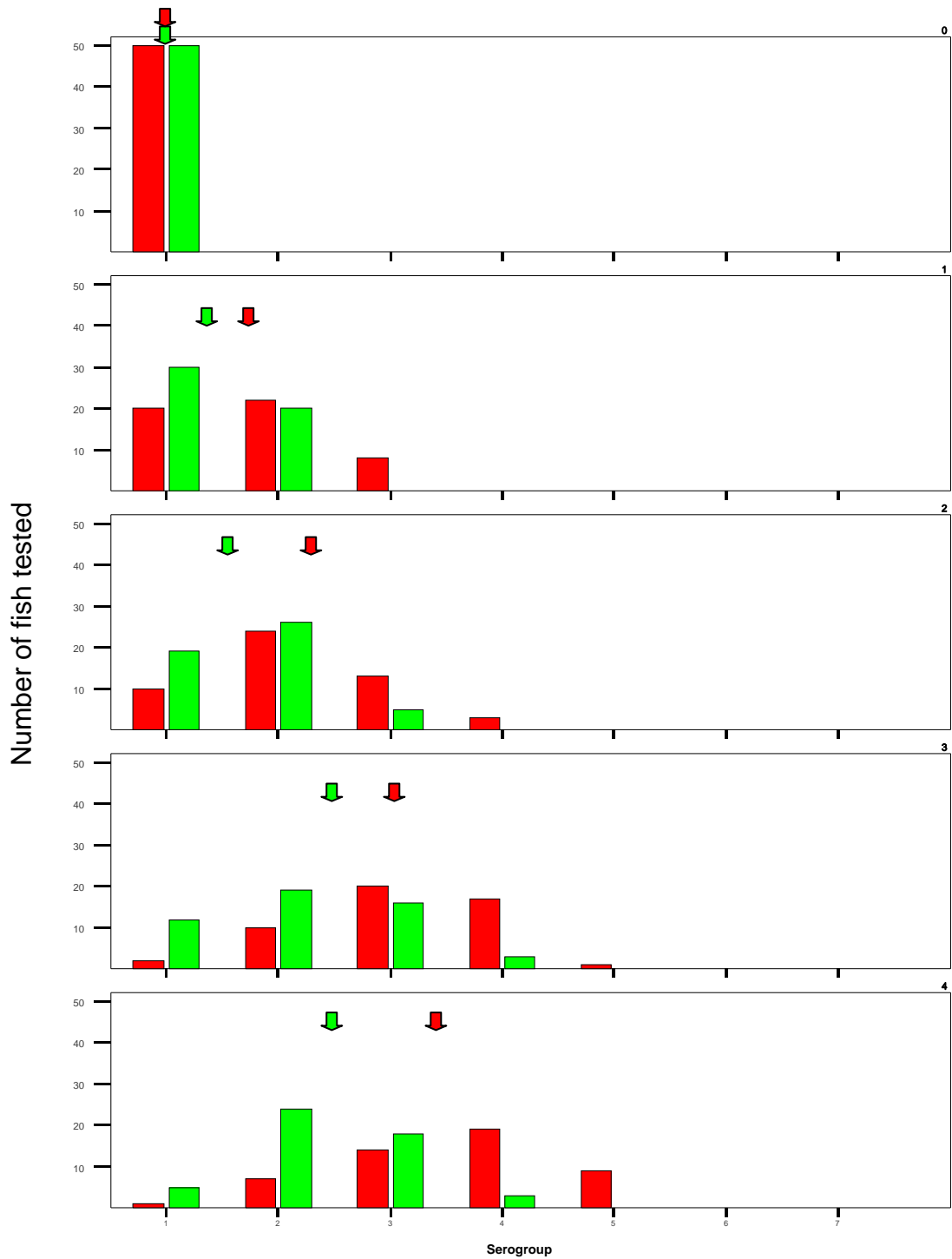
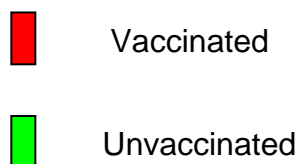


Figure 9.2 The monthly serum profile of sea-cage barramundi that have been vaccinated with *S. iniae* by bath immersion. The arrows denote the average sero-group observed at each time point. The months represented are from September (top) to January (bottom).



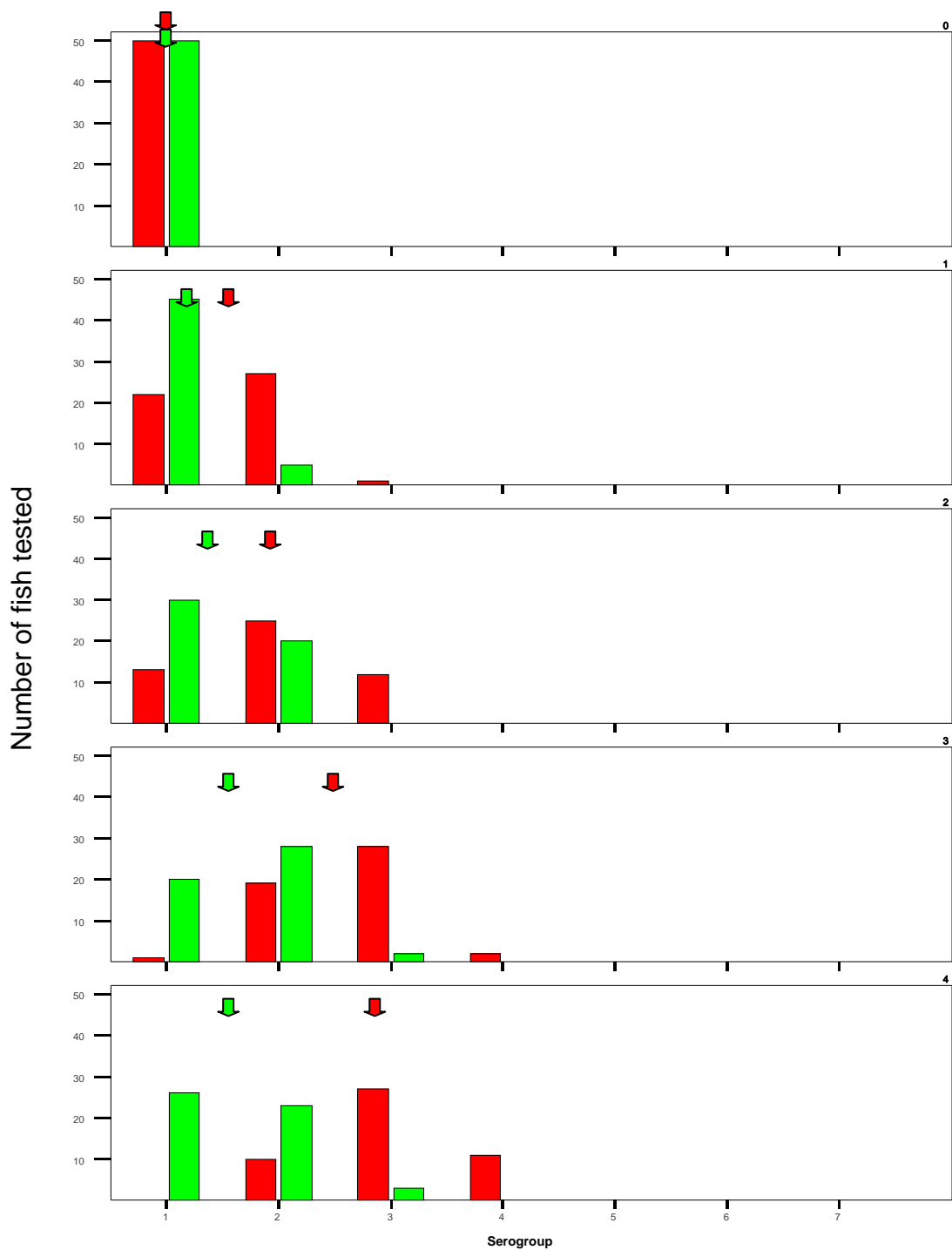
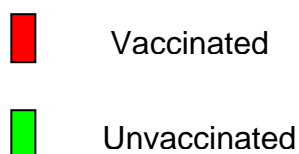


Figure 9.3 The monthly serum profile of barramundi maintained at a freshwater recirculation facility (constant 27°C water temperature) that have been vaccinated with *S. iniae* by bath immersion. The arrows denote the average sero-group observed at each time point. The months represented are from May (top) to September (bottom)



9.4 Discussion

In the present study I demonstrated dramatic serological differences in barramundi maintained at two different facilities that suffer from epizootics due to *S. iniae*. Barramundi maintained at the sea-cage facility demonstrated a high level of autologous serum antibodies against whole preparations of *S. iniae*, while fish held at the freshwater facility possessed lower overall levels of specific antibody. There was a strong correlation between specific antibody titre and protection against a lethal dose of *S. iniae* when challenged via a natural route of infection. Vaccination of fish facilitated sero-conversion at both sites, which resulted in improved protection over the unvaccinated controls.

The serological differences between the monitored farms were striking. The fish maintained at the sea-cage facility possessed a high level of specific humoral immunity to *S. iniae* throughout the period of the study. This is in contrast to both of the freshwater facilities where the fish were grouped mainly into the lower serogroups, especially at the *S. iniae*-free facility. The observed difference between the sea cage farm and the freshwater control farm was not surprising. It is intuitive to expect that animals continually exposed to an antigen will mount a specific humoral immune response. I have previously demonstrated (Bromage and Owens 2002) that barramundi at this facility are continually exposed to *S. iniae* through infected feral fish found co-inhabiting the cages. In contrast, the control freshwater facility is not exposed to the same level of feral aquatic animals as the marine facility; therefore the potential for exposure to *S. iniae* was restricted.

It is difficult to explain why the fish at the freshwater facility possessed such a low serum titre. It could be hypothesised, that these fish are not subject to continuous low-level challenge, but rather episodic high level challenges that does not allow these barramundi to develop humoral immunity. The use of antibiotics helps eliminates the bacterium from the surviving barramundi, and the husbandry techniques used at this facility (regular cleaning of tanks, flow

through system etc) do not allow reservoirs of the bacteria to persist. The point source for subsequent re-infections at this facility has yet to be determined.

The specific humoral immune response of fish undergoing natural exposure to pathogens has been documented a number of times previously. Smith and co-workers (1994) examined the humoral immune response of hybrid striped bass (female striped bass *Morone saxatilis* x male white bass *M. chrysops*) that had recently been exposed to a natural infection of *Amyloodinium ocellatum*. Following the parasitic infection, Smith and co-workers (1994) demonstrated that fish possessed specific antibody levels similar to that observed in vaccinated fish. A similar situation was observed in rainbow trout (*O. mykiss*) that had undergone exposure to infectious hematopoietic necrosis virus and survived (LaPatra *et al.* 1993; Ristow *et al.* 1993). In this case the authors found that the increase in specific antibodies provided protection when the fish were experimentally challenged.

There has been little evidence to date to suggest there is a relationship between serum antibody levels and protection against any streptococcal pathogen. It has been demonstrated that following IP vaccination with a whole cell vaccine (Klesius *et al.* 2000), there was a significant improvement in relative percent survival of Nile tilapia, *Oreochromis niloticus*, following a challenge with *S. iniae*. In a more extensive study, Eldar and co-workers (1997) demonstrated that following IP vaccination, *O. mykiss* mounted a specific humoral immune response, and that these fish were protected from infection upon lethal challenge. In this series of experiments, I classified barramundi according to their level of specific antibody to *S. iniae* that was obtained via natural exposure to the pathogen. Upon challenge it was evident that fish possessing a high level of specific antibody (serogroup 7 or 8) were protected from lethal challenge. Those fish possessing a low-level serum titre (group 2 and 3) had a lower overall survival than the higher serogroups, but their survival was improved compared to the naïve fish exposed to challenge. These data demonstrated for the first time that there is a relationship between serum antibody levels and protection against lethal challenge.

This finding was significant for the potential control of streptococcal infections in barramundi. The data indicated that vaccination, resulting in the generation of specific antibody will protect barramundi against natural challenge. Previously, it was demonstrated that specific serum antibody could be generated via IP inoculation and to a slightly lesser extent via immersion using a whole cell bacterin (Bromage 1997). It was important in the field trials to minimise the risk, cost and time to the farmers involved. Therefore, the delivery method used in the vaccine field trials was developed to incorporate into the current husbandry techniques in place at both farms. The use of sorting bins for the delivery of the vaccine during grading eliminated extra handling and therefore extra stress on the fish, as well as cost to the farmer. The only potential problem with this method was that the exposure to the vaccine would vary depending on the size of the fish.

The vaccination of the barramundi resulted in marked improvement in survival at both the facilities, in particular at the sea-cage facility where the mortality was reduced from 7.2% to 0.6% over the monitoring period. The improvement in survival was not as dramatic at the freshwater facility, but mortalities were halved in the vaccinated group. The different improvement in protection observed between the two facilities may be attributable to the bacterium used for the vaccine. The bacterium was isolated from the sea-cage facility and is thus likely to be serologically and phenotypically similar to the *S. iniae* causing mortality at this facility resulting in the higher protection observed.

In future studies it will be important to determine the effect of the bacterial strain on the protection it affords to vaccinated fish. Indeed (Barnes *et al.* 2003c) highlighted the importance of phenotypic characteristics on the ability of immune serum to recognise various *S. iniae* strains. However the results from this study demonstrate that the development of an effective vaccine is viable that will provide protection against infection with *S. iniae*.

CHAPTER 10

PRODUCTION AND USE OF MONOCLONAL ANTIBODIES GENERATED AGAINST *STREPTOCOCCUS INIAE*

10.1 Introduction

The Lancefield grouping system for streptococci is based on the antigenicity of the cell wall polysaccharide present on the cell surface (Lancefield 1932). Monoclonal and polyclonal antibodies have been developed to recognise these antigens and are used in the serotyping of *Streptococcus* spp. *Streptococcus iniae* is regarded as a Lancefield ungroupable bacteria that is associated with severe mortality in numerous species of fish (for review see Chapter 2), and has been associated with morbidity in human patients (Lau *et al.* 2003). The fact that current grouping reagents do not recognise *S. iniae* suggests that it possesses a unique cell wall carbohydrate profile, and new serological tools to aid in rapid classification of *S. iniae* would be useful.

There have been no previously published attempts to generate monoclonal antibodies against *S. iniae*, and relatively few reports of antisera raised against fish pathogenic streptococci (Kitao 1982). Barnes and co-workers (Barnes and Ellis 2003; Barnes *et al.* 2003a; Barnes *et al.* 2003b) have conducted considerable research of the cell surface properties of many isolates of *S. iniae*. Their research reported the emergence of a new strain that is arginine dihydrolase negative (ADH –ve), that has modified its cell surface and is no longer recognizable by immune trout serum generated against the type I strain. However, there is however some cross-reactivity with antisera generated against type II *S. iniae* with the type I isolate.

In this chapter I am attempting to generate polyclonal and monoclonal antisera that may aid in the rapid identification of *S. iniae* as well as identify newly emergent serotypes. The production of such tools may also allow *in situ* disease progression studies.

10.2 Methods

10.2.1 Antibody production

10.2.1.1 Preparation of *S. iniae* for vaccination

A heat killed bacterial culture (#28, Chapter 3.5) was prepared as a water-in-oil emulsion with FCA (Sigma, St. Louis MO) for the primary vaccination. A booster vaccination was prepared in FIA (Sigma, St. Louis MO).

10.2.1.2 Immunisation of chickens

One half ml of the *S. iniae* emulsion, containing approximately 10^8 cells, was injected into the breast muscle of six, 12-week-old chickens. Three weeks following the initial vaccination, the chickens were boosted with the FIA with complex (10^7 cells). Due to the poor immunogenicity of the antigen, booster inoculations were repeated monthly for five months. At the time of the boost, 1 ml of blood was collected from the wing vein of each animal in order to monitor serum titres. When the titre reached an acceptable level, 25 ml of blood was collected from the jugular vein of each animal. The blood was processed (as per Chapter 3.2) and stored at -20°C until required.

10.2.1.3 Immunisation of Balb/c mice

Ten Balb/c mice were injected with 250 μl of the FCA bacterial emulsion (4.5×10^8 cells). Due to the poor immunogenicity of the antigen, booster inoculations of 10^6 bacterial cells in FIA were administered at week 4, 10, 15,

and 26. Serum titres were monitored regularly from serum obtained via tail bleed, and specific response monitored via Western blotting.

10.2.1.4 Monitoring of specific antibody via ELISA

Nunc ELISA plates were coated with killed *S. iniae* (as described in Chapter 5). The serum to be tested (mouse or chicken) was added to the plate in duplicate and sequentially diluted to determine titre. Preceding a 60-minute incubation and subsequent washing step, the appropriate secondary antibody (goat-anti-mouse HRPO, BioRad, Australia or rabbit anti chicken HRPO, Jackson Immunochemicals, USA) was added to the plate (1:5000) and incubated at room temperature for 1 h. Following the final washing step, 100ul of ABTS substrate (KPL, Maryland) was added to each well and the reaction allowed to proceed for 1 h before being read spectrophotometrically at 405nm.

10.2.2 Monoclonal antibody production

As per Chapter 3.4

10.2.3 Monoclonal antibody characterisation

10.2.3.1 Isotyping

Isotyping of the monoclones was performed using the Sigma isotyping kit (Sigma-Aldrich, St. Louis USA) following the manufacturers instructions.

10.2.3.2 Recognition of bacterial proteins via Western blotting

Five different isolates (#28, #16, #44, #46, and the ATCC type strain of *S. iniae*; Appendix 2) of live *S. iniae* cells (0.5 g wet weight) were placed into a glass tube with 100 mg of glass beads (2 mm diameter, Sigma, St Louis, MO). The contents of the tube were disrupted with a handheld sonicator (Vibracell, Danbury USA) set at 200 Watts for 30 s. The tube was immediately placed on ice to cool the bacterial products. This process was repeated a further 3 times. Following treatment, the tubes were spun at low speed (500 g) for 5 minutes to remove the glass bead and bacterial debris. Protein concentration of each sample was determined by BCA (Pierce Biochemicals, Rockford, IL). Ten micrograms of each solution was added to a lane of 10% SDS-PAGE gel (Chapter 3.2), and electrophoresed under reducing conditions. The contents of the gel were transferred to PVDF (Chapter 3.5), the membrane allowed to dry, and stored at -20°C until required. An identical second gel was prepared and stained via Commassie blue (BioRad, Australia) to visualise the bacterial proteins.

The PVDF possessing the bacterial proteins was added to a Petri dish (Pyrex, USA) and the following steps performed at room temperature. Cell culture supernatant from each monoclonal was tested against separate membranes, diluted in PBS (1:50), and added to the dish and allowed to incubate for 1 h. The membrane was washed 3 times with PBS, before the secondary antibody (goat-anti-mouse HRPO, BioRad Australia) was added to the plate (1:10,000) and incubated for 1 h. The membrane was washed extensively with PBS before the addition of DAB rapid stain (Sigma, St Louis, MO). When the desired development was achieved, the reaction was stopped by washing the membrane with ddH₂O.

10.2.3.3 Cross reactivity with other strains of *S. iniae*

The panel of monoclonal antibodies generated were tested against 14 isolates of *S. iniae* previously isolated from healthy and diseased barramundi, local waterways (Bromage 1997), as well as one isolate from Israel (#46) and the ATCC type strain (Appendix 2). Each bacterial isolate was coated to ELISA plates following the method described above, and the titre determined for each antisera.

10.2.3.4 Agglutination reaction with other bacterial species

The panel of monoclonal antibodies produced were tested via the standard agglutination reaction (Lanyi and Bergan 1978) for their cross reactivity with other bacterial species (Table 10.2). Bacteria were obtained from stocks maintained in the Discipline of Microbiology and Immunology culture collection. In short, bacterial agglutination was performed using heat-killed bacteria that had been centrifuged and re-suspended in PBS at a concentration of 5×10^8 cells / ml. Cell preparations (100 μ l) were incubated against 2-fold serial dilutions of cell culture supernatant (100 μ l) obtained from monoclonal antibodies generated against *S. iniae*. Each reaction was performed for 1 h at 37⁰C in V-bottomed 96 well plates (Nunc, Australia). The agglutination of cells was characterised by a coarse granular bacterial clumping and each is scored on a scale of – ve (no agglutination), +1 (weak agglutination <1:2 dilution) to +4 (strong reaction >1:1024 dilution).

10.2.4 Immunohistochemistry

10.2.4.1 Slide preparation

The intestine, kidney, spleen, liver, heart and brain of experimentally infected barramundi (obtained from a previous study, Bromage and Owens 2002) were

fixed in 10% neutral buffered formalin, embedded in paraffin and processed (Bucke 1989). Sections were cut to a thickness of 5 µm on a microtome (American Optical 820), applied to silanised microscope slides (Sigma, St Louis, MO), and the paraffin removed via heat (67°C for 1 h).

10.2.4.2 *In-situ* detection of *S. iniae* in experimentally challenged barramundi

Slides were immersed for 5 minutes in a solution of 0.1% hydrogen peroxide to block endogenous peroxidase. Following this treatment, the slides were immersed in the cell culture supernatant (4F4) and incubated at room temperature for 2 h. The slides were washed 3 times in PBS, before goat-anti-mouse HRPO, diluted 1:3000, was applied to each slide and incubated for 1h. The slides were washed again, and the substrate 3-amino-ethylcarbazol (AEC, Sigma, St Louis, MO) was added to each slide and allowed to react for 10 minutes before the reaction was stopped with PBS. Slides were counterstained with 0.001% fast green (Sigma, St Louis, MO) before being mounted using aqueous mounting medium.

10.3 Results

10.3.1 Antibody production

The immunisation of chickens resulted in the production of highly variable antibody titres. Only 2 out of the 5 birds vaccinated produced antiserum with a titre in excess of 2000, and there was little difference in the serum titre of the remaining birds and control sera collected from birds in the same flock. There was a high degree of non-specific binding in all birds (control and vaccinated animals) and this resulting antiserum was determined to be unsuitable for further evaluation.

Repeated vaccinations in mice also failed to produce a high serum titre to whole bacterial cells. Specifically the animals failed to produce a detectable IgG response to the antigen. However there was no detectable serum titre in control animals, and therefore a fusion was attempted. The result was the production of 4 hybridomas, all of the IgM isotype (Table 10.1).

Table 10.1 The characteristics of the hybridomas produced against a whole cell preparation of *S. iniae*.

Antibody ID	Titre for 50% OD Max	Isotype	Western Positive
4F4	2000	IgM	Yes
7F6	1000	IgM	Yes
7F11	1000	IgM	Yes
66	500	IgM	Yes

10.3.2 Monoclonal antibody characterisation

10.3.2.1 Protein profile and Western blotting

Western blotting of the monoclonal antibody (4F4) against the sonicated bacterial proteins indicated that the antiserum was able to bind to the same 21 kDa protein from five isolates of *S. iniae* tested (Figure 10.1). The other 3 monoclones all recognised the same bacterial protein.

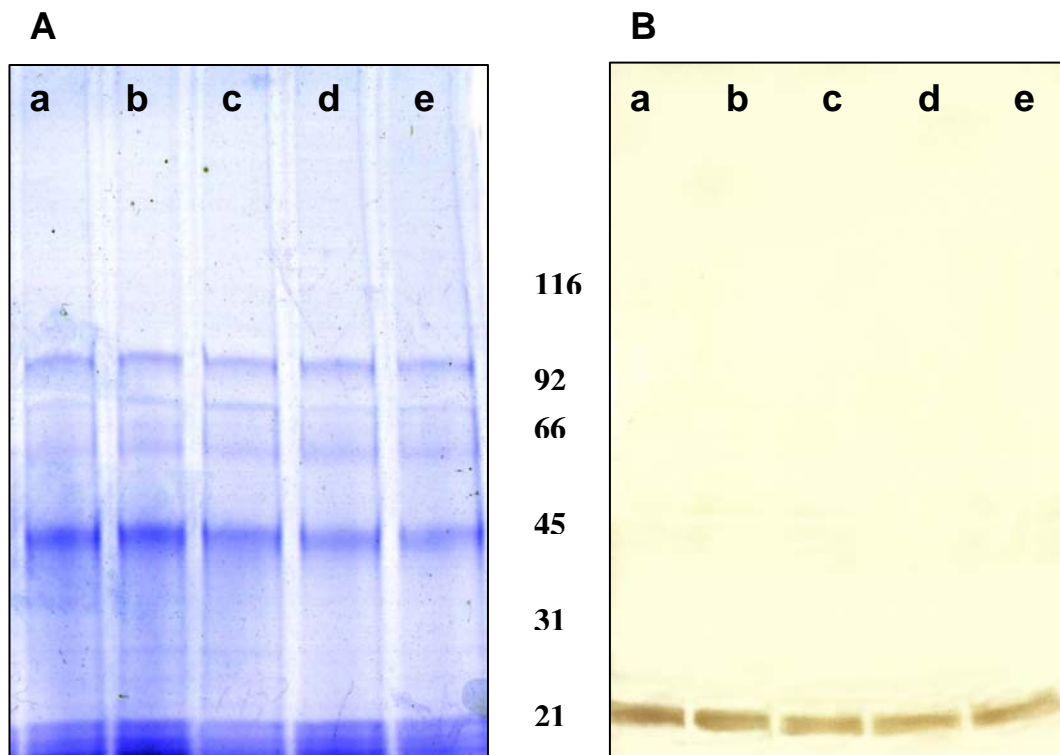


Figure 10.1 The SDS-PAGE protein profile of 5 isolates of sonicated *Streptococcus iniae* ((a) #28 (b) #16 (c) #44 (d) #46 (e) ATCC type strain), and the Western blotting profile against these isolates using the monoclonal antibody (4F4) generated against *S. iniae* isolate #28.

10.3.2.2 Intra species-specific recognition of monoclonal 4F4

When the monoclonal 4F4 was tested via ELISA against the other isolates of *S. iniae* (Figure 10.2), it displayed strong reaction against most of the isolates. Notably, it displayed a strong reaction with the original ATCC type strain, as well as all of the isolates obtained from barramundi. There was slightly poorer recognition of the *S. iniae* strain isolated from a water sample (#44), and the single isolate from the freshwater recirculation facility (#47). The monoclonal also reacted to the isolates from Israel (#46).

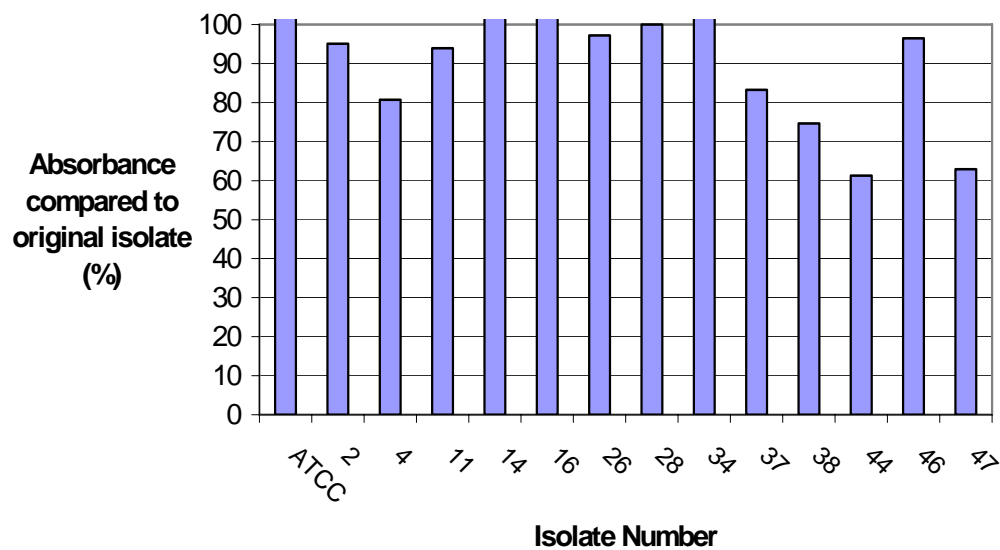


Figure 10.2 The ability of the monoclonal antisera (4F4) to recognise various isolates of *S. iniae* via ELISA. Each isolate is compared to the reaction achieved when using isolate #28 from which the monoclonal was generated.

10.3.2.3 Agglutination reaction of the monoclonal antibodies against various bacterial isolates.

The agglutination reactions indicated that all the monoclones had minimal to moderate cross-reactivity with *Streptococcus equi*, and two of the monoclones had moderate reactions with *Streptococcus canis*, and *Streptococcus agalactiae*. There was no agglutination observed with any of the other bacterial species tested (Table 10.2).

Table 10.2 The observed agglutination reactions against various species of bacteria using the monoclonal antisera generated against *Streptococcus iniae* (#28). (-) no reaction (+) low (++) medium (++++) high

Agglutination Reaction				
	4F4	7F6	7F11	66
<i>Streptococcus agalactiae</i>	-	+	-	++
<i>Streptococcus pyogenes</i>	-	-	-	-
<i>Streptococcus faecalis</i>	-	-	-	-
<i>Enterococcus faecium</i>	-	-	-	-
<i>Staphylococcus epidermis</i>	-	-	-	-
<i>Streptococcus canis</i>	-	++	++	-
<i>Streptococcus equi</i>	++	+	+++	+
<i>Staphylococcus aureus</i>	-	-	-	-
<i>Enterococcus faecalis</i>	-	-	-	-
<i>Vibrio harveyi</i>	-	-	-	-
<i>Lactococcus garvieae</i>	-	-	-	-
<i>Flavobacterium</i> sp.	-	-	-	-
<i>Klebsiella</i> sp.	-	-	-	-
<i>Campylobacter</i> sp.	-	-	-	-

10.3.3 Immunohistochemistry

The monoclonal antisera displayed specific cell surface staining of bacteria located in the various organs. There was no staining observable in any of the control fish. High levels of *S. iniae* were observed throughout the brain of experimentally infected fish (Figure 10.3). At higher magnification the bacteria could be seen located in the circulation (Figure 10.4), however, no stained bacterial cells were observed intracellularly. Very high levels of bacteria were also observed in the intestine of infected fish, in particular the lamina propria (Figure 10.5)

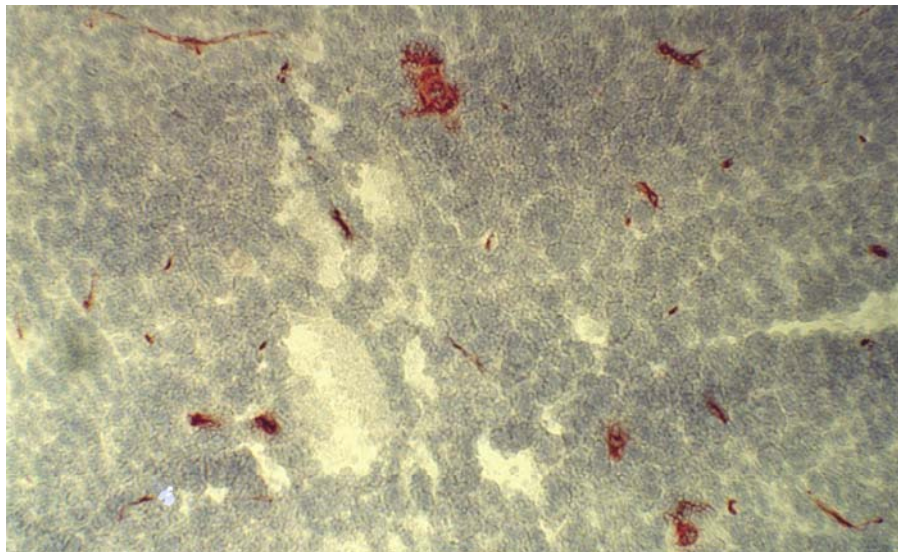


Figure 10.3 The specific staining (AEC) of bacteria in the brain of an experimentally challenged barramundi using monoclonal antiserum (4F4) against *S. iniae*. The slide was counterstained with fast green (300x).



Figure 10.4 The specific staining (AEC) of bacteria in the circulation of the brain of an experimentally challenged barramundi using monoclonal antiserum (4F4) against *S. iniae*. The slide was counterstained with fast green (800x).

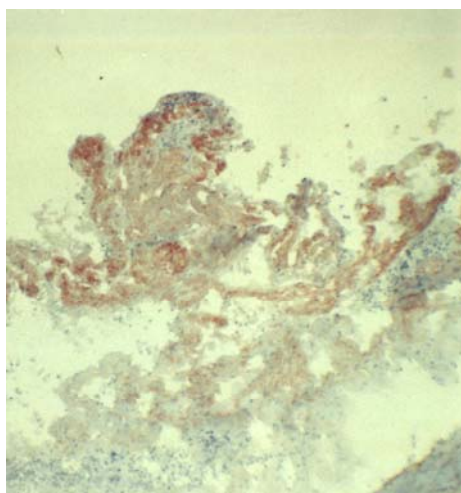


Figure 10.5 The specific staining (AEC) of bacteria in the intestine of an experimentally challenged barramundi using monoclonal antiserum (4F4) against *S. iniae*. The slide was counterstained with fast green (400x).

10.4 Discussion

Infections due to *Streptococcus iniae* have caused widespread losses throughout the aquaculture industry; however, technology for its identification has remained limited to standard techniques, which can be time consuming and laborious. Here I describe the production of monoclonal antibodies that readily recognize *S. iniae* and have minimal cross-reactivity with other bacterial species. One particular hybridoma 4F4 displayed particular promise for its use in ELISA-based assays and immuno-histochemistry.

Despite a number of bacterial challenge protocols attempted (varying dose and boosting frequency, data not shown), vaccination of chickens and mice with *S. iniae* resulted in low and variable production of specific antibody. While low level challenges of mice with live bacteria resulted in death of challenged animals (data not shown). Only high doses and repeated inoculations of killed bacterial resulted in a minimal antibody response against the bacterial isolate in both chickens and mice. It is well recognized that many streptococci have very low immunogenicity which is usually associated with cell wall structures that allow streptococci to evade the immune system of the host (Hayman *et al.* 2002, Haanes *et al.* 1992). There is recent evidence to suggest that *S. iniae* possesses a cell capsule that may also help it evade the immune system of infected fish (Barnes *et al.* 2003), but it is unclear whether the bacteria would still retain this ability once it had been killed.

Despite multiple boosting, it was disappointing that the immunisation of mice with *S. iniae* did not result in a high antibody response or isotype switching in any individuals. It is hypothesised that the low immunogenicity was due to the inability of mice and chickens to efficiently process the bacterium and present it to B and T-cells for an effective immune response to develop (Kotwal 1997; Staats *et al.* 1997; Anzai *et al.* 1999; Allen 2003). However this hypothesis would require considerable further research to establish if this was the case for *S. iniae*.

There have been very few successful attempts at making monoclonal antibodies using whole streptococci cells as the immunogen. Typically, the epitope of interest (protein, polysachharide etc) are purified or artificially synthesised and injected into mice (Ota *et al.* 1990; Moyo *et al.* 1999). This method overcomes the ability of the bacteria to evade the immune system, resulting in the production of antisera against the target antigen. Due to time and monetary limitations, this procedure was not attempted, although it is reasonable to assume that if the 28 kDa protein was purified, it may serve as an excellent immunogen for the creation of IgG monoclonal antibodies.

Despite the poor immunogenicity of *S. iniae* in mice, four monoclonal antibodies were able to be generated and were utilised in this study. One particular monoclonal antibody, 4F4, readily recognised all the *S. iniae* isolates from Australia, as well as the ones from Israel and America (ATCC type strain). It also displayed limited cross reactivity with other bacterial species and thus was the monoclonal antibody of choice for further research. The ability of this hybridoma to efficiently recognise all the isolates of *S. iniae* is somewhat surprising. A number of reports have indicated that there is minimal cross-reactivity of polyclonal anti-serum between the between various strains of *S. iniae* (Bachrach *et al.* 2001; Zlotkin *et al.* 2003). There has been recent evidence to suggest that *S. iniae* vary in presence or absence of a cell capsule, as well as variability in ADH activity (Eldar *et al.* 1999; Fuller *et al.* 2001; Barnes *et al.* 2003b). All Australian isolates used in this study were ADH +ve (Bromage *et al.* 1999), but it is not known whether they are encapsulated. The isolates from Israel were a mixture of encapsulated and un-encapsulated as well as ADH +ve and -ve isolates. The monoclonal antibody 4F4 did show some minor variability in the titre between isolates, but no pattern was observed between the various strains of the bacterium (data not shown).

The agglutination reactions using the monoclonal antibodies indicate that *S. iniae* may share a similar cell-surface epitope with *S. equi*. This is not an unexpected finding. I have previously reported that biochemically, *S. iniae* is very similar to *S. equi* subspecies *equisimilis* (Bromage 1997). *Streptococcus equi* also has also been shown to possess a cell capsule (Anzai *et al.* 1999).

which may share similarity to that observed on *S. iniae*. These findings may also account for the elevated background titre observed in unvaccinated chickens. Their exposure to environmental bacteria, particularly bacteria associated with farm animals (horses, pigs, sheep and cattle) that are maintained in close proximity to the chicken enclosure, may facilitate exposure, resulting in the high background.

The antiserum from 4F4 was also extremely useful for specific staining of *S. iniae* in formalin-fixed sections of experimentally challenged barramundi. The results highlighted the previous findings (Bromage and Owens 2002) of the neurotropic nature of *S. iniae*, with its presence in the meninges, subarachnoid spaces and the microcirculation of the brain. However the immunohistochemistry results also indicate that *S. iniae* was dispersed throughout the intestine in bath challenged fish. Using standard histological techniques, I previously reported *S. iniae* in the intestine of orally challenged fish, but not in the other experimental groups (Bromage and Owens 2002). However, this monoclonal antibody demonstrated that *S. iniae* is also located in the intestine of bath challenged fish.

The development of this tool provides rapid means for the identification of *S. iniae in-vitro* or *in-situ*, and is the first monoclonal antibody created for fish pathogenic streptococci. These antisera will also provide an excellent means for following the progression of the bacterium through the fish's system following infection. It may also be possible to utilise the Mab's produced to detect the level of *S. iniae* in waterways. However, the results would have to be verified by other methods to ensure that they were not biased by cross-reactions with other bacteria.

CHAPTER 11

GENERAL DISCUSSION

The establishment of *Streptococcus iniae* as a threat to Australian aquaculture has been imminent for a number of years. The bacterium was first isolated in Queensland in 1992, in a remote sea-cage facility. The initial mortality was chronic, but over the following years became more severe. The origin of the bacterium remains in question. This facility had been in operation for 10 years prior to the first outbreak, so it is likely that the bacterium was introduced from an exotic source. It is unfortunate that more was not known about *S. iniae* in the early 90's, as this period represented the best opportunity to control the spread of the bacterium. Adequate monitoring and quarantine procedures were not employed, primarily due to the lack of knowledge, and *S. iniae* soon became established in other facilities throughout the state.

The late 1990's provided another opportunity to control of the spread of *S. iniae* throughout other states, where production of barramundi in intensive recirculation facilities was flourishing. All barramundi fry production was conducted in northern Queensland, and it was evident that the bacterium was being transported with fingerlings to other facilities. There had been extensive publication on the impact and treatment of the disease in other countries (Eldar *et al.* 1997; Hurvitz *et al.* 1997; Ghittino *et al.* 1998), and the potential impact on barramundi culture had been highlighted (Bromage 1997; Bromage *et al.* 1999). However, the problem was largely ignored as it was thought to be only of regional significance.

More recently, data was presented (Bromage 2000) indicating the spread of *S. iniae* to 21 farms throughout Australia (13 Queensland, 4 South Australia, 2 New South Wales, 1 Victoria, and 1 hatchery in the Northern Territory). In spite of the data, the impact of *S. iniae* to the barramundi aquaculture industry was questioned by an officer from the Queensland Department of

Primary Industries, Northern Division (Appendix 5). It could be said that if *S. iniae* infections were taken more seriously by the people responsible for the control of agricultural diseases, the current situation may have been avoided. However, the disease is now present in every state of Australia, except Tasmania, and the recent loss of \$2 million worth of barramundi at one single farm, representing 25% of the total value of the industry in 2002, must now highlight the importance of *S. iniae* to Australian aquaculture. Further, *S. iniae* stands out as the only pathogen that will cause severe morbidity and mortality in a wide range of species, including many species of fish, mice, frogs and leeches, while also possessing the ability to infect humans.

The control of *S. iniae* now lies with efficient management, antibiotic usage and the administration of vaccines. The desire to minimise antibiotic usage in the industry led to the framework of this thesis, which was to garner a greater understanding of what initiates *S. iniae* outbreaks, as well as the potential for control through the use of a vaccine. In order to perform the second goal, an equally important aim of characterising the barramundi immune system needed to be achieved.

The management of *S. iniae* outbreaks through good husbandry may provide producers with an opportunity to limit mortality. The data presented in this thesis indicate that environmental temperature, and to a lesser extent pH, are crucial to the establishment of lethal infection. While it is impossible to manage temperature in outdoor facilities, the ability to regulate temperature in indoor recirculation facilities is readily achievable. There is also considerable information that the spread of *S. iniae* in susceptible species is enhanced at high stocking densities (Shoemaker *et al.* 2000), and poor water conditions (Hurvitz *et al.* 1997). Both of these are factors that can be easily managed in all culture facilities.

Recently published data also highlighted that efficient management of an outbreak could also limit mortality. It was discovered that barramundi are highly susceptible to infection when they ingest the bacteria (Bromage and

Owens 2002), and epizootics are accentuated when the fish are exposed through the water. Early recognition of infected fish and their removal would aid significantly in reducing the spread of the disease, while increased water flow will decrease the bacterial load of *S. iniae* present in the surrounding environment.

As discussed above, early recognition of *S. iniae* infection is vital if an outbreak is to be managed. Current techniques for its identification can be both laborious and lengthy. The development of monoclonal antibodies that recognised a dominant cell surface protein may aid in improving diagnosis and limit delays, and may substantially reduce the cost of initial screening. The Mab's were shown to work effectively detecting the bacteria in formalin-fixed sections and displayed limited cross reactivity with other bacterial species. It is unfortunate that the monoclones were not tested in a sandwich ELISA format that may have allowed the detection of *S. iniae* in water samples or body fluids. Similar monoclones have been developed for specific for cell surface proteins of bacterial kidney disease and have been used in a rapid detection tool for its diagnosis (Rockey *et al.* 1991).

The second aim of this thesis was build on the current knowledge of the barramundi's immune system (Chao and Chong 1986; Bryant *et al.* 1999; Crosbie and Nowak 2002), and to compare it to the available knowledge on other teleosts. It is known that fish possess the same basic elements that underpin the immune system of higher vertebrates. However, there are two striking differences from the mammalian arrangement; they do not possess bone marrow and only have one immunoglobulin isotype. These unique differences in the immune system of teleosts, have led many researchers to the conclusion that it is the primitive cousin of the mammalian immune system. This misconception has recently started to change, primarily due to intensive research with catfish (*I. punctatus*) and rainbow trout (*O. mykiss*). The research has shown that these fish possess a powerful and complex immune system that was previously unprecedented.

One of the most intriguing discoveries has been in the assembly of teleost immunoglobulin. Early research indicated that catfish (*I. punctatus*) serum immunoglobulin was not always completely polymerised, and under denaturing conditions various structural isotypes of Ig were observed. These isotypes, or redox forms, were readily observed in Ig isolated from barramundi serum, and appear to be a characteristic of all teleost species (Bromage *et al.* 2004). It is hypothesized that the structural diversity displayed in teleost Ig may provide rigidity or flexibility in form that may allow for differential binding and possibly effector function. Additionally, the development of effective vaccines may rely on the preferential production of one of these isotypes, for the production of long term and protective immunity.

The process of the primary antibody response is probably the most well researched system in fish immunology. Key to its study is the development of serological reagents that are used to monitor the levels of total or specific antibody present in the circulation. A number of research groups have developed polyclonal antiserum against barramundi Ig (Chao and Chong 1986; Bryant *et al.* 1999; Crosbie and Nowak 2002), and have been used to monitor the barramundi immune response following challenge. The production of monoclonal antibodies against barramundi immunoglobulin was a logical progression from the previous research, and was achieved in this study. The Mab's were effectively employed in various assays to monitor barramundi humoral immunity following exposure to *S. iniae*, as well as quantifying total serum antibody levels. Their use in characterising the development of humoral immunity in barramundi fry highlighted the physiological differences between barramundi and many other teleost species. Barramundi are prolific reproducers, with one female capable of producing 5 million eggs in a spawning season. She invests very little energy into each egg, relying on the rapid development of fry. The disappearance of maternally derived antibodies coinciding with the absorption of the yolk eliminated the possibility of maternal vaccination. Maternal vaccination was considered a possibility for the protection of fry against hatchery diseases, notably VNN. But as our results indicated, a complete loss of maternal

antibody occurred with 3 days of spawning. This would render the fry susceptible to infection, thus be an ineffective method of control of this disease.

The rapid development of humoral immunity in barramundi does provide a great opportunity to producers. The research indicated that fry are able to respond to vaccination as early as 6 weeks post-hatch. At this stage, the fry would still be in the confines of the hatchery, relatively small, and are easily and regularly handled. The controlled exposure to potential pathogens, through the use of vaccines, would provide an effective and efficient mechanism for future loss minimisation. This could be supplemented with booster vaccinations at the growout facility to achieve long lasting protection.

The temperature dependence of the humoral immune response was highlighted in adult barramundi. Specifically, low temperatures retarded the development of specific antibody development. This finding was not unexpected, but again highlighted the physiological differences of barramundi from the most well researched species. Specifically, the lower limit of a B-cell response in barramundi was determined to be at or near 18⁰C, some 14⁰C above the limit of rainbow trout, and 17⁰C above Atlantic salmon. An interesting observation was the heterogeneity in the immune response at the lower temperature threshold. This was visualised via sero-grouping, a novel method for examining serological parameters of large populations of animals. This technique highlighted the complete absence of a specific immune response in some barramundi, as well as the depressed and delayed sero-conversion in others.

A key finding in this thesis was the link between specific serum antibody titre and protection against lethal infection with *S. iniae*. This was demonstrated when a greater portion of barramundi possessing high antibody titre survived lethal challenge, versus the high mortality observed in fish that had a low titre or were naïve to *S. iniae* exposure. The link between antibody and protection against *S. iniae* has been previously alluded too (Eldar *et al.* 1997; Bromage

et al. 1999; Klesius *et al.* 2000), but no link had yet been definitively established.

Further support for serum antibody providing protection was observed from the 'native' *S. iniae* antibody levels observed at the various farms.

Barramundi held at the sea-cage facility, previously shown to be exposed to *S. iniae* from the environment (Bromage *et al.* 1999; Bromage and Owens 2002), possessed a higher serum antibody titre than those held a facility that lacked constant exposure to *S. iniae*. While chronic losses to *S. iniae* were observed at the first facility, it was hypothesised that large scale epizootics were not seen in this population due to the high levels of specific antibody protecting most animals from infection. This was not the situation at the freshwater facility. Barramundi at this farm possessed very low serum anti-*S. iniae* antibody titres. Thus, when barramundi were exposed to *S. iniae*, mortality was rapid, and losses high.

These data unequivocally determined that exposure to *S. iniae* via a method that induces specific serum antibody production will provide protection to that individual. This is the foundation for vaccine development; exposure to a non-lethal form of antigen will provide protection against a lethal challenge in later life. Utilising this premise, various isolates of *S. iniae* were screened for their ability to generate serum antibody (Bromage 1997) and provided protection in a laboratory challenge. Following the initial success of these trials, the vaccine was tested in a large multi-farm field experiment. The results indicated that vaccination significantly improved serum titre, which resulted in a decrease in mortality in the vaccinated fish.

This finding resulted in the application of the vaccine to all barramundi held at the facilities involved in the field trial. The results, which can be seen in Figure 7.2, demonstrate that the vaccination of fish held at the sea-cage facility resulted in a dramatic decrease in mortality over a 2 year period (1998, 1999). Similar results were achieved at the freshwater facility; however, the operators of this farm requested that the mortality data for this facility not be published.

This research has shown that barramundi possess a humoral immune system that is both complex and able to respond to vaccination. It is anticipated that these studies will serve as an impetus for further research to more fully characterise the functions of the barramundi immune system. If future research can lead to methods for complete protection of barramundi in aquaculture from *S. iniae*, then perhaps such devastating losses of stock such as the most recent and costly loss in can be avoided in the future.

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APPENDIX 1

BUFFERS AND SOLUTIONS

1.1 Transfer Buffer

Tris Base	3.03 g
Glycine	14.41 g
Methanol	200 ml

Make up to 1 L, do not pH, Store at 4⁰C

1.2 Phosphate Buffered Saline (10 X)

Ingredient	Quantity g / L
NaCl	400
KH ₂ PO ₄	10
Na ₂ HPO ₄	57.5

Make up to 1 with distilled water and adjust to pH 7.2

1.3 SDS-PAGE Loading Buffer

ddH ₂ O	4.0 ml
0.5 M Tris-HCl (pH 6.8)	1.0 ml
Glycerol	0.8 ml
10% (w/v) sodium-dodecyl-sulphate	1.6 ml
2-beta-mercaptoethanol	0.4 ml
0.05% (w/v) bromophenol blue	<u>0.2 ml</u>
	8.0 ml

APPENDIX 2

2.1 The Strain Number and Source of *Streptococcus iniae* Isolates

Strain number	Source
ATCC 29177	<i>Iniae geoffrensis</i>
2	Moribund <i>L. calcarifer</i>
4	Moribund <i>L. calcarifer</i>
11	Water sample
13	Moribund <i>L. calcarifer</i>
16	Water sample
26	Moribund <i>L. calcarifer</i>
28	Moribund <i>L. calcarifer</i>
34	Healthy <i>L. calcarifer</i>
37	Healthy <i>L. calcarifer</i>
38	Moribund <i>L. calcarifer</i>
44	Moribund <i>L. calcarifer</i>
46	Moribund <i>O. mykiss</i> (Israel)
47	Moribund <i>L. calcarifer</i>

APPENDIX 3

3.1 Least Significant Difference (LSD) Comparison of Mortality (dependent variable) and Temperature.

	Group								
	1	2	3	4	5	6	7	8	9
2	.835								
3	.849	.691							
4	.021*	.034*	.013*						
5	.037*	.058	.024*	.816					
6	.372	.487	.284	.163	.243				
7	.966	.802	.882	.019*	.034*	.350			
8	.847	.686	.999	.012*	.022*	.275	.881		
9	.967	.803	.881	.019	.034*	.350	.999	.880	
10	.981	.854	.831	.022*	.039*	.385	.947	.828	.948

* denotes significant difference

APPENDIX 4

Table 4.1 Pair-wise comparison of survival using the Wilcoxon (Gehan) statistic for barramundi challenged with *Streptococcus iniae* at different physical variables. (a) Temperature (b) pH (c) Salinity. (G, d.f., p value)

a)

	18	21	24	27	30
21	0.01, 1, 0.94				
24	11.22, 1, <0.01	13.09, 1, <0.01			
27	21.63, 1, <0.01	25.05, 1, <0.01	2.70, 1, 0.10		
30	8.26, 1, <0.01	10.72, 1, <0.01	0.42, 1, 0.52	5.13, 1, 0.02	
33	1.45, 1, 0.23	1.97, 1, 0.16	6.70, 1, 0.01	15.50, 1, <0.01	4.12, 1, 0.04

b)

	pH 6	pH 7	pH 8
pH 7	6.66, 1, 0.01		
pH 8	5.74, 1, 0.02	0.10, 1, 0.75	
pH 9	2.48, 1, 0.12	1.10, 1, 0.30	0.56, 1, 0.46

c)

	0 ppt	10 ppt	20 ppt	30 ppt
10 ppt	0.02, 1, 0.88			
20 ppt	0.54, 1, 0.46	0.37, 1, 0.55		
30 ppt	3.17, 1, 0.55	2.55, 1, 0.11	1.03, 1, 0.31	
40 ppt	0.12, 1, 0.73	0.08, 1, 0.77	0.14, 1, 0.71	2.39, 1, 0.12

APPENDIX 5

From: "XXX XXXXXXXXX" <XXXXXXXX@prose.dpi.qld.gov.au>
To: "belly-up@affa.gov.au" <belly-up@affa.gov.au>
Subject: FISH KILLS AND STREPTOCOCCUS INIAE
Date: Fri, 15 Oct 1999 09:45:35 +1000
X-Mailer: Internet Mail Service (5.5.2448.0)

With the recent circulation of information on streptococcosis following Mr Bromage's posting on ProMed, it appears necessary to supply some accurate information. It is unfortunate that such information is being broadcast widely without reference to the appropriate authorities in Queensland. It is incumbent on any scientist to ensure any public information is scientifically supportable and appropriate diagnostic tests are carried out to confirm a diagnosis.

Fish kills along the Queensland coast did increase through the period from late 1997 until early 1999. The Environmental Protection Agency (EPA) has the primary responsibility for the investigation of fish kills in Queensland and the Fish Health Services group within the Queensland Department of Primary Industries (QDPI) actively support EPA investigations by offering pathology services to determine any role for infectious pathogens. During 1998 EPA in northern Queensland investigated, in detail, over 30 fish kills. The majority were determined to be caused by depleted dissolved oxygen levels in the water. The balance were associated with EUS which has been reported in appropriate Quarterly Disease Reports. In none of the fish kills was a streptococcus septicaemia diagnosed.

The increased incidence of fish kills is considered to be associated with climatic changes seen throughout the State. After three years of serious drought, the rains returned late 1997 with high rainfall early in 1998 and 1999. The movement of surface water would have carried large amounts of organic matter and other material that had accumulated on land and urban areas during the drought period. This suspended organic material caused rapid oxygen depletion, so much so that in small creeks and rivers fish died from suffocation. Another significant factor in many of the fish kills that occurred in small streams and creeks was the submersion of weeds and grasses which had grown on the banks and bottoms of the dried-up water courses. After submersion the vegetation rotted, again causing marked oxygen depletion.

The EPA and QDPI now have a significant base of information on these fish kills and none of that information supports Mr Bromage's claim that fish kills have been caused by *S. iniae*. The isolation of a ubiquitous, environmental bacteria from moribund fish does not constitute a diagnosis in our opinion. At all times in tropical aquatic animals, isolation of a bacteria must be supported by confirmatory histopathology. Streptococcosis is easily confirmed histologically by the presence of large numbers of Gram-positive cocci in all haematopoietic organs. We have never seen this in our examinations of fish kill specimens. Nor is there any suggestion that the fish kills were in anyway associated with aquaculture.

For those specialists with any knowledge of tropical aquatic animal disease, it has been known for sometime that *S. iniae* can cause disease in marine cage barramundi.

QDPI first diagnosed this condition in 1992. Since that time information on streptococcosis has been widely disseminated in information on the diseases of barramundi (eg., the Proceedings of the Australian Barramundi Farming Workshop, 15 & 16th June 1995, Walkamin).

This fact has also been reported at the national level. If one considers the Japanese experience in marine fish culture, we would expect streptococcosis to be a common disease of marine finfish in the warm water and the tropics. The prevalence of streptococcosis has not changed in marine cage cultured barramundi in Queensland but has, in the last year, been reported from barramundi cultured in freshwater, for the first time.

S. iniae is a soil-associated bacteria which was thought to first enter the marine culture systems following heavy rain. The two barramundi marine cage farms (now only one) had sites close to shore and would be directly affected by freshwater run-off following rain, reducing salinity and exposing fish to suspended material (including *S. iniae*) from the land. From Mr Bromage's research and our own examination of clinical material, it is clear that following the initial epizootic, carrier hosts exist in the cultured barramundi population and the wild fish living around cages. As streptococcosis epizootics in marine systems are always associated with a stressor eg., heavy rain, neap tides etc., it is assumed existing infections in barramundi become active due to immuno-depression and/or the wild fish are a source of a new infection in the stressed barramundi population. That is to say, with effective management and appropriate stocking densities, the disease can be managed except when the weather causes stressful changes in water quality. We also see a resistance developing in older animals, that is to say, the high mortalities are more commonly seen in fingerlings held in nursery cages. This indicates that a vaccine could be useful and we support Mr Bromage's research in this area.

Only one occurrence of streptococcosis in freshwater-reared barramundi has ever been diagnosed in northern Queensland. The affected farm uses recirculation technology for nursery rearing and floating cages in freshwater ponds for grow-out. The epizootic occurred following severe flooding of the grow-out ponds. It is thought that the *S. iniae* was introduced with suspended soil in the flood water or by tilapia from the near-by creek which appeared in the ponds after flooding subsided. The epizootic was effectively controlled by antibiotic therapy. Subsequent problems were seen in the nursery system. It was determined the farm would hold market-sized fish prior to processing in holding tanks in the nursery. Due to poor hygiene practices, the nursery tanks would become contaminated and epizootics in the young fish would occur. Ending this practice and improved hygiene resulted in complete prevention of streptococcosis. The farm has never had another outbreak of streptococcosis.

It is critical in recirculation systems to prevent cross contamination between tanks to effectively manage streptococcosis. After an outbreak all information available indicates there will be carriers in the surviving population. Any new fish introduced into the recirculation system must be strictly isolated from the older fish. There is no evidence that the infection is introduced from the hatchery. We understand Mr Bromage has attempted isolation of *S. iniae* from hatchery-held fry, with no success. We remain of the opinion that initial infections arise from environmental

contamination.

Clearly streptococcosis is an important pathogen of barramundi (and all marine finfish) and has been for some time. It is not necessarily more important than columnaris disease, bacterial enteritis or other opportunistic bacterial infections to the barramundi farming industry. It is also worth noting farmed barramundi production in Queensland continues to increase from 328 tonnes in 1995/96, to 349 in 1996/97 and to 434 tonnes in 1997/98.

QDPI offers a complete diagnostic service for aquatic animal disease (including bacterial taxonomy) by a team with over 35 years combined experience in the area. Fish Health Service staff operate from veterinary laboratories in Brisbane and Townsville.

XXX XXXXXXXXX

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APPENDIX 6