CHARACTERISATION OF THE IMMUNE RESPONSE OF THE STRIPED CATFISH (*Pangasianodon hypophthalmus,* Sauvage) FOLLOWING IMMUNOMODULATION AND CHALLENGE WITH BACTERIAL PATHOGENS

THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN AQUATIC VETERINARY STUDIES

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To my family - without your support, I never would have got this far.

Many thanks x

ขอบคุณสำหรับกำลังใจจากครอบครัวที่ทำให้สามารถมาจนถึงวันนี้

Declaration

I declare that this thesis has been compiled by myself, and is the result of my own work and that it has not been submitted for any other degree and all sources of information have been duty acknowledged.

Wanna Sirimanapong

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Abbreviations

AChE	Acetylcholinesterase
ADCC	Antibody-dependent cellular cytotoxicity
AGD	Amoebic gill disease
APCs	Antigen-presenting cells
B-cells	B lymphocytes
BCP	1-Bromo-3-chloropropane
BCR	B-cell receptor
BCWD	Bacterial cold water disease
BNP	Bacillary Necrosis of *Pangasianodon*
BSA	Bovine serum albumin
C	Constant region
CHNV	crucian carp haematopoietic necrosis virus
COI	Cytochrome oxidase subunit 1
CRP	C-reactive protein
CTLs	Cytotoxic T-cells
CYP1B	Cytochrome P4501B
D	Diversity
DEAE	Diethylaminoethly cellulose
d.p.i.	Day post infection
ECPs	Extracellular products
ESC	Enteric septicaemia of channel catfish
EST	Expressed sequence tag
FASL	Fas ligand
GCCHDV	Grass carp haemorrhage disease
G-CFB	Gelatine-complement fixation buffer
GMO	Genetically Modified Organism (GMO)
NGS	Next-generation sequencing)
HOCl	Hypochlorous acid
h.p.i.	Hour post infection
HPSEC	High-performance size-exclusion chromatography
HSP70	Heat shock protein 70
HSWB	High salt wash buffer
IFNγ	Interferon-γ
IgM	Immunoglobulin M
IHNV	Infectious hematopoietic necrosis virus
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
i.p.	Intraperitoneal injection
IPN	Infectious pancreatic necrosis
ISAV	Infectious salmon anemia virus
J	Joining
LAMP	Loop-mediated isothermal amplification method
LPS	Lipopolysaccharide

LGBP	LPS/b-glucan binding protein
LSWB	Low salt wash buffer
LTα	Lymphotoxin-α
MAI	Motile Aeromonas infection
MAS	Motile Aeromonas septicaemia
MBL	Mannose-binding lectins
MASPs	MBL-associated serine proteases
MAITs	Mucosal associated invariant T-cells
MHC II	Major histocompatibility complex class II
MPO	Myeloperoxidase
NBT	Nitroblue tetrazolium
NCBI	National centre for biotechnology information
NK cells	Natural killer cells
NKT cells	Natural killer T-cells
NO	Nitric oxide
OD	Optical density
PBS	Phaosphate buffer saline
PCA	Principal component analysis
PDV	Pancreas disease virus
PMA	Phorbol myristate acetate
p.f.	Post feeding
poly I:C	Polyinosinic:polycytidylic acid
proPO	Prophenoloxidase
RI	Refractive index
ROS	Reactive oxygen species
RSIV	Iridoviral disease virus
RT	Room temperature
SAP	Amyloid P component
SOD	Superoxide dismutase
SOMO	SOlution MOdeller
SRBC	Sheep red blood cells
SV	Sedimentation velocity
SD	Standard diviation
T-cells	T lymphocytes
TCR	T-cell receptor
T_{CM}	Central memory T cells
T_{EM}	Effector memory T cells
Th cells	Helper T-cells
TMB	3’3’5’5’-tetramethylbenzidine dihydrochloride
TNFα1	Tumor necrosis factor-α1
TNFα2	Tumor necrosis factor-α2
T_{REG}	Regulatory T-cells
T_{RM}	Resident memory T cells
T_{SCM}	Stem cell memory T cells
USD	US Dollar
V	Variable region
VASEP	Vietnam Association of Seafood Exporters and Producer

Abstract

In Southeast Asia, the family Pangasiidae is important for commercial fisheries and aquaculture. *Pangasianodon hypophthalmus* (striped catfish) is the most economically important species farmed in Vietnam, with a total export value of 1.7 billion USD in 2012. Intensive aquaculture can lead to problems with major outbreaks of disease and *Edwardsiella ictaluri* and *Aeromonas hydrophila* represent two important bacterial pathogens in *P. hypophthalmus* aquaculture. Immunostimulants have proven to be a very useful food additive for the aquaculture industry, since they can be easily fed to fish to enhance their immune response at times of stress and to improve resistance to disease.

The immune system of pangasius catfish has not been fully described, despite the recent growth in aquaculture for this species, and little is known about the effects of immunostimulants on disease resistance. Understanding the immune response is very important in order to evaluate the health status of the fish and assist in control of disease (including prevention) so that production levels by the aquaculture industry can be sustained. The aims of this thesis were to develop and standardise methods to elucidate and measure immune responses in *P. hypophthalmus* and then to use these with relevant disease models (*A. hydrophila* and *E. ictaluri*) and immunomodulators (*β-glucan*s from different sources and at different doses) to determine if bacterial diseases can be controlled, and which functional immune responses and immune genes could be correlated with disease resistance.

As a variety of different species from family Pangasiidae are economically important for aquaculture, initial work focused on the characterisation of the immunoglobulin IgM molecule in these species, and anti-P. *hypophthalmus* IgM mAbs were tested to determine if they cross-reacted between different Pangasiidae species (Chapter 2). Although affinity purification of IgM from the different fish species resulted in a purer preparation ammonium sulphate precipitation (14% w/w), the latter proved faster and easier to perform. The heavy (H) and light (L) chains of IgM from *P. hypophthalmus* were estimated to be 70-72 kDa and 25-26 kDa, respectively, using SDS-PAGE (12.5%). The L chains of IgM in the other Asian fish species examined were similar in molecular weight to *P. hypophthalmus*, while the H chains varied (*P.*

gigas and *P. larnaudii* 76kDa, *P. sanitwongsei* 69kDa, *H. filamentus* 73kDa, *P. borcoti* and *H. wyckioides* 75kDa, *C. bactracus* 74kDa, *C. macrocephalus* 73kDa and *C. carpio* 70kDa), as did the native IgM molecules. Sedimentation velocity ultracentrifugation was used to determine the molecular weight of the whole IgM molecule from *P. hypophthalmus* as an alternative to the more commonly used native gels that are run under non-denaturing conditions, although this technique proved more complex. Anti–*P. hypophthalmus* IgM monoclonal antibodies (mAbs) cross reacted with all of the Pangasiidae species and were successfully applied in an enzyme-linked immunosorbent assay (ELISA) using mAb 23 to measure serum antibody response of *P. hypoophthalmus* following experimental infection with *A. hydrophila* by interperitoneal (I.P.) injection in Chapter 3 and *E. ictaluri* by immersion in Chapter 4.

As *P. hypophthalmus* is a relatively new aquaculture species, there are few reports evaluating its immune response to pathogens. Thus, functional assays were standardised to evaluate both innate and adaptive immune responses of this species and then these assays used to compare immune response following stimulation with live and killed *A. hydrophila.* (Chapter3). Four treatment groups of 40 fish per group (53.2 ± 14.8g.) consisting of an untreated control group, a group injected I.P. with adjuvant (Montanide ISA 760 VG) only, a group injected with heat-killed *A. hydrophila* (1 x10^{9} cfu ml^{-1} mixed with adjuvant), and a group injected with a subclinical dose of live *A. hydrophila* 2.7 x10^{5} cfu ml^{-1} were used in the study. Samples were collected 0, 1, 3, 7, 14 and 21 days post injection (d.p.i.) to assess the immune response of fish. The results indicated that challenge with live or/and dead bacteria stimulated the immune response in *P. hypophthalmus* significantly above control groups with respect to specific antibody titre, lysozyme activity, phagocytosis and plasma peroxidase at 7 or/and 14 d.p.i. Moreover, on 21 d.p.i. total IgM, specific antibody titre and lysozyme activity from both live and dead *A. hydrophila* challenge groups were significantly different to the control groups. Differential immune responses between live and dead bacterial challenges were also observed as only live *A. hydrophila* significantly stimulated WBC counts and plasma peroxidase at 3 d.p.i. with the greatest increase in WBC counts noted at 21 d.p.i. and in phagocytosis at 14 d.p.i. By 21 d.p.i. only the macrophages from fish challenged with dead *A. hydrophila* showed significantly stimulated respiratory burst activity.

Abstract

Immunostimulants are food additives used by the aquaculture industry to enhance the immune response, and β-glucan is now commonly used for this purpose in aquaculture. In Chapter 4 the effect of the prebiotic β-glucan on the immune response and disease resistance of *P. hypophthalmus* was evaluated. The fish (60.3 ± 11.7 g.) were fed with a basal diet (control) or diets supplemented with fungal derived β-glucan at concentrations of 0.05 %, 0.1 %, or 0.2 % g/kg for four weeks. Fish fed 0.1 % commercial yeast derived β-glucan were also included as a positive control group. Samples were collected from fish on Days 0, 1, 3, 7, 14, 21 and 28. The results showed that fish fed with the highest two levels of fungal derived β-glucan had enhanced immune responses compared to the control group, with respiratory burst activity on all days examined and lysozyme activity on 7 days post feeding (d.p.f.) being significantly elevated (*P<0.05*) in the group fed with 0.2 % fungal derived β-glucan, while plasma anti-protease activity on 21 d.p.f., natural antibody titre on 3 d.p.f. and complement activity 7 d.p.f. and 14 d.p.i. were significantly enhanced (*P<0.05)* in the group fed 0.1 % fungal derived β-glucan. The lowest dose of fungal derived β-glucan (0.05 %) appeared insufficient to effectively stimulate the fish's immune response. WBC count, respiratory burst, lysozyme activity and complement were useful as an early indication of immunostimulation (1 to 7 days). Four weeks after feeding with the different diets, the fish were experimentally infected with *E. ictaluri* by immersion using 8 x10^4 cfu ml^{-1} for 1 h and mortalities were monitored for 14 days. There was a great deal of variation in the level of mortalities within the four replicate tanks for each dietary group. Although the *in vivo* challenge results showed no statistical differences between the groups fed on the different diets, the highest mortalities were observed in group fed with the control diet and the lowest mortalities were observed in the groups fed with commercial yeast derived β-glucan and 0.2 % fungal derived β glucan.

Immune gene expression following stimulation with β-glucan and challenge with *E. ictaluri* was investigated in Chapter 5. The *P. hypoophthalmus* (36 ±0.34 g) were fed 0.1% of a fungal-derived β-glucan, a commercial yeast derived β-glucan or a basal diet (control). After 14 days, liver, spleen and kidney tissues were collected and processed for expression analysis of seven immune genes [acute phase response (transferrin, C-reactive protein and precerebellin like protein), complement (C3 and factor B), adaptive response (2a MHC class II) and cytokine (interleukin-1β)] by

quantitative real time PCR. Translation elongation factor-1α, 18s rRNA and β-actin were used as house-keeping reference genes. Twenty-five fish from each of the four replicate tanks of the three treatment groups were then either experimentally infected with 1 x10^6 cfu ml^{-1} of *E. ictaluri* by immersion for 30 min and the remaining twenty five fish per tank were mock infected with the culture medium. At 24 h.p.i., tissue samples were again collected for immune gene expression and the challenge monitored for 2 weeks. The relative percentage mortality at 14 d.p.i. was statistically significantly different between the control diet (30 ±12%), and the 0.1% fungal derived β-glucan (17 ±8%) and commercial yeast-derived β-glucan diets (16 ±5 %). There was no obvious difference in relative gene expression for the genes examined between the different dietary treatments after feeding fish for 14 days, while there were clear differences between the infected and uninfected groups at 24 h.p.i. The expression pattern of the immune genes in liver, spleen and kidney with respect to the immunostimulation and the infection varied with diets. Overall, principal component analysis with 11 variables (liver [C-reactive protein, transferrin, complement factor B and C3, precerebellin, IL-1β and MHC class II], the kidney [IL-1β and MHC class II] and the spleen [IL-1β and MHC class II]) showed significant differences between fish fed with control diet and immunostimulant diet in challenged or/and unchallenged with *E. ictaluri* (*P_mc<0.05*).

A variety of functional immune assays and gene expression methods for *P. hypophthalmus* were developed and standardised during this study, and these provide useful useful tools and basic information on the immune response in striped catfish that can be applied for the health control of this species. Furthermore, the identification of striped catfish immune genes during this work will be very useful for further genomic research relating to disease. Future work on the *P. hypophthalmus* immune system should focus on full immunological transcriptomic analysis to enable a more complete understanding of the gene expression and regulatory networks involved in the immune response of *P. hypophthalmus* to disease.

Table of Contents

List of Tables

List of Figures

Chapter 1 General introduction

1.1 Pangasius aquaculture

Species of fish belonging to the family Pangasiidae are important for both commercial fisheries and aquaculture. Four genera, with a total of 23 different species, are reported to be associated with this family worldwide (Pouyaud*, et al.* 2004). In Southeast Asia, twelve species from four different genera have been identified in Thailand and the Indochinese region *i.e. Helicophagus waandersii*; *Pteropangasius pleurotaenia and P. micronema*; *Pangasianodon gigas* and *P. hypophthalmus*; *Pangasius bocourti*, *P. krempfi, P. larnaudii, P. macronema, P. pangasus, P. polyuranodon* and *P. sanitwongsei* (Vidthayanon 1994). Many of these species have economic potential for aquaculture including *P. bocourti*, *P. gigas*, *P. hypophthalmus*, *P. larnaudii* and *P. sanitwongsei* (Kwantong and Bart 2003; Sriphairoj*, et al.* 2010). *Pangasianodon hypophthalmus,* the most economically important species of these to be farmed, has many different common names associated with it (*e.g.* Iridescent Shark-Catfish, Striped Catfish, Suchi Catfish, Thai Catfish in the UK; Pla Sawai in Thailand; Cá Tra, and Cá Tra yêu in Vietnam (BayScience Foundation 2013; FAO 2013). The natural distribution of this riverine freshwater species is limited to the Mekong River, the Chaopraya River and possibly the Mekong basins in Cambodia, Lao People's Democratic Republic, Thailand and Vietnam, the Irrawaddy or Ayeyawady basin of Myanmar (within a range of 19^{o}N to 8^{o}N), as shown in Figure 1.1. The fish migrates over a long distance (several hundred kilometres) between its spawning habitat located upstream and its feeding and nursery habitat downstream. It is omnivorous, feeding on algae, higher plants, zooplankton, and insects, while larger individuals are also known to eat fruit, crustaceans and fish (FAO 2013).

Figure 1.1. The distribution of wild *P. hypophthalmus* in Southeast Asia countries; Myanmar in Irrawaddy or Ayeyawady basin, Thailand in Chaopraya river and Mekong basins in Cambodia, Lao People's Democratic Republic, Thailand and Vietnam

Pangasianodon hypophthalmus is an important aquaculture species in the Mekong riparian countries. The culture of this species has developed rapidly in recent years, and it is considered to be a valued food fish of economic significance to many countries, especially to Vietnam (Nguyen 2009). Recorded production of *P. hypophthalmus* culture was in the region of 683,000 tons in 2007, valued at around 645 million USD, and represents one of the largest single species - based farming systems in the world, restricted to a very small geographical area. Although Vietnam is described as one of the most successful primary food production sectors in the world

(Phan*, et al.* 2009), data from the Vietnam Association of Seafood Exporters and Producers for the period from January to December 2012 reported that Vietnam's total export value for pangasius declined to USD 1.7 billion, 3.4 % down from 2011 (Globefish 2013). This may have been due to a restriction imposed by Europe, Asia and Latin America on the import of this species from Vietnam. Nevertheless, Vietnam is still the world's largest producer of pangasius catfish (Globefish 2011). Intensive fish culture in Vietnam's Mekong Delta started in 1960 with *P. bocourti* in cages and in 1999 with *P. hypophthalmus* in ponds. Both species are mostly destined for export to international markets. In 2000, the production of fish for export was more than 62,000 tons from more than 5,000 cages in the Mekong Delta region. Cage sizes vary from 200 m^3 to over 1,500 m^3, and have a productivity of 90-150 kg of fish per cubic meter during a culture period of 10-12 months. Mass production of *P. hypophthalmus* fingerlings is successful in intensive culture in ponds ranging from 1,000 to 10,000 m^2 and reaches production levels of 300-500 tons/ha/year (Wilder and Phuong 2002; Phan*, et al.* 2009). The product is sold almost totally for export to over 100 different countries as frozen fillets, where it is an acceptable alternative to more expensive locally sourced white fish.

Intensive aquaculture can lead to problems with major outbreaks of disease. The diseases associated with this species tend to be bacterial in nature caused by the *Edwardsiella ictaluri*, *Aeromonas hydrophila* and *Pseudomonas dermoalba,* or parasitic caused by *Dactylogyrus, Trichodina, Epistylis, Myxobolus, Henneguya* and Nematoda (Ferguson*, et al.* 2001; Crumlish*, et al.* 2002; Vu and Campet 2009).

1.1.1 Important diseases

Two of the most important diseases in intensive Pangasius culture are *A. hydrophil*a and *E. ictaluri*.

1.1.1.1 Aeromonas hydrophila

Aeromonas hydrophila is Gram-negative, rod shaped, fermentative bacterium, which uses flagella for motility (Austin and Austin 2007). It causes motile aeromonas septicemia (MAS) in a variety of aquatic (freshwater and marine) and land animals, and can be zoonotic in nature (Aoki 1999; Ye*, et al.* 2013). It also can be a primary, secondary and opportunistic pathogen in both animals and humans (Esteve*, et al.* 1993; Lio-Po*, et al.* 1996; Joice*, et al.* 2002). The clinical signs in fish infected with MAS include ulcerative lesions in the skin (ranging from superficial to deep lesions in the skin) tail and fins, and haemorrhagic septicaemia, exophthalmos, distention of the abdomen, petechiation and haemorrhaging of the visceral organs, and swollen lower intestine, vent, liver and spleen (Inglis*, et al.* 1993; Noga 2010; Austin and Austin 2007). A variety of morphological changes can be seen in tissue, including skin lesions with dermatitis and myositis, necrosis of the renal and splenic haematopoietic tissue, necrotic intestinal mucosa and focal necrosis in the heart, liver, pancreas, and gonads (Noga 2010).

Many fish species can be affected by MAS, including eel (Esteve*, et al.* 1993), Indian major carp (Chandran*, et al.* 2002) , goldfish (Viji*, et al.* 2013), catfish (Griffin*, et al.* 2013), tilapia (Abdel-Tawwab*, et al.* 2010), gilthead seabream (Reyes-Becerril*, et al.* 2011), rainbow trout (Saavedra*, et al.* 2010) and striped catfish (Kumar and Ramulu 2013).

The disease can produce significant losses to the aquaculture industry because of reduced growth and the unmarketable appearance of infected fish. When virulent strains of *A. hydrophila* become endemic in a fish population, it is difficult to introduce new fish into the water body without significant mortalities resulting. The diagnosis of MAS is through culture and identification of the bacterium by bacteriology (Noga 2010), or a variety of other methods *e.g.* agglutination assays, (Toranzo*, et al.* 1987), fluorescent antibody techniques (De Figueiredo and Plumb 1977), monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) (Austin*, et al.* 1986), quantitative polymerase chain reaction (Griffin*, et al.* 2013), multiplex polymerase chain reaction (m-PCR) (Panangala*, et al.* 2007; Altinok*, et al.* 2008), or multiplex nested-polymerase chain reaction (m-nested PCR) (Chang*, et al.* 2009).

1.1.1.2 Edwardsiella ictaluri

Edwardsiella ictaluri is a Gram negative, rod shaped, facultative anaerobic bacterium that possesses peritrichous flagella (Buller 2004; Austin and Austin 2007). It is the causative agent of enteric septicemia of channel catfish (ESC), and is one of the most important diseases of channel catfish (*Ictalurus punctatus*) aquaculture (Wagner*, et al.* 2002). The others species reported to be susceptible to *E. ictaluri* infections include striped catfish (*P. hypophthalmus*)(Ferguson*, et al.* 2001; Crumlish*, et al.* 2002; Yuasa*, et al.* 2003), rainbow trout (*Oncorhynchus mykiss*)(Seçer*, et al.* 2004), danio (*Danio devario*)(Waltman*, et al.* 1985), chinook salmon (*Oncorhynchus tshawytscha*)(Baxa*, et al.* 1990), European catfish (*Silurus glanis*) (Plumb and Hilge 1987), yellow catfish (*Pelteobagrus fulvidraco*) (Ye*, et al.* 2013), walking catfish (*Clarias batrachus*) (Kasornchandra*, et al.* 1987), hybrid catfish (*Clarias macrocephalus* (Günther) x *Clarias*

gariepinus (Burchell))(Suanyuk*, et al.* 2013), tadpole madtom (*Noturus gyrinus*) (Klesius*, et al.* 2003), Southern catfish (*Silurus meridionalis*) (Geng*, et al.* 2013), zebrafish (*Danio rerio*) (Hawke*, et al.* 2013), brown bullheads (*Amieurus nebulosus*) (Iwanowicz*, et al.* 2006) and Nile tilapia (*Oreochromis niloticus*) (Soto*, et al.* 2012; 2013).

The clinical signs vary including listless swimming in a vertical position at the surface of the water, or in spiralling circles (Austin and Austin 2007; Inglis*, et al.* 1993). Gross lesions are present as petechial haemorrhages, on the skin area around the jaw, the base of all fins, the operculum and the abdomen, the gills appear pale and exophthalmia is also evident. The skin lesions appear as small white de-pigmented areas (1-3 mm) that can progress into cutaneous ulcers with inflammation. Internally, the kidney and spleen appear swollen and the peritoneal cavity can be filled with bloody or clear yellow ascites. Haemorrhaging and necrosis can be present in the liver and petechial haemorrhages can be seen in internal muscle walls (Noga 2010; Inglis*, et al.* 1993). Reports of *P. hypophthalmus* infected with *E. ictaluri* describe multifocal irregular white lesions of varying sizes on the internal organs (*i.e*. liver, spleen and kidney). Histopathologically, enteritis, hepatitis, myositis, interstitial nephritis, chronic foci, inflammation in the olfactory sac and the telecephalon of the brain (meningoencephalitis), were seen together with multifocal areas of necrosis and pyogranulomatuous inflammation in liver, kidney and spleen (Noga 2010; Ferguson*, et al.* 2001). Gram-negative bacteria were observed in target tissues. During the acute phase of the infection, the kidney is the organ of choice to sample, while during the chronic phase the brain is reported the best organ for bacterial isolation. These can be

identified using bacteriological methods (Noga 2010; Austin and Austin 2007) and a variety of other techniques *e.g.* Fluorescent Antibody Test (Ainsworth*, et al.* 1986), ELISA, *in situ* hybridization, the loop-mediated isothermal amplification (LAMP) and multiplex PCR (m-PCR) (Yeh*, et al.* 2005; Austin and Austin 2007; Panangala*, et al.* 2007; Noga 2010; Suanyuk*, et al.* 2013).

1.2 Interaction between host, pathogen and environment

The ability of pathogens to infect their host depends on a complex interaction between the pathogen, their host and the environment. The virulence of the pathogens depends on a variety of factors including the strain, biotype, serotype, and genotype, route of entry and duration of the exposure to the agent (Hedrick 1998). Various routes of infection by *E. ictaluri* in fish have been suggested *e.g*. through the olfactory organ via the nasal opening, from where it migrates into the olfactory nerve and then to the brain of the fish (Inglis*, et al.* 1993; Wolfe*, et al.* 1998); or through ingestion, entering the blood via the intestine (Inglis*, et al.* 1993). The gastrointestinal tract of fish has also been reported as the route of infection for *A. hydrophila* in crucian carp (*Carassius carassius*) (Zhang*, et al.* 2013).

Several parameters have been associated with the host's risk to developing the infection. These factors include the genotype, age, size and developmental stage of the host, as well as it's nutritional, reproductive and behavioural states, in addition to general health status (Hedrick 1998).

The environment is one of the main factors affecting the interaction between host and pathogen, particularly chemical and physical factors of the surrounding water body, such as dissolved gases, pH, toxin, temperature, flows, turbidity and water

quality (Hedrick 1998) all contributing to increased levels of stress in the fish. Also, specific temperature profiles are associated with particular infections (e.g. *E. ictaluri* is 20-27^{o}C (Inglis*, et al.* 1993) and *A. hydrophila* is between 18–30^{o}C (Camus*, et al.* 1998). A summary of the interactions between the host, pathogen, and environment, which can lead to an increased risk of disease outbreaks in striped catfish, is shown in Figure 1.2

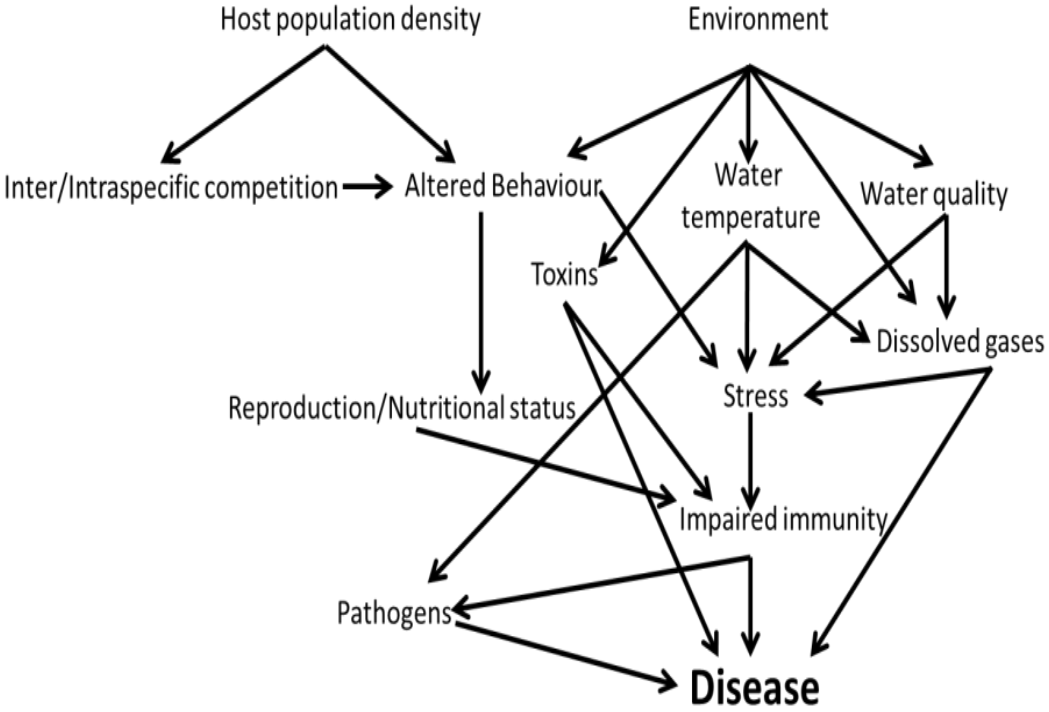


Figure 1.2. A web of causative factors increasing the risk of disease in fish, modified from Hedrick (1998)

1.3 Disease prevention and control

Chemotherapy to control MAS has included the use of antibiotics such as oxytertacycline, sulphamerazine, chloramphenicol and nifurpirinol, while, oxytetracycline, sulphonamides (Inglis*, et al.* 1993), and cefaperazone, cinoxacin,

kanamycin, moxalactam, neomycin, nitrofurantoin, oxolinic acid, streptomycin, ticarcillin and trimethoprim have been used to treat ESC (Austin and Austin 2007). The worldwide growth of aquaculture has been rapid, and although use of antibiotics is well controlled with restricted application in some countries (*e.g.* UK and Norway), this is not the case for all countries. Therapeutic and prophylactic use of antibiotics to control diseases in aquaculture has resulted in the development of plasmid mediated resistance to antibiotic compounds in both the fish and the aquatic environment (Cabello*, et al.* 2013). For example, Dung *et al.*(2008) found 64 Vietnamese isolates of *E. ictaluri* from *P. hypophthalmus* displaying acquired resistance to streptomycin (83%), oxytetracycline (81%), trimethoprim (71%), flumequin (8%) and oxolinic acid (6%), while antimicrobial resistance of *A. hydrophila* has been reported to streptomycin (57%), tetracycline (48%) and erythromycin (43 %) (Son*, et al.* 1997), methicillin (100%), rifampicin (100%) and novobiocin (99%) (Vivekanandhan*, et al.* 2002).

Cabello, *et al.* (2013) reported that genetic elements and resistance determinants for quinolones, tetracyclines, and β-lactamases are shared between aquatic bacteria, fish pathogens, and human pathogens, which appear to have originated in aquatic bacteria. The use of antimicrobials in aquaculture should therefore be restricted to reduce drug residues developing further in the aquatic environment and potentially threatening animal and human health. Methods for the prevention of bacterial diseases in aquatic animals without using antibiotics have/are being developed and tested in many species of fish. A holistic approach, which considers the pathogen, its host and the environment, would be most suitable in the

long term as suggested by Defoirdt *et al.* (2011) as shown in Figure 1.3. Biological control to prevent disease is an important health management strategy that should be considered (Subasinghe*, et al.* 2001). The use of immunostimulants and vaccines has become the norm for the prevention of infectious diseases in aquaculture (Dong*, et al.* 2013; Evensen and Leong 2013). In addition, the combined use of immunostimulants or adjuvants with vaccines has been shown to increase the efficacy of vaccines (Tafalla*, et al.* 2013).

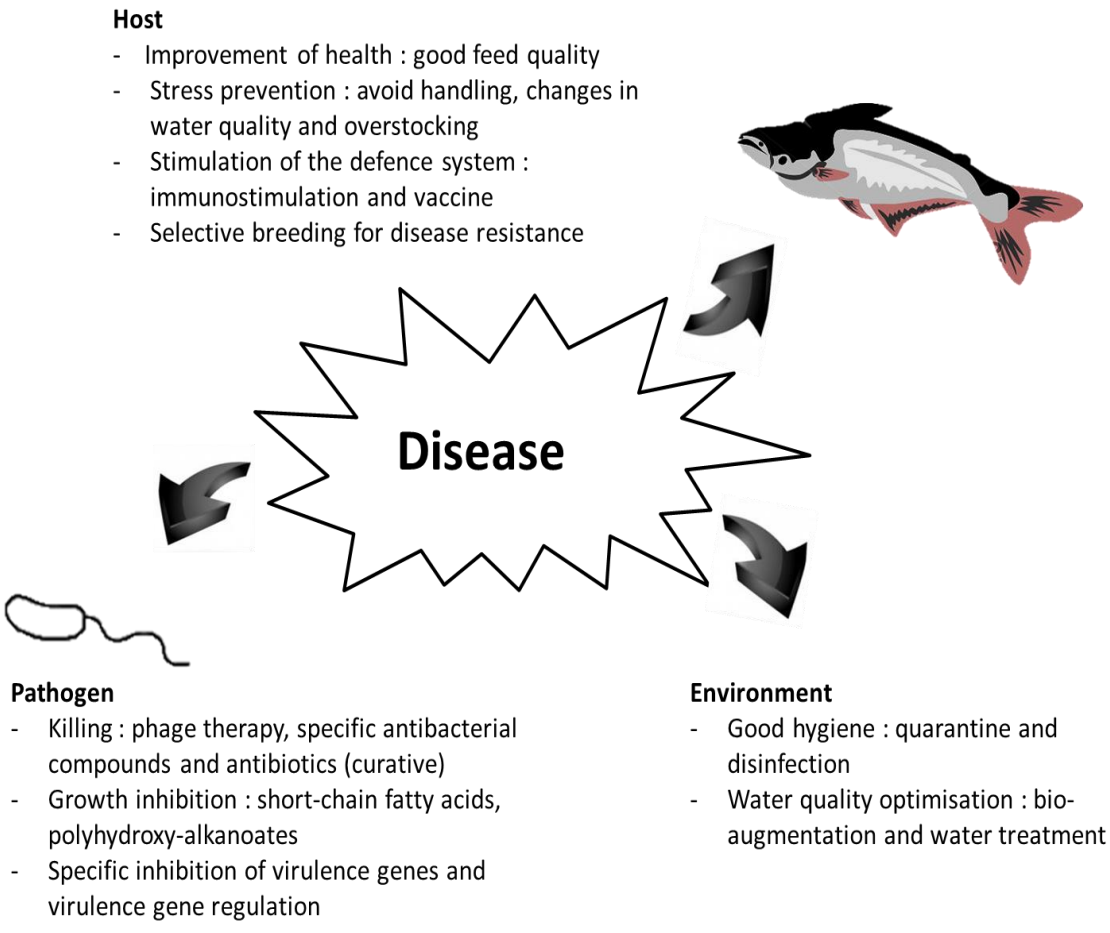


Figure 1.3. The interaction between host, pathogen, and environment that can increase the risk of disease outbreaks in fish. Strategies to prevent and control bacterial disease in aquaculture should ideally take into account the different aspects of the pathogen-host-environment continuum, data from Defoirdt, *et al.* (2011).

Immunomodulation is a change in the fish's immune response so as to enhance or reduce its activity. For example, immunostimulants are substances that can stimulate the immune response of fish by inducing or increasing immune activity. There are two main types of immunostimulants; specific immunostimulants that act via an antigenic specific response such as vaccines or via a non-specific action, which increase the immune response independent of antigenic recognition, such as adjuvants and non-specific immunostimulators (Kumar*, et al.* 2011). For example, β-glucans, chitin, lactoferrin, levamisole, vitamins B and C, growth hormone and prolactin have all been reported to be immunostimulators in teleost fish (Sakai 1999). Adjuvants are often used in conjunction with vaccines, to help generate a stronger protective response to the antigens in the vaccine, providing a higher degree of protection against the pathogen. Cytokines, produced by the cellular immune system also act as immunostimulators and are able to enhance immune function (Kumar*, et al.* 2011).

Fish immune cells have been shown to have enhanced phagocytic activity following administration of immunostimulants such as β-glucan (Chen and Ainsworth 1992; Verlhac*, et al.* 1998; Ai*, et al.* 2007), vitamin C (Verlhac*, et al.* 1998; Ortuno*, et al.* 1999), lactoferrin (Kamilya*, et al.* 2006), chitin and chitosan (Esteban*, et al.* 2001; Lin*, et al.* 2011), nisin (Villamil*, et al.* 2003) and various kinds of probiotics (Nayak 2010). The activities of phagocytic cells can be assessed by examining their phagocytic, killing and chemotactic activities. The process of pathogen killing seems to be heightened in the macrophages of fish treated with immunostimulants. The mechanism of macrophage killing involves both oxygen-dependent and/or oxygen-independent processes. The oxygen-dependent killing process, through the production of reactive

oxygen species (ROS), can be detected by chemiluminescence or the reduction of Nitroblue tetrazolium (NBT) (Lunden*, et al.* 1999; Vera-Jiménez*, et al.* 2013). Lymphocytes in Nile tilapia have been reported to be activated by immunostimulants such as *Saccharomyces cerevisiae*, β-glucans and laminaran (El-Boshy*, et al.* 2010), and by tuftsin in Indian Major carp (Misra*, et al.* 2006a). Complement activity is also activated by immunostimulants, as shown by Pionnier *et al.* (2013), who found that complement activity was stimulated by β-glucan in common carp. Furthermore, lysozyme activity is influenced by treatment with immunostimulants (Yin*, et al.* 2009; Dong*, et al.* 2013; Paredes*, et al.* 2013).

1.4 The teleost immune system

The immunology of fish has been useful to investigate the evolution of the immune response between lower vertebrates and mammals (Corbel 1975; Plouffe*, et al.* 2005; Saurabh and Sahoo 2008). Fish are the first animals in the phylum to possess both an innate and adaptive immune system (Magnadottir 2010). The innate response is the first line of defence in fish against invading pathogens (Ellis 2001), while the adaptive immune response is slower to develop and is important for the specific recognition and destruction of pathogens (Cooper and Alder 2006). A comparison of the humoral and cellular components of the innate and adaptive immune systems is shown in Figure 1.4 (Bayne and Gerwick 2001). Knowledge of the function of the immune system is essential for the successful development of disease prevention strategies for fish, such as the development of vaccines, selection for increased disease resistance and identification of genes suitable for transgenesis (Watts*, et al.* 2001).

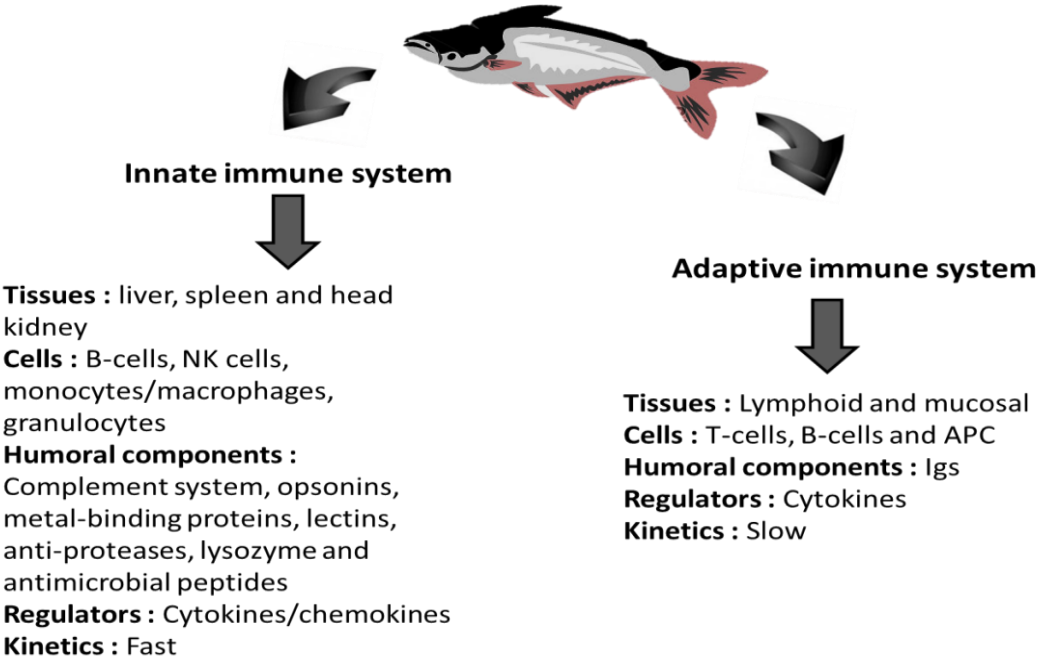


Figure 1.4. Components of the innate and adaptive arms of the fish immune system (Modified from Bayne and Gerwick (2001))

1.4.1 Innate immune response

The innate immune system is the first line of defence against infectious disease (Janeway*, et al.* 2001). There are three important features associated with the innate response; it provides non-specific protection, it responds quickly against pathogens over the first few days of the infection; and it is relatively temperature-independent (Ellis 2001). It is comprised of physical barriers (*i.e*. skin, scales and mucus), and cellular and humoral components, a summary of which can be found in Figure 1.5. The mucus contains many proteins which help in its ability to prevent pathogens from entering *e.g*. immunoglobulins, pentraxins, lysozyme, complement proteins and antibacterial peptides (Rombout*, et al.* 1993; Aranishi and Nakane 1997).

Cells of the innate immune system include monocytes/macrophages, granulocytes, cytotoxic NK-like cells (Ellis 2001). Phagocytic, cytolytic and anti-

microbial properties are the activities associated with the cells of the innate immune response (Woods*, et al.* 1999). Pattern recognition receptors present on the cells bind to pathogen-associated molecular patterns (PAMPs) associated with different groups of pathogens.

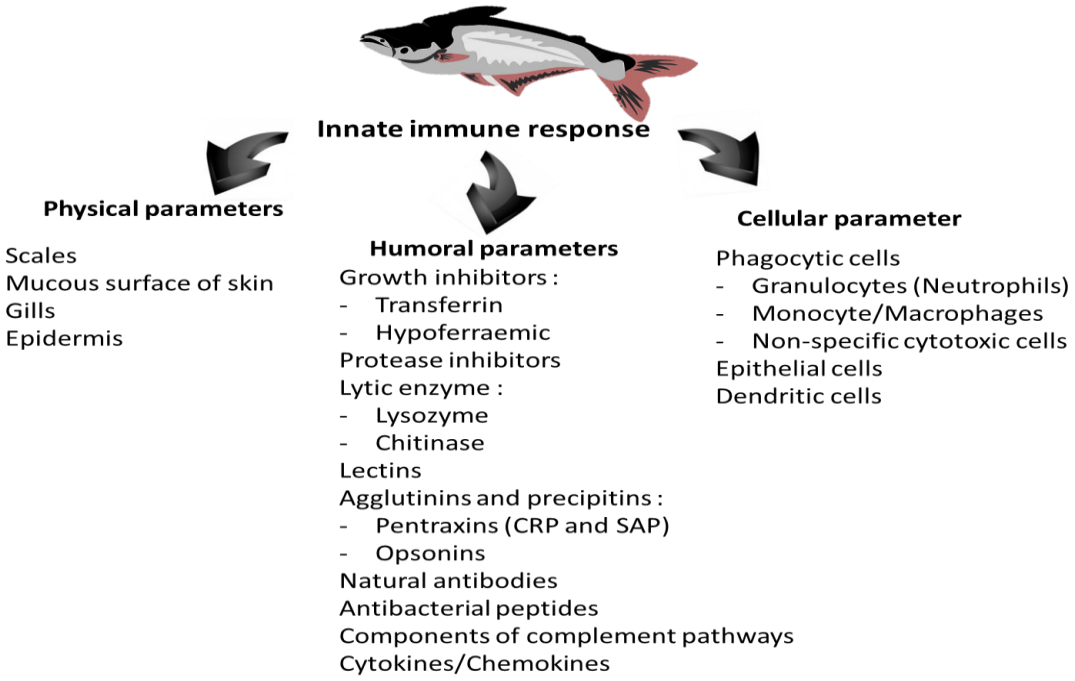


Figure 1.5. Summary of innate immune response in fish (Modified from Magnadóttir (2006) and Ellis (2001)).

1.4.1.1 Humoral immune response

The humoral response of the innate immune system has a variety of different types of proteins and glycoproteins that are able to inhibit and destroy the pathogen, which it does through anti-bacterial peptides (pleurocidin and ceropin), complement, lectins (hemagglutinin), pentraxins (serum amyloid protein (SAP) and C-reactive proteins (CRP)), lysozymes, bacterial growth inhibitor transferrins, proteases (trypsin-like proteases and cathepsin L and B), cytokines, and antiviral interferons (Ellis 2001;

Magor and Magor 2001; Secombes*, et al.* 2001; Angeles Esteban*, et al.* 2006; Magnadóttir 2006; Whyte 2007). Other important enzymes included in the innate immune response include cyclooxygenase, which is responsible for the production of prostaglandins and inducible nitric oxide synthase that generates nitric oxide (Secombes*, et al.* 2001).

Anti-microbial peptides have been found in integument secretions of both plants and the animals, and are also classified as a component of the innate immune response (Whyte 2007). The antimicrobial peptides isolated from fish are a heterogenous group, the majority of which are known to form amphipathic α–helices (Plouffe*, et al.* 2005). In teleost fish, a number of antimicrobial peptides have been reported, such as cathepsin D in skin mucosa of catfish (Cho*, et al.* 2002a), hepcidin in liver of gilthead seabream (*Sparus aurata* L.), histone H1 in liver of Atlantic salmon (*Salmo salar*) (Richards*, et al.* 2001), hipposin in skin mucus of Atlantic halibut (*Hippoglossus hippoglossus*) (Birkemo*, et al.* 2003) and piscidin in mast cells of striped bass (*Morone saxatilis*) (Campagna*, et al.* 2007).

The functions of complement are many fold; they induced lysis of pathogens, opsonise (coat) bacteria to enhance phagocytosis by macrophages and clearance of potentially damaging immune complexes (antigen-antibody complex), enhance antigen presentation and the production of various peptides involved in vasodilation, phagocyte adhesion and phagocyte chemotaxis, and signalling the production of an inflammatory response and regulation of complement activation (Mak and Saunders 2005).

The complement system of bony fish is similar to that of higher vertebrates in that it can be activated through three different pathways of activation as detailed in Figure 1.6. The first is the classical pathway activated by antigen antibody complexes. The alternative complement pathway is activated by surfaces mortifies on bacteria and fungi. The lectin binding pathway is mediated by protein complexes containing mannose-binding lectins (MBL) binding to carbohydrates on cell surfaces of the microorganism, such as mannan, and because it is associated with serine proteases it is therefore called MBL-associated serine protease (MASP). There are two types of MASPs *i.e.* MASP-1 and MASP-2. The MBL pathway is activated through MASP1 and MASP-2 binding to mannans on the pathogen's surface. After activation of the C3 convertase from both the classical and the alternative pathway, C3b is produced and it in turn leads to the production of the C5 convertase molecule (C4bC2a). The C5 convertase is converted to C5a and C5b. The small fragment of C5a is an anaphylatoxin, involved in the inflammatory process by phagocytic cells. The large fragment of C5b attaches to surface-bound C3b and stimulate the production of the membrane attack complex (MAC) by the subsequent binding of C6, C7, C8 and C9. The MAC is able to lyse the cell and results in the death of pathogen by creating a channel or pore in the cell membrane (Holland and Lambris 2002; Zhou*, et al.* 2012). There are many published studies relating to the complement system in teleosts, and more recently examining gene expression of complement components by qRT-PCR (Gonzalez*, et al.* 2007; Pionnier*, et al.* 2013).

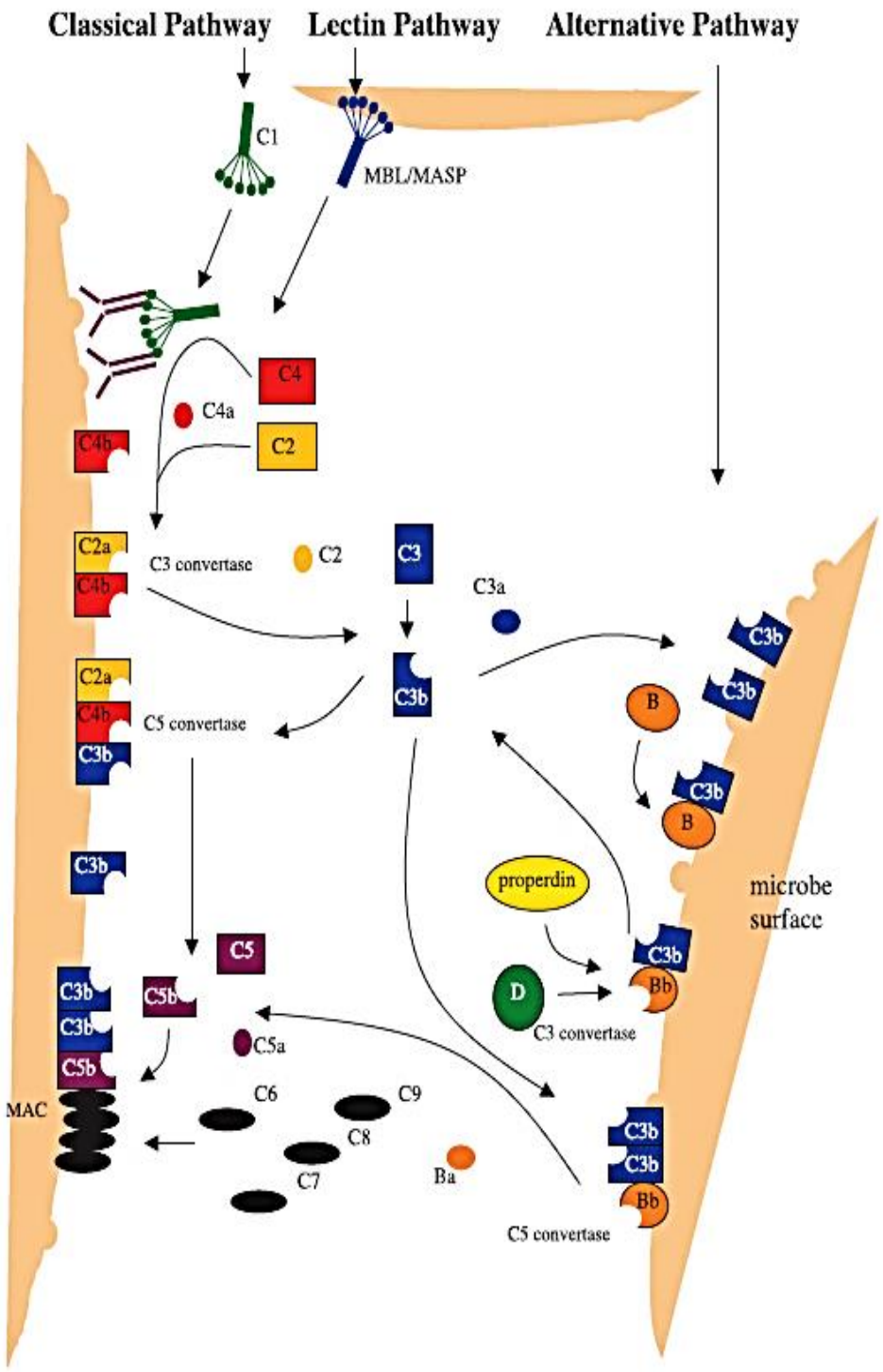


Figure 1.6. The three complement activation pathways in mammals (Data from Holland and Lambris 2002)

Lectins are carbohydrate-binding proteins that interact non-specifically with structures on the surface of the pathogen. They act as opsonins to promote phagocytosis or are able to activate the complement cascade (Magnadóttir*, et al.* 2005). There are two major classes of lectins, C-type (which is an extracellular protein with a disulphide-rich Ca^{2+}-binding carbohydrate-recognition domain) and S type (which is both and intra- and extracellular protein, with no disulphide bonds and which recognizes predominantly galactose) (Ewart*, et al.* 2001). The C-type lectin has been found in oocytes of gibel carp (*Carassius auratus gibelio*) (Dong*, et al.* 2004), in serum and kidney of Atlantic salmon (Richards *et al.* 2003) and has been shown to enhance Atlantic salmon macrophage anti-*A*. *salmonicida* activity (*i.e*. respiratory burst, phagocytic and bactericidal activity, and opsoniszation of bacteria for phagocytosis), and thus can inhibit the growth of *A. salmonicida* within the fish (Ottinger*, et al.* 1999).

C-reactive protein (CRP) and serum amyloid P component (SAP) are classical pentraxins, associated with the acute phase response (Magnadóttir*, et al.* 2005). They are involved in the initiation of the lectin binding complement pathways as shown by Cook *et al*. (2003a) for snapper (*Pagrus auratus*) (Magnadóttir 2006). They have been found in the serum Atlantic salmon and rainbow trout (Jensen*, et al.* 1995; Lund and Olafsen 1999) and common carp (Cartwright*, et al.* 2004).

Lysozyme is a hydrolytic enzyme that cleaves the β-(1,4)-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan, the main bacterial cell membrane polymer in Gram positive bacteria (Figure 1.7(A)). In Gram negative bacteria its action starts once the outer cell membrane of the bacterium has

been destroyed by complement to expose the inner peptidoglycan layer in the bacterium (Saurabh and Sahoo 2008) (Figure 1.7(B)).

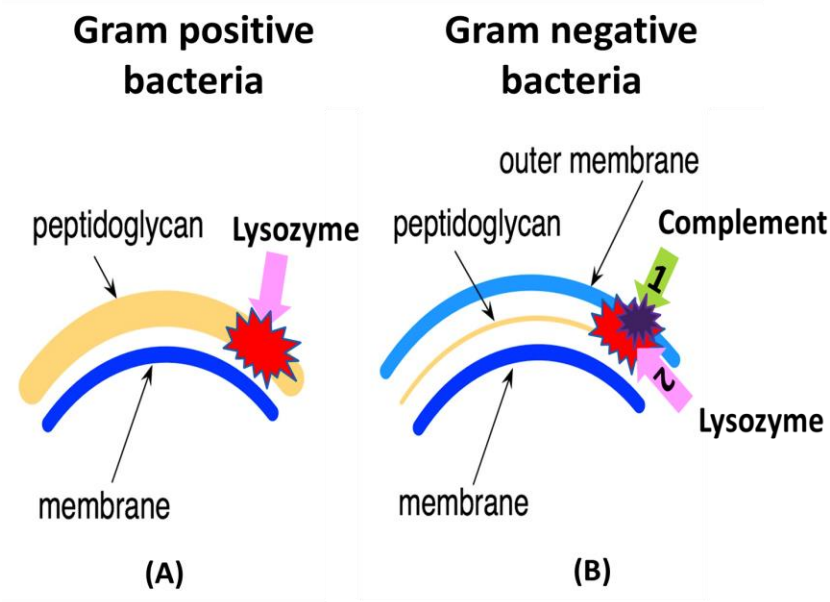


Figure 1.7. Lysozyme activity acts on the peptidoglycan layer of microbial cell walls and causes cell lysis. (A) Direct action of lysozyme to peptidoglycan layer of Gram positive bacteria. (B) Indirect action of lysozyme on the peptidoglycan, where complement first destroys the outer membrane of Gram negative bacteria to allow the action of lysozyme. (Modified from (Tort*, et al.* 2003; Saurabh and Sahoo 2008; Fabia 2013)

There are three types of lysozyme in the animal kingdom. Fish, including Japanese flounder, common carp, Atlantic salmon, Atlantic cod, Chinese perch, orange-spotted grouper and mandarin fish have goose lysozyme type-g which is also found in birds and mammals. Chicken or conventional type (c-type) lysozyme is present in most vertebrates, including some fish *e.g.* Japanese flounder, common carp, turbot, rainbow trout and zebrafish, in the phylum Chordata, and different classes of Arthropoda). The third type of lysozyme is the invertebrate type (i-type). In teleost fish, lysozyme is one of the most important components of the innate immune response. It has been found in mucus, ova, lymphoid tissue, plasma and the body fluids of fish, and its activity

depends on the age, sex and size of the fish, and external influences such as season, water temperature, pH, toxicants, infections and the degree of stress (Tort*, et al.* 2003; Saurabh and Sahoo 2008).

Natural antibodies also play an important role in the innate immune response and are linked to the adaptive immune response. They give broad protection against invading pathogens (Whyte 2007), and are involved in maintaining homeostasis, clearing apoptotic cells, and in tumour defence (Magnadóttir 2006). Variations in natural antibody specificity has been reported between different fish species (Whyte 2007), and Magnadóttir*, et al.* (1999; 2001) showed high levels of natural antibodies in cod with increasing age, environmental temperature and during infection. An interesting study by Sinyakov, *et al*. (2002) showed that the activity of natural antibodies of goldfish could divided into two groups; a group with high activity (which elicited protection against a virulent strain of *A. salmonicida*) and a group with low activity (resulting in 100 % morbidity in fish with these low levels after challenging them with *A. salmonicida).*

Transferrin is a protein that binds iron; iron is important to organisms for as an enzyme co-factor involved in DNA replication. Transferrin also has many other functions *e.g*. antimicrobial activity, acts as a growth factor, is involved in differentiation activities (myotrophic, embryo morphogenic, proliferative, mitogenic, neurotrophic, chemotactic and angiogenic activity) and in cytoprotection (Gomme*, et al.* 2005). This high-affinity iron-binding protein is produced by the host, so that the availability of iron to pathogenic bacteria is reduced (Ellis 2001), and the bacteria are therefore unable to replicate in the host tissue. However, pathogenic bacteria have

evolved several ways of overcoming this defence including the production of high-affinity iron sequestering mechanisms of their own (Ellis 1999).

Proteases are enzymes that catalyze the hydrolysis of proteins, and are classified into serine, cysteine, aspartic and metalloproteases, based on the chemical nature of their substrate (Hartley 1960; García-Carreño 1992). Fish mucus and/or muscle have been found to contain serine proteases, such as trypsin (Hjelmeland*, et al.* 1983), cysteine proteases (Cathepsin B and L) (Aranishi 1999), aspartic proteases (cathepsin D) (Cho, *et al.* 2002a) and metalloproteases (Bracho and Haard 1995). Protease can also found in other fish tissues such as serum, liver, kidney, spleen, ovary and intestine (Goetz and Garczynski 1997; Brooks*, et al.* 1997; Whang*, et al.* 2011). Proteases have been shown to cleave bacterial proteins by directly damaging the bacterium and indirectly activating complement, and through the production of immunoglobulins and antimicrobial peptides (Hartley 1960; Aranishi 1999; Cho*, et al.* 2002b).

Anti-proteases (protease inhibitors) have also been found in fish plasma/ serum and other body fluids (Hjelmeland*, et al.* 1983; Ciereszko*, et al.* 1998) to restrict the ability of bacteria to invade and grow in their host (Rao and Chakrabarti 2004). In teleost fish, the main types of anti-protease found are alpha-1-anti-protease (α1-protease inhibitor), alpha-1-anti-trypsin (α1-trypsin inhibitor) and alpha-2-macroglobulin (α2-macroglobulin) (Zuo and Woo 1997; Jones 2001).

1.4.1.2 Cellular immune response

Innate cellular defence responses of teleost fish involve phagocytic cells (*i.e*. neutrophils, monocyte/macrophages, dendritic cells, epithelial cells and non-specific

cytotoxic cells) (Graves*, et al.* 1984; Press*, et al.* 1994; Ganassin and Bols 1996; Frøystad*, et al.* 1998; Ernst and Stendahl 2006; Fischer*, et al.* 2006). Phagocytosis is initiated by the binding of pathogens to receptors on the surface of the phagocyte's surface, after which engulfment of the bacterium takes place as shown in Figure 1.8. The phagocyte has many receptors on its surface to which the pathogen can bind, such as opsonin receptors, scavenger receptor and toll-like receptors (Sompayrac 2008; Delves*, et al.* 2011). The killing mechanism of the phagocyte differs depending on pathogen; killing can occur within the phagocyte (intracellular killing) or outside of the phagocyte (extracellular killing) (Hampton*, et al.* 1998; Mak and Saunders 2005; Delves*, et al.* 2011).

There are two types of oxygen-dependent intracellular killing (Fang 2004). The first type is the oxygen-dependent production of a superoxide anion, which is bactericidal. The superoxide is converted to hydrogen peroxide and singlet oxygen by superoxide dismutase, from which hydroxyl radicals result and assist in the killing process (Mak and Saunders 2005). The second type uses myeloperoxidase found in neutrophil granules. The granules fuse with the phagosome and myeloperoxidase is released into the phagolysosome. The enzyme uses hydrogen peroxide and chlorine to create hypochlorite (toxic to bacteria)(Hampton*, et al.* 1998).

There are four types of oxygen independent intracellular killing. The first uses electrically charged proteins that damage the bacterial membrane. The second type uses lysozyme to degrade bacterial cell walls. The third type uses lactoferrins to remove essential iron from bacteria and the fourth type uses proteases and hydrolytic enzymes to digest the proteins of destroyed bacteria (Delves*, et al.* 2011)

The extracellular killing of phagocyte occurs through the production of nitric oxide (NO) stimulated by interferon gamma produced by $CD4^+$ T cell, $CD8^+$ T cells, natural killer cells, B cells, natural killer T cells, monocytes, macrophages, or dendritic cells) (Schroder*, et al.* 2004). Nitric oxide is released from the activated macrophage to kill the bacteria (Mak and Saunders 2005; Delves*, et al.* 2011).

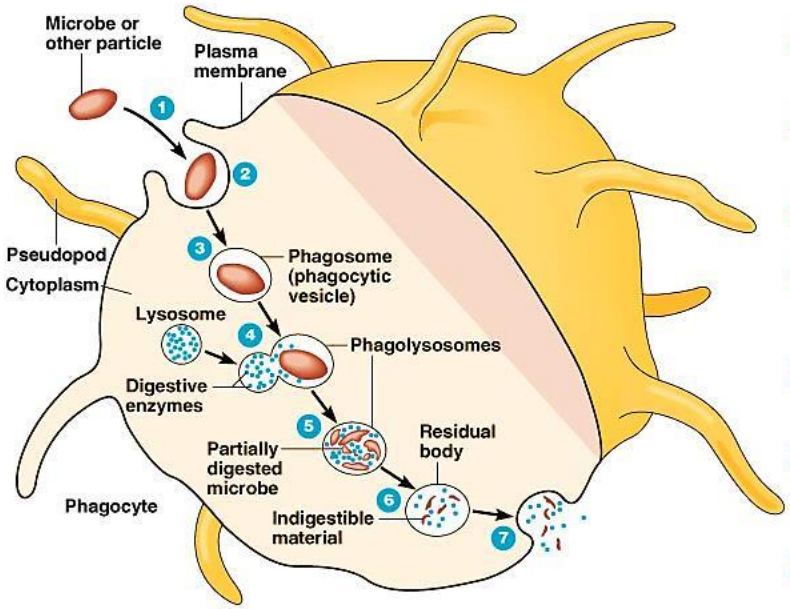


Figure 1.8. Phagocytosis is mediated by macrophages and polymorphonuclear leucocytes and involves the ingestion and digestion of microorganisms, insoluble particles, damaged or dead host cells, cell debris and activated clotting factors. The stages of phagocytosis include (1) Chemotaxis and adherence of microbe to phagocyte, (2) Ingestion of microbe by phagocyte, (3) Formation of a phagosome, (4) Fusion of the phagosome with a lysosome to form a phagolysosome, (5) Digestion of ingested microbe by enzymes, (6) Formation of residual body containing indigestible material and (7) Discharge of waste materials. From Cotter (2006)

1.4.2 Adaptive immune response

The lymphoid system (home to the adaptive response) of teleost fish is different from mammals, with fish lacking bone marrow, lymph nodes and Payer's patches. Fish lymphoid tissues include the thymus, anterior kidney, spleen, gut associated lymphoid tissue and mucosa-associated lymphoid tissue (Morrison and Nowak 2002).

The adaptive immune system is specific, selective, has a memory component and either repels a second invasion or quickly eliminates the recurrent invader by mobilizing a faster and more efficient immune response (Cooper and Alder 2006). It is divided in to cellular and humoral components as shown in Figure 1.9. The humoral component of the adaptive immune system involves antibodies, or immunoglobulins (Ig), while the cellular component, or cell-mediated immunity, in composed of T-cells, B-cells and antigen presenting cells (APCs) (Miller*, et al.* 1998).

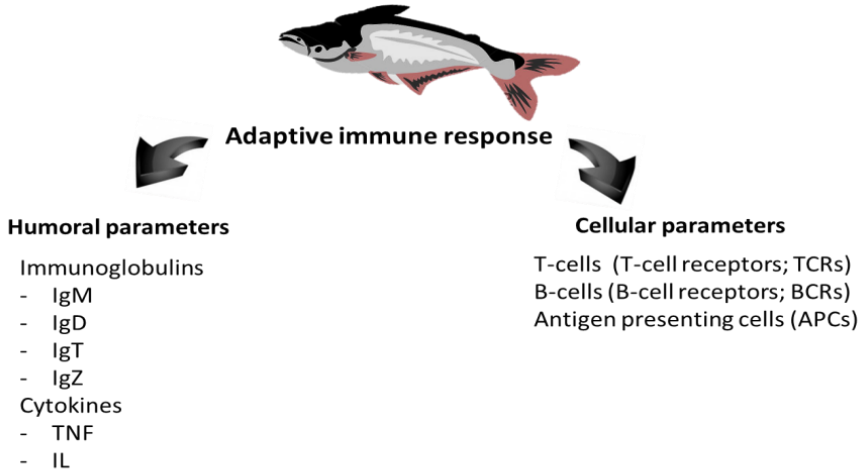


Figure 1.9. Summary of the components of the fish's adaptive immune response (Modified from Miller, *et al.* (1998) and Watts, *et al.* (2001).

The general components of the adaptive immune system are B-cell and T-cell receptors (BCR and TCR), recombination activator genes (RAG1 and RAG2), major

histocompatibility complex (MHC I and II), and specialized primary and secondary lymphoid tissues (Flajnik and Du Pasquier 2004; Watts*, et al.* 2001). The T-cells and B-cells circulate throughout the body in search of antigens and use their cell-surface receptors to recognize specific antigenic configurations. Binding of antigen triggers clonal amplification and cellular differentiation. The B-cells then produce antibodies with the same antigen binding specificity (Cooper and Alder 2006). The TCRs recognize peptide fragments of antigens presented by other cells within cell-surface molecules encoded by the major histocompatibility complex (MHC) class I and class II genes. The BCRs are Igs that recognise the intact or macromolecular complexes on the antigen. Cytokines orchestrate the events of the immune response by having important regulatory roles within the response. Many fish cytokines have been identified based on their functional similarity to mammalian cytokine activities (Manning and Nakanishi 1996), and have been grouped based on general names such as lymphokines, monokines, chemokines, interleukins, interferons, tumour necrosis factors and colony stimulating factors, on functions such as pro- and anti-inflammatory or innate and adaptive immunity-related, on structure such as short and long chain cytokines, and on receptors used (immunoglobulin superfamily), hematopoietic growth factor (type 1-family), interferon family (type 2-family), tumour necrosis factor (type 3-family) and chemokine receptors (7 transmembrane helix family) (Aoki*, et al.* 2008). Interferons (IFNs) are secreted proteins, which induce vertebrate cells to produce an anti-viral response. There are three families of IFNs (type I IFN, type II IFN and IFN-λ). Type I IFNs include the classical IFN-α and IFN-β and are induced by the presence of virus within cells, while type II IFN *i.e*. IFN-γ, is produced by natural killer cells and T lymphocytes in

response to interleukin-12 (IL-12), IL-18, mitogens or antigens. The IFNs of fish show structural and functional properties similar to mammalian type I IFNs, have been cloned from Atlantic salmon, channel catfish, pufferfish, and zebrafish (Whyte 2007; Robertsen 2006). Interleukins in teleosts, IL-1 (IL-1α, IL-1β and IL-1 receptor (IL-1ra)), IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12, IL-18, IL-21 and IL-25 have been identified in rainbow trout, carp, catfish, flounder, tilapia, salmon and fugu (Whyte 2007; Aoki, et al. 2008; Randelli, et al. 2008; Wang and Secombes 2013).

Tumor necrosis factor-α (TNF- α)-like gene has been identified in number of fish species such as Japanese flounder, rainbow trout, brook trout, gilthead sea bream, carp, channel catfish and zebrafish. Chemokines are a superfamily of cytokines. They are produced by a variety of different cell types and their functions include, chemo-attractant properties stimulating the recruitment, activation, and adhesion of cells to sites of infection or injury. Chemokines have been identified in a wide variety of fish species, with Interleukin-8 (IL-8) the first chemokine to be identified. This chemokine stimulates the activation and migration of neutrophils, T lymphocytes and basophils (including their degranulation).

1.4.2.1 B-cells and antibodies

The B cells are a group of lymphocytes involved in adaptive immunity. The major function of B-cells is the production of antibodies against antigens; they also perform the role of antigen-presenting cells (APCs) and evolve into memory B-cells. Each B-cell has a unique BCR on its surface, which is specific for one particular antigen. The B-cell is activated by a signal from T-helper cells and develops into two types of cells (plasma B cells and memory B cells) (Figure 1.10). Activated B lymphocytes differentiate into

antibody-producing plasma cells. The plasma cells are characterized by the large amounts of intracytoplasmic, immunoglobulins that are rapidly synthesized and secreted (Ellis 1977). Memory B cells are formed from activated B cells and are specific for the same antigen encountered during the primary immune response (Mak and Saunders 2005). The B-cells also possess additional immune functions, including the production of cytokines and the ability to function as a secondary antigen presenting cells (APCs) (Mizoguchi and Bhan 2006).

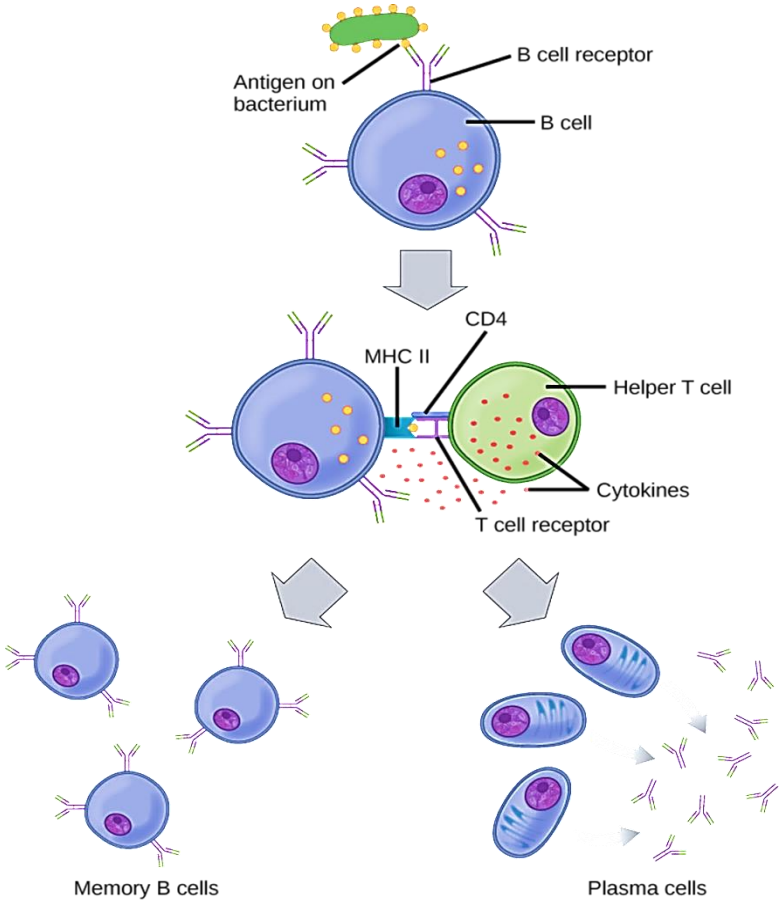


Figure 1.10. B-cell activation in mammals by antigen binding to the B–cell receptor. The B cell internalizes the antigen and presents it with MHC class II antigens to a helper T cell which recognizes the MHC class II antigen complex and activates the B cell which differentiate into memory B cells and plasma cells (Bear and Rintoul 2013a).

Immunoglobulins can be found in blood (plasma and serum), skin, gut, gill mucus and bile of teleost fish (Morrison and Nowak 2002). Immunoglobulin (Ig) morphology is Y-shaped and each tip contains a paratroop, which is specific to the epitope of the antigen. An antibody is composed of two identical L-chain and H-chains, and the specific genes are found in the Variable (V) region and the constant (C) regions. The V-region in the H-chain has three segments- variable (V), diversity (D) and joining (J) (VDJ recombination), and this functions to produce a unique variable domain in the immunoglobulin (Figure 1.11.). The V-region in the L-chain has two segments V and J.

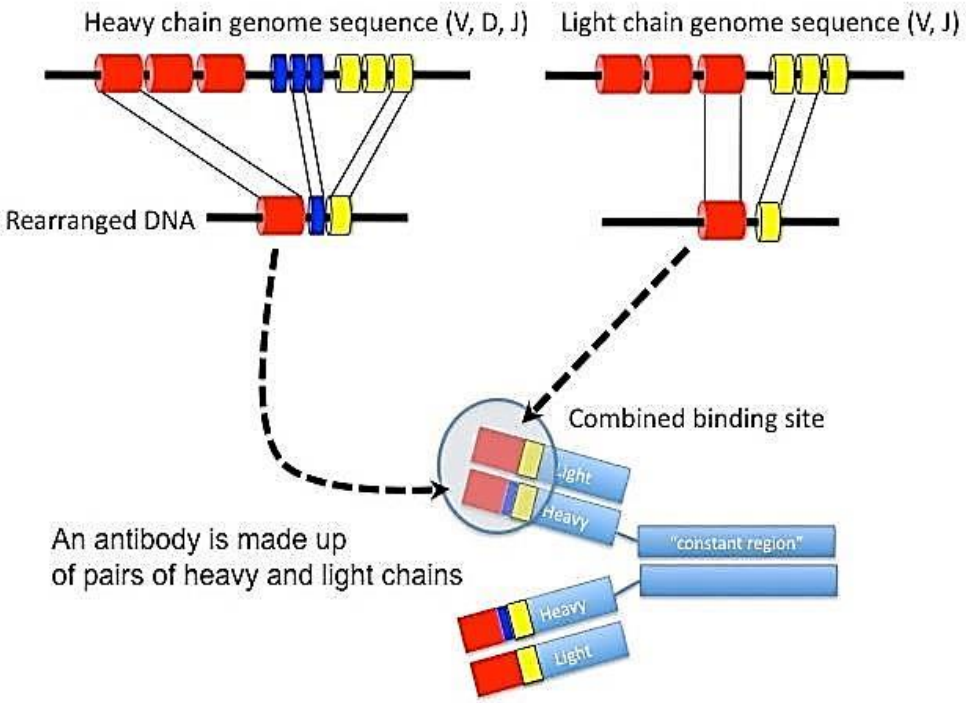


Figure 1.11. Immunoglobulin structure. The antigen binding domain is composed of three separate segments, the V (variable) D (diversity) and J (joining) segments. Each antibody is made up of two copies each of two chains of different sizes, called "heavy" and "light", and each chain uses its own set of gene segments (VDJ for heavy, VJ for light) (Ward 2011).

Antibodies can be categorized into different isotypes based on the structure of their heavy chain. In mammals, there are five different isotypes present (IgA, IgD, IgE, IgG and IgM (Sompayrac 2008; Pier*, et al.* 2004; Geisberger*, et al.* 2006). Fish do not have IgG, IgA or IgE, and IgM is the main immunoglobulin in fish. Phylogenically, it is the first Ig to appear in lower vertebrates. In teleost fish IgM, IgD, IgZ or IgT and IgM-IgZ chimera have been reported. In mammals, IgM is pentameric, however in teleost fish, IgM is tetrameric in structure, and IgD has been found in catfish, Atlantic cod and Atlantic salmon, while IgZ, IgT and IgM-IgZ chimera have been identified from common carp, fugu, stickleback (*Gasterosteus aculeatus*), catfish, medaka (*Oyzias latipes*), rainbow trout and zebrafish (Stenvik and Jørgensen 2000; Savan*, et al.* 2005; Randelli*, et al.* 2008; Tian*, et al.* 2009; Gambón-Deza*, et al.* 2010; Castro*, et al.* 2013). Some of the functional activities of immunoglobulins are highlighted below in Figure 1.12.

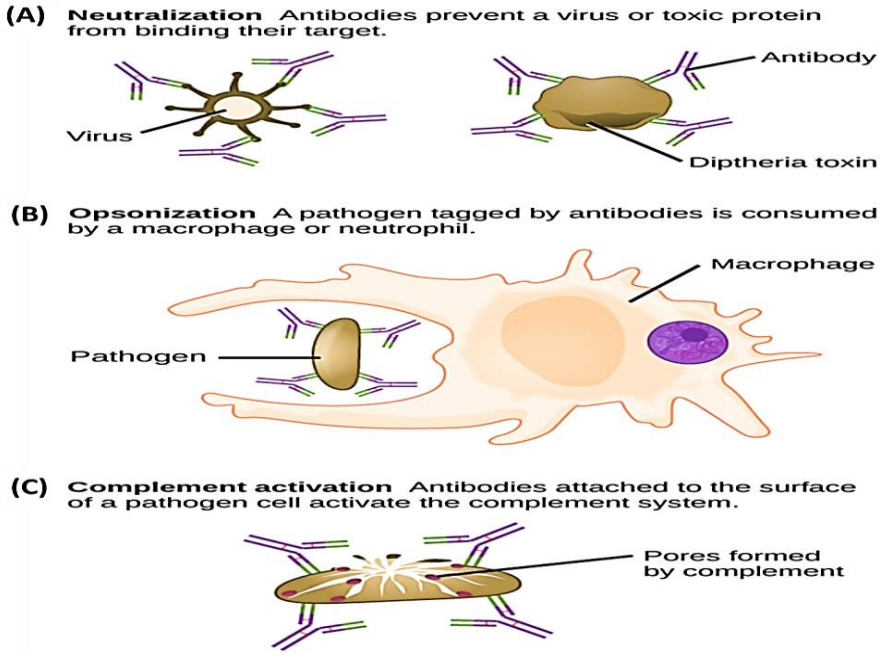


Figure 1.12(A-C). The function of immunoglobulins (A) preventing the antigen from binding its target by neutralising the antigen, (B) tagging a pathogen for destruction by macrophages or neutrophils, or (C) activating complement (Bear and Rintoul, 2013a)

1.4.2.2 T-cells and cytokine

The T-cells are a type of lymphocyte acting in cell mediated immunity. The T-lymphocytes are classified into helper T-cells (Th cells), cytotoxic T-cells (CTLs), memory T-cells, regulatory T-cells (T_{REG} cells), natural killer T-cells (NKT cells) and mucosal associated invariant T-cells (MAITs) (Mak and Saunders 2005). In mammals memory $CD4^+$ or CD8 T cells represent a small fraction of antigen-specific T cells present in the circulation

The Th cells are involved in activation of B-cell proliferation and differentiation, known as T-cell-dependent B-cell activation (Noelle and Snow 1992). The Th cells express CD4 glycoprotein on their surface and are referred to as $CD4^+$ T-cells. The Th cells are activated by the presentation of peptide antigens to the TCR by antigen presenting cells (APCs) linked to a MHC class II molecules, as shown in Figure 1.13(A). The activated Th cells then rapidly divide and release cytokines, which regulate or promote the immune response. The Th cells can differentiate into Th1 or Th2 cells which secrete different cytokines profiles. The Th1 cells mediate cellular immunity against intracellular bacteria and viruses, whereas the Th2 cells promote humoral immunity against extracellular pathogens. The effector functions of Th1 cells are exerted in part by production of IFN-γ, IL-2 and lymphotoxin-α (LTα) and those of Th2 cells by IL-4, IL-5, IL-10 and IL-13. The Th cells can also activate a CTL response, which destroy infected cells and tumor cells, and are known as $CD8^+$ T cells because of the CD8 glycoprotein they express on their surface membrane. These cells recognize their target by binding to antigen associated with MHC class I, which is present on the surface of all nucleated cells as shown in Figure 1.13(B).

There are three mechanisms of target cell destruction by $CD8^+$ CTLs, *i.e.* the granule exocytosis pathway, the Fas pathway and the release of cytolytic cytokines (TNF, LTα and IFN-γ). In the granule exocytosis pathway, granules containing granzymes and perforin are released in close proximity to the target cell membrane. Granzymes and perforin enter the cell by an as yet uncharacterised mechanism and are captured in endolysosomal vesicles. Then apoptosis is activated by either a mitochondrial pathway or an unknown caspase-independent mechanism. Granzymes can also access the nucleus to induce DNA fragmentation directly. In the Fas pathway, Fas ligand (FasL), expressed on the surface of the activated CTL, binds to Fas expressed on the target cell, inducing caspase-8 activation and apoptosis mediated by either the mitochondrial pathway or direct caspase-3 cleavage (Mak and Saunders 2005).

Regulatory T-cells (T_{REG} cells), also known as suppressor T cells, are involved in the secretion of soluble factors including IL-10, TGF-β, fibrinogen-like protein-2 (FLG-2), granzyme and adenosine. There are two major classes of $CD4^+$ T_{REG} cells, natural occurring T_{REG} cells and adaptive T_{REG} cells. Naturally occurring T_{REG} cells ($CD4^+$, $CD25^+$, $FOXP3^+$ T_{REG} cells) are thymus-derived. Adaptive T_{REG} cells or inducible T_{REG} cells (Tr1 cells secrete IL-10; Th3 cells secrete TGF-β and IL-10 and $FoxP3^+$ T_{REG} cells) (Peterson 2012).

Much research has been performed on T-cell activity in teleost fish. CTLs act to eliminate virus and are important for anti-viral adaptive immunity in teleost fish; the mRNA expression of CTL molecule such as CD8, TCR and MHC class I has been assessed in many fish species (Somamoto*, et al.* 2013). One such example is by Somamoto *et. al*. (2002), who found that cytotoxic cells from crucian carp were activated by crucian carp

haematopoietic necrosis virus and showed that virus-specific cytotoxic cells have a significant role in controlling the viral infection.

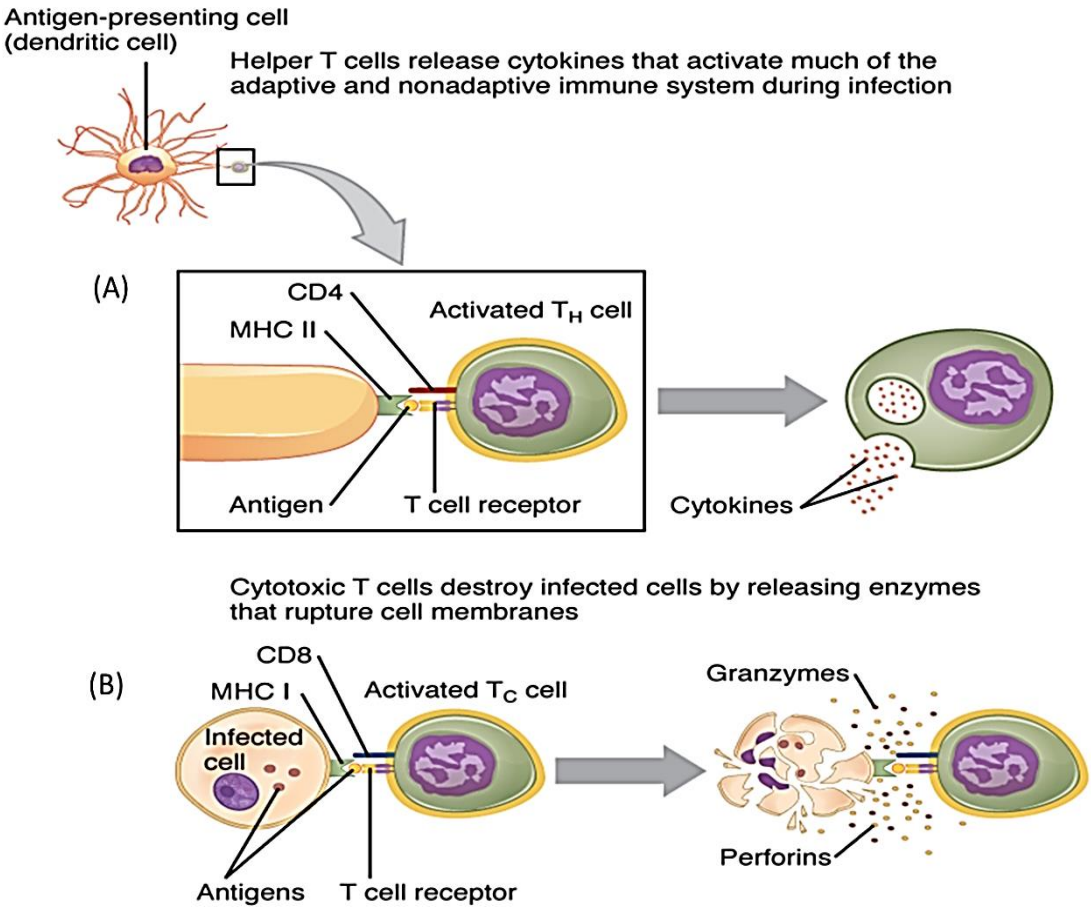


Figure 1.13(A-B). T–cell activation by antigen presenting cells. (A) CD4 is associated with helper and regulatory T cells. An extracellular pathogen is presented in association with the CD 4 molecule with antigen displayed in the binding deft of a MHC class II molecule. (B) CD8 is associated with cytotoxic T cells. An intracellular pathogen is presented by MHC class I molecules, which interact by CD8 (Bear and Rintoul 2013b)

1.4.2.3 MHC (response to bacteria and viruses)

Antigen-presenting proteins, also known as MHC molecules, are cell surface molecules and are highly polymorphic heterodimeric glycoproteins (Klein*, et al.* 1997). Antigen processing is a mechanism that enzymatically cleaves the antigen into smaller pieces.

The antigen fragments are then brought to the cell's surface and are associated with a specialized type of antigen-presenting protein. In humans, the MHC genes are divided into three subgroups, class I, class II and class III. MHC class I includes peptide-binding proteins, which select short sequences of amino acids for antigen presentation, one chain (α) whose ligands are the CD8 receptor borne by cytotoxic T-cells, and inhibitory receptors borne by NK cells. MHC class II is compound extracellular proteins of peptide-bindings protein and proteins assisting antigen loading onto MHC class II peptide-binding proteins, two chains (α and β), whose ligands are the CD4 receptors borne by helper T-cells. MHC class III are the other immune proteins outside antigen processing and presentation, such as components of the complement cascade, the cytokines of immune signalling and heat shock proteins buffering cells from stresses (Mak and Saunders 2005).

Adaptive cell-mediated cytotoxicity (CMC) requires key molecules expressed on cytotoxic T lymphocytes (CTLs) and target cells. The CTLs kill host cells harbouring intracellular pathogens by binding of their T cell receptor (TCR) and its co-receptor CD8 to a complex of MHC class I and bound peptide on the infected host cell. Alternatively, extracellular antigens are taken up by professional antigen presenting cells such as macrophages, dendritic cells and B cells to process those antigens and present the resulting peptides in association with MHC class II to $CD4^+$ T helper cells.

In teleost fish, the first MHC molecule was identified in 1990 using PCR with degenerate primers (Grimholt and Lie 1998). In bony fish MHC class I and class II loci form separate linkage groups (Sato*, et al.* 2000). The activation of MHC depends on the pathogen *i.e.* whether it is an intracellular or extracellular. For example, MHC class I

was found to be stimulated following infection with the intracellular bacterium *E. ictaluri* in blue catfish (Peatman*, et al.* 2008). Moreover, virus infection with infectious salmon anemia virus (ISAV) in Atlantic salmon induced expression MHC class I pathway genes, but not MHC II-β. It was also found that acute phase infections by infectious hematopoietic necrosis virus (IHNV) induced MHC class Ia in rainbow trout (Hansen and La Patra 2002). On the other hand, Morrison, *et al*. (2006) found *Neoparamoeba* sp. associated with amoebic gill disease (AGD) induced MHC class II-β expression in Atlantic salmon, indicative of a Th_2 type response associated with an antibody response. Extracellular bacteria (*e.g*. *A. hydrophila*) have also been shown to stimulate MHC class II-α production in purse red common carp (*Cyprinus carpio* Linnaeus) (Liu*, et al.* 2013). During recent years, genes encoding MHC class I and II, TCR and its co-receptors CD8 and CD4 have been cloned in several fish species and antibodies have been developed to study protein expression in morphological and functional contexts (Fischer*, et al.* 2006).

1.4.3 Cooperation between innate and adaptive immune responses

The initial immune response against the invading pathogens is through innate immunity, and this provides an early defence until the lymphocytes of the adaptive immune response are activated. The advantage of innate immunity is that it acts as the first line of defence allowing time for the adaptive immune response to come into play. With some infections, the innate immune response can stop the infection before the adaptive immune has time to come into play (Mak and Saunders 2005). Pro-inflammatory cytokines induce the activation of the adaptive immune response, *e.g*. TNFα and IL1β produced by neutrophils and macrophages, to induce the migration of

phagocytic cells to the site of infection, which in turn present antigen to cells of the adaptive immune response (Secombes*, et al.* 2001).

The adaptive immune response is a more complicated system than the innate immune response, and can trigger many of the same effector cells employed by the innate system to remove pathogens. The cellular response of the innate immune system recognizes certain conserved antigens *i.e* PAMPs on a wide variety of pathogens and having recognised the pathogen then lyses it. However, this action is limited to a number of pathways. The development of the adaptive response is though the recognition of a pathogen, and which then results in the specific proliferation of lymphocytes directed against that particular pathogen. The activation of lymphocytes induces differentiation into other types of lymphocytes, such as effector lymphocytes for destroying the pathogen, or the secretion of cytokines that stimulate the innate response (Mak and Saunders 2005). A summary of cooperation between innate and adaptive immunity is shown in Figure 1.14.

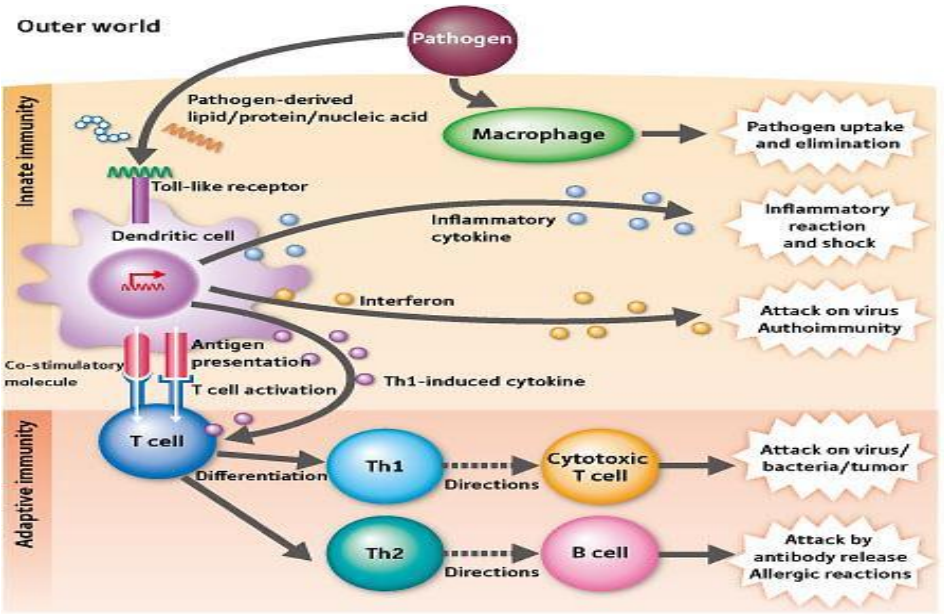


Figure 1.14. Cooperation between innate and adaptive immune responses (From Kaisho, 2007)

1.5 Aims of the study

Despite *Pangasianodon hypophthalmus* being an economically important fish species in aquaculture there is little information available on the basic immune response of this species. Such information is essential if effective methods are to be developed to prevent and control diseases in the farming systems for this species.

Thus, the specific objectives of this thesis were to:-

1. Characterize IgM from different species within the family Pangasiidae with respect to molecular weight (whole molecule and H and L chains), and to determine if monoclonal antibodies developed against *Pangasianodon hypophthalmus* IgM cross reacted with IgM from the other Pangasiidae species.
2. To develop and standardise functional assays to evaluate both innate and adaptive immune responses of *P. hypophthalmus,* and to use these assays to compare the response of *P. hypophthalmus* to live and killed *A. hydrophila.*
3. To evaluate the effects of feeding different levels of fungal derived β-glucan on the immune response of *P. hypophthalmus* and compare this response to that obtained with commercial β-glucan derived from yeast. The effect of feeding these immunostimulants on the disease resistance of *P. hypophthalmus* to *E. ictaluri* was also examined. Ultimately, the purpose of this study was to formulate and test β-glucan-containing diets for use by the Pangasius aquaculture industry.
4. To identify immune genes and develop primers for immune gene expression in *P. hypophthalmus*, to compare the expression of immune genes in *P. hypophthalmus* fed with the different β-glucan supplemented diets and examine the gene

expression profiles in relation to immunostimulation with the β-glucan and their disease resistance against *Edwardsiella ictaluri.*

Chapter 2 Characterisation of immunoglobulin M (Ig M) from fish within the family Pangasiidae

2.1 Introduction

Fish within the family Pangasiidae are important for both commercial fisheries and aquaculture in Southeast Asia, with *Pangasius bocourti*, *P. gigas*, *P. hypophthalmus*, *P. larnaudii* and *P. sanitwongsei* having particular economic potential for aquaculture (Kwantong and Bart 2003; Sriphairoj*, et al.* 2010). Of these *Pangasianodon hypophthalmus,* in particular, is the most important species for aquaculture, with its culture developing rapidly within the Mekong riparian countries. It is considered a valued food fish of economic significance, especially in Vietnam (Nguyen 2009). Vietnam is currently one of the most successful primary food production sectors globally (Phuong and Oanh 2010), however, sever disease outbreaks as a result of farming pangasius in intensive culture systems means that a deeper knowledge of fish health and immunity is required for the development of methods to control these disease outbreaks. For example basic knowledge of their antibody response, a component of their humoral immune response, would be useful for vaccine development and to examine the host's response to pathogens. Little is known about this response in pangasius or the structure and activity of their immunoglobulins.

The immunoglobulin molecule itself is a heterodimer composed of two immunoglobulin heavy (H) chains and two light (L) chains. There are five different isotypes present in mammals based on the composition of the H chain (*i.e.* IgM (μ), IgD (δ), IgG (ɣ), IgA (α) and IgE (Ɛ)) (Zaccone*, et al.* 2008). The main immunoglobulin in fish is IgM (Wilson and Warr 1992). Dooley and Flajnik (2006) found three isotypes in cartilaginous fish *i.e* IgM, IgNAR, IgR in Little skate (*Raja erinacea*) and Clearnose skate (*Raja eglanteria*), and IgW in Nurse shark (*Ginglymostoma cirratum*), Sandbar shark

(*Carcharinus plumbeus*) and Horn shark (*Heterodontus francisci*). In teleost fish, various isotypes have been reported, namely IgM in Atlantic salmon, halibut, haddock and cod (Magnadóttir 1998), Indian major carp (Bag*, et al.* 2009) and African catfish (Rathore*, et al.* 2006), IgD in Atlantic salmon (Hordvik*, et al.* 1999) and rainbow trout (Ramirez-Gomez*, et al.* 2012), IgT in rainbow trout (Hansen*, et al.* 2005) and Atlantic salmon (Tadiso*, et al.* 2011) and IgZ in zebrafish (Danilova*, et al.* 2005).

Immunoglobulin M in higher vertebrates and cartilaginous fish is pentameric in structure (Kunihiko*, et al.* 1984), whereas teleost fish have tetrameric IgM (Acton, Weinheimer, Hall*, et al.* 1971; Magnadóttir 1998). In teleost fish, each monomer of IgM contains two heavy (H) chains and two light (L) chains with molecular weights ranging between 66-77 kDa and 23-26 kDa, respectively (Muiswinkel and Woo 1995), depending on the fish species (Table 2.1). The molecular weight of the whole IgM molecule of teleost fish is reported to range from 600-870 kDa by (Jurd 1985; Pilström and Bengtén 1996) and 608-900 kDa by (Muiswinkel and Woo 1995), again depending on the fish species being examined (Table 2.1)

A variety of techniques have been used to purify and characterize fish IgM. Common methods for purification include affinity column chromatography (Magnadóttir 1998; Håvarstein*, et al.* 1988; Palenzuela*, et al.* 1996; Morrison and Nowak 2001; Rathore*, et al.* 2006; Suresh Babu*, et al.* 2008; Bag*, et al.* 2009; Choudhury and Prasad 2011; Huong Giang*, et al.* 2012) and partial purification using ammonium sulphate precipitation (Rombout*, et al.* 1993; Magnadóttir 1998; Uchida*, et al.* 2000; Huong Giang*, et al.* 2012).

Table 2.1. Comparison of the molecular weight of Immunoglobulin M heavy and light chains and whole IgM molecule for a variety of teleost fish

Species	Immunoglobulin M (kDa)			References
	Heavy chain	Light chain	Total MW	
African catfish (*Clarias gariepinus*)	74.8	27.2	840	(Rathore*, et al.* 2006)
Asian sea bass (*Lates calcarifer*)	83	27	896	(Choudhury and Prasad 2011)
Atlantic cod (*Gadus morhua* L.)	81	27.5	-	(Pilström and Petersson 1991)
	-	-	700-832	(Magnadóttir 1998)
Atlantic salmon (*Salmo salar*)	72	27	800	(Håvarstein*, et al.* 1988)
	-	-	850-870	(Magnadóttir 1998)
Channel catfish (*Ictalurus punctatus*)	70	23	-	(Lobb and Clem 1983)
Common carp (*Cyprinus carpio* L.)	70	25	-	(Rombout*, et al.* 1993)
European sea bass (*Dicentrarchus labrax* L.)	78	27.5, 28.5	855	(Palenzuela*, et al.* 1996)
	-	-	883	(Bourmaud*, et al.* 1995)
Gilthead sea bream (*Sparus aurata*)	70	25	-	(Navarro*, et al.* 1993)
			830	(Palenzuela*, et al.* 1996)
Halibut (*Hippoglossus hippoglossus*)	72	26.5	830-933	(Magnadóttir 1998)
Haddock (*Melanogrammus aeglefinus*)	72	26.5	700-840	(Magnadóttir 1998)
Indian catfish/Asiatic catfish (*Clarias batrachus*)			863	(Swain*, et al.* 2004)
Indian major carp or rohu (*Labeo rohita* (Ham.))	85	23	850	(Suresh Babu*, et al.* 2008)
Japanese eel (*Anguilla japonica*)	72	25	790	(Uchida*, et al.* 2000)
Snapper (*Pagrus auratus*)	67.7-71.8	29-30.2	766	(Morrison and Nowak 2001)
Striped catfish (*Pangasianodon hypophthalmus*)	72	24,26 and/or 28-29	900	(Huong Giang*, et al.* 2012)
	70.1	26	798	(Sudhagar*, et al.* 2013)
Strip trumpeter (*Latris lineata*)	86	28	926	(Covello*, et al.* 2009)
Tilapia (*Oreochromis niloticus*)	90	30	900	(Rajavarthini*, et al.* 2000)
Turbot (*Scophthalmus maximus*)	78	27	820	(Estevez*, et al.* 1993)
White sturgeon (*Acipenser transmontanus*)	73	27-30	870	(Adkison*, et al.* 1996)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has been widely employed to determine the molecular weight of the H chain and L chains and the molecular weight of whole IgM using 10-14 % polyacrylamide gels under reducing condition, or 3-5 % polyacrylamide, sometimes containing 0.5-0.6% agarose, under non-reducing conditions (Håvarstein*, et al.* 1988; Navarro*, et al.* 1993; Magnadóttir 1998; Rajavarthini*, et al.* 2000; Uchida*, et al.* 2000; Morrison and Nowak 2001; Rathore*, et al.* 2006; Bag*, et al.* 2009; Covello*, et al.* 2009). Gradient gels have also been used under both reducing (10-15%) and non-reducing (2-16%) conditions (*i.e* native gels) to determine the molecular weight of H/L chains (Pilström and Petersson 1991; Bourmaud*, et al.* 1995; Uchida*, et al.* 2000; Choudhury and Prasad 2011) and whole IgM (Morrison and Nowak 2001; Håvarstein*, et al.* 1988; Palenzuela*, et al.* 1996; Choudhury and Prasad 2011; Suresh Babu*, et al.* 2008), respectively. Two-dimensional gel electrophoresis (2-DE) has also been used to determine the molecular weight of Olive flounder (*Paralichthys olivaceus*) IgM (Shin*, et al.* 2007).

Western blotting of IgM following the SDS-PAGE process, has been used to help characterise fish IgM for a variety of fish species using both monoclonal and polyclonal antibodies raised against the IgM being investigated (Pilström and Petersson 1991; Navarro*, et al.* 1993; Rombout*, et al.* 1993; Magnadóttir 1998; Uchida*, et al.* 2000; Bag*, et al.* 2009; Choudhury and Prasad 2011).

Determining the molecular weight of the whole IgM molecule accurately can be difficult using native gels and therefore a variety of alternative approaches have been reported. For example gel filtration chromatography (Acton, Weinheimer, Dupree*, et al.* 1971; Håvarstein*, et al.* 1988; Bourmaud*, et al.* 1995; Palenzuela*, et al.* 1996;

Magnadóttir 1998; Rajavarthini*, et al.* 2000; Morrison and Nowak 2001; Covello*, et al.* 2009; Huong Giang*, et al.* 2012) and sedimentation velocity analytical ultracentrifugation (SV) (Acton*, et al.* 1971; Acton*, et al.* 1971; Hall*, et al.* 1973; Kunihiko*, et al.* 1984) have been used.

Analytical ultracentrifugation (AUC) is used for the quantitative analysis of macromolecules in solution and is used to study bio-macromolecules in a wide range of solvents and over a wide range of solute concentrations. There are two types of AUC (Cole*, et al.* 2008), sedimentation velocity (SV) and sedimentation equilibrium (SE). The former is performed at speeds high enough for the centrifugation of solute away from the centre of rotation to be monitored as the rate of movement of a sedimentation boundary (Harding and Winzor 2001), and is used to determine the sedimentation coefficient - a measure of molecular shape, mass and hydration. The SE generates equilibrium concentration gradients at lower centrifugal fields, and these are analysed to determine molecular mass, assembly stoichiometry and association constants (Cole*, et al.* 2008).

The aim of the present study was to purify and fully characterize the IgM molecule from the family Pangasiidae with respect to molecular weight (whole molecule and H and L chains), and to determine if anti-*P. hypophthalmus* IgM monoclonal antibodies cross reacted with a variety of other Pangasiidae species IgM.

2.2 Materials and methods

2.2.1 Fish and serum collection

Four serum samples were collected from *P. hypophthalmus* obtained from a local fish farm in Supanburi province, Thailand and a local fish farm in Can Tho province, Vietnam, respectively, weighing 73 ±10 g and 55 ±6 g respectively. Serum from *P. borcoti* (105 ±29 g) was also collected in Can Tho Province, Vietnam. The other fish species were collected from three local fish farms in Nakornsawan, Supanburi and Rachaburi Provinces in Thailand. Four serum samples were collected per species for *P. gigas* (52 ±6 g), *P. hypophthalmus cross breed* (73 ±10 g), *P. larnaudii* (30 ±10 g), *P. sanitwongsei* (42 ±8 g), *Hamibragus wyckioides* (58 ±6 g), *Hamibragus filamentus* (65 ±8 g), *Clarias bactracus* (383 ±115 g), *Clarias Macrocephalus* (21 ±3 g) and *Cyprinus carpio* (51 ±22 g).

Fish were anesthetized with 100 mg/L benzocaine and blood was collected from the caudal vein. After clotting, the blood was centrifuged at 3,000 xg for 5 min and the serum was stored at -80^{o}C until used.

2.2.2 Immunoglobulin purification

Immunoglobulins were purified from the serum samples by sodium sulphate (Na_2SO_4) precipitation and/or affinity column chromatography.

2.2.2.1 Sodium sulphate precipitation

Three different percentages of Na_2SO_4 (BDH Laboratory supplies, England), 14%, 16% and 20%, were used to precipitate the serum of *P. hypophthalmus.* The serum samples were defrosted and warmed to 25^{o}C for 5 min (in a water bath) before adding 14%, 16% or 20% (w/v) Na_2SO_4 to the sample. These were incubated with Na_2SO_4 at 25^{o}C

for 5 min, then centrifuged at 17,000 xg for 15 min. The supernatant was removed and the precipitate was washed twice with 14%, 16% or 20% of Na_2SO_4, warmed to 25^{o}C and centrifuged, as described above. The precipitate was finally dissolved in phosphate buffer saline (PBS; 0.02M Phosphate, 0.15M NaCl, pH 7.2) to its original volume. The serum samples from the other species were precipitated with 14% Na_2SO_4. Samples were stored at -20^{o}C until required.

2.2.2.2 Affinity column chromatography purification

A HiTrap affinity column (GE Healthcare, UK) was used to purify IgM from the serum of *P. hypophthalmus, P. hypophthalmus cross breed*, *P. larnaudii*, *P. sanitwongsei*, *P. borcoti, Hamibragus filamentus, Hamibragus wyckioides, Clarias bactracus*, *Clarias Macrocephalus* and *Cyprinus carpio*. The procedure was conducted according to the manufacturer's instructions. Binding buffer (20 mM sodium phosphate; BDH Laboratory Supplies, 0.8 M ammonium sulphate, pH 7.5), elution buffer (20 mM sodium phosphate, pH 7.5) and regeneration buffer (20mM sodium phosphate, 30% isopropanol, pH 7.5) were pumped through the column to equilibrate the system. The serum samples were diluted 1:50 in binding buffer and passed through a 0.22 µm filter (Fisherbrand Inc., Ireland) to remove particulates. The sample was loaded on to the column followed by the binding buffer to increase the specificity of binding and the IgM was eluted from the column into fractions using the elution buffer (20mM sodium phosphate, pH 7.5).The sample fractions containing the IgM were then pooled and placed in a 10 kDa centrifuge concentrating tube (Amicon, Millipore Inc., Ireland) and washed 3 times with elution buffer by centrifuging at 3,000 xg at 4^{o}C for 20 min. Purified samples were frozen at -20^{o}C until required.

2.2.3 Protein determination

The protein concentration of the purified IgM was determined using a BCA™ Protein Assay Kit (Thermo scientific, UK) using the manufacturer's instructions. A standard curve of bovine serum albumin was used as a reference to determine the protein concentration (BSA, Sigma, UK).

2.2.4 Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weights of the H and L chains of purified IgM, using 12.5% Next-Gel™ (Amresco Inc., USA), following the basic procedure described by Laemmli (1970). Briefly, reservoir buffer was added to the inner and outer chamber of the gel electrophoresis apparatus (Mighty small II, Hoefer Inc., USA). Samples (approximately 1 mg ml^{-1}) were mixed in ratio of 1:1 with 2x sample buffer (0.5 M Tris-HCl; 20% glycerol; 4% Sodium dodecyl sulfate (SDS); 0.3% dithiothreitol (DTT); 0.002% (w/v) bromophenol blue). The samples were then boiled in a water bath for 4 min and centrifuged at 16,000 xg for 2 min. Pre-stained protein molecular weight markers were used in a range of 10-250 kD (Bio-Rad Laboratories, USA). Gels were run at 150 V until the dye front reached the bottom of the gel, after which the gels were removed from the cassettes and stained with Coomassie blue stain (Triple Red Laboratory Technology; England) overnight or silver stain (Sigma, UK). Gels were stored in BioDesignGelWrap™ membrane (FisherScientific, BioDesign Inc., New York) and scanned to determine the molecular weights of the bands.

2.2.5 Western blot

The SDS gels were run as described above and proteins from the gels were transferred onto nitrocellulose paper (Amersham^{TM},GE Healthcare, UK) using trans-blot buffer (0.2 M glycine; 0.025 M Tris; 20% methanol, pH 8.3). The gels were blotted onto the membrane using a trans-blotter (ThermoFisher, UK), following the manufacturer's instructions and blots were run at 60 V for 30 min. The nitrocellulose paper was then incubated with 1% w/v bovine serum albumin (BSA; Sigma, UK) in Tris buffered saline (TBS; 10 mM Tris, 0.5 M NaCl, pH 7.5) for 60 min to block non-specific binding sites. After blocking, the paper was washed three times with 5 min washes of Tris buffered saline with Tween 20 (TTBS; 0.1% Tween 20 in TBS). The membrane was then incubated with appropriate anti-*P. hypophthalmus* IgM monoclonal antibodies for 1 h, following by biotin anti-mouse IgG (1:250 in PBS; 0.02M Phosphate, 0.15M NaCl pH 7.2) (Sigma, UK) for 1 h and Neutravidin-HRP (1:250 in PBS) (Sigma, UK) for 1 h, washing between steps as described above. Finally, the nitrocellulose paper was washed with TBS for 1 min and the 4CN peroxidase substrate kit (KPL, USA) was adding until bands were visualized. The reaction was stopped with distilled water for 10 min.

2.2.6 Monoclonal antibody production

The hybridoma cells (SP1) producing antibody specific for *P. hypophthalmus* IgM were provided by the Aquatic Vaccine Unit, Institute of Aquaculture and University of Stirling. The hybridoma cells were cultured and supernatants screened in this project to characterize the antibodies, which were then used in Western blot and ELISA to assist in the characterisation of IgM from different fish species.

Supernatants were screened by an indirect ELISA, as described below (Section 2.2.8), and positive hybridoma cells were grown and subcultured every 2-4 days. Hybridoma cells were subcloned 3 times and screened by ELISA. Selected clones provided for this project were expanded in 150 cm^2 cell culture flasks and cultured for 10 days. Cells and cell debris were removed by centrifugation at 4^oC, 2000 xg for 10 min and supernatant collected. After that, supernatants were concentrated using a Pall LV centramate and the anti-*P. hypophthalmus* IgM monoclonal antibodies were purified using a HiTrap Protein G affinity column (GE Healthcare, UK), as described in Section 2.2.2.2, but using the following binding buffer (20 mM sodium phosphate; BDH Laboratory Supplies, pH7.5) and elution buffer (0.1 M glycine-HCL; Sigma, pH2.7) for purification.

2.2.7 Monoclonal antibody isotyping

Mouse monoclonal antibody isotype test kits (AbD Serotec, UK) were used to isotype the monoclonal antibodies. Anti-*P. hypophthalmus* IgM monoclonal antibodies were diluted in PBS containing 1% w/v bovine serum albumin (BSA; Sigma, UK) to a final concentration of 1 µg ml^{-1}. Then 150 µl of the diluted sample was added to the development tube and incubated at 22^oC for 30 sec. Tubes were vortexed briefly to ensure that the coloured microparticle solution was completely re-suspended. One isotyping strip was then placed with the solid red end at the bottom, into each development tube and incubated to 5-10 min until the positive flow control bands appeared.

2.2.8 Enzyme-Linked Immunosorbent Assay (ELISA)

An indirect ELISA was used to screen the anti- *P. hypophthalmus* monoclonal antibodies, and to determine if these cross reacted with IgM from the other fish species.

2.2.8.1 Indirect ELISA using *P. hypophthalmus* IgM and anti-*P. hypophthalmus* IgM monoclonal antibodies

ELISA plates (Immulon Inc., USA) were coated with 100 µl of purified *P. hypophthalmus* IgM at 10 µg/ml in coating buffer (0.05 M carbonate-bicarbonate, pH 9.6; Sigma, USA). The plates were incubated overnight at 4^{o}C and then washed three times with low salt wash buffer (LSW; 0.02M Tris base, 0.38 M NaCl, 0.05% v/v Tween-20, pH 7.3). Plates were post-coated with 3% w/v Casein (dried milk) in distilled water, 250 µl well^{-1} to block non-specific binding. The ELISA plates were incubated for 2 h at 22^{o}C, and then washed 3 times with LSW. After washing, the appropriate anti-*P. hypophthalmus* IgM monoclonal antibody supernatant, together with PBS used as a negative control, were added (100 µl well^{-1}) and incubated for 1 h at 22^{o}C. The ELISA plates were washed 5 times with high salt wash buffer (HSW; 0.02M Tris base, 0.5 M NaCl, 0.1% v/v Tween-20), incubating for 5 min on the last wash. Goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma, UK), diluted 1:4,000 in conjugate buffer, was added to the wells (100 µl well^{-1}) for 1 h at RT, and the plate was then washed with HSW as previously described. Chromogen (42 mM 3'3'5'5'-tetramethylbenzidine dihydrochloride (Sigma, UK), in acetic acid (BDH Laboratory Supplies): distilled water (1:2) was added to substrate buffer (0.1 M citric acid; BDH Laboratory Supplies, 0.1 M sodium acetate; Sigma,UK, pH 5.4) (150 µl chromogen and 15 ml substrate buffer and

5 µl H_2O_2 substrate) was added to the wells of the ELISA plate (100 µl $well^{-1}$) and incubated for 10 min at 22°C. The reaction was stopped with 50 µl $well^{-1}$ of 2M H_2SO_4 (BHD Laboratory Supplies). Finally, the ELISA plate was read at 450 nm using an ELISA reader (Dynex Technologies Inc., USA)

2.2.8.2 Indirect ELISA to determine cross-reaction between anti-*P. hypophthalmus* monoclonal antibodies and IgM from different fish species

ELISA plates (Immulon Inc., USA) were coated with 100 µl of purified IgM from different fish species, using *P. hypophthalmus* IgM as the positive control. Affinity purified IgM from *P. hypophthalmus*, *P. hypophthalmus* cross breeding *P. gigas*, *P. larnaudii, P. sanitwongsei*, *P.borcoti, Hamibragus filamentus, Hamibragus wyckioides, Clarias bactracus*, *Clarias Macrocephalus* and *Cyprinus carpio* and 14% Na_2SO_4 precipitation IgM from *P. hypophthalmus*, *P. hypophthalmus* cross breeding *P. gigas*, *P. larnaudii, P. sanitwongsei*, *P.borcoti, Hamibragus filamentus, Hamibragus wyckioides, Clarias bactracus*, *Clarias Macrocephalus* and *Cyprinus carpio* was used. All other procedures were as previously described in Section 2.2.8.1.

2.2.9 Analytical ultracentrifugation (AUC)

A number of assumptions and calculations were made during sedimentation velocity analytical ultracentrifugation (SV-AUC) to take into account the temperature during centrifugation, the buffer composition and amino acid composition of the protein molecule being analysed.

2.2.9.1 Partial specific volume and buffer density and viscosity calculations

The amino acid sequence (H and L chain) of catfish IgM was used to calculate the partial specific volume ($\bar{v}$) of the IgM protein (Table 2.2), and density (ρ) and viscosity

(η) were calculated for the buffer (PBS) at two temperatures using the SENTERP program (Laue*, et al.* 1992), as shown in Table 2.3.

Table 2.2. The amino acid composition of heavy and light chains of channel catfish (*Ictalurus punctatus*) immunoglobulin M (Acton*, et al.* 1971b).

Amino acid	Residues per molecule		Total
	Heavy chain	Light chain	
Lysine	40	11	51
Histidine	8	3	12
Arginine	18	5	23
Aspartic acid	54	18	72
Threonine	51	20	71
Serine	62	31	93
Glutamic acid	67	24	91
Proline	41	18	59
Glycine	39	22	61
Alanine	34	12	46
Valine	43	18	61
Methionine	6	1	7
Isoleucine	25	5	30
Leucine	43	18	71
Tyrosine	18	7	25
Phenylalanine	22	6	28
S-Carboxy-methyl-cysteine	12	5	17
Tryptophan	15	3	18

Table 2.3. The partial specific volume, density and viscosity of protein at 4°C and 20°C calculated using the SENTERP program' (Laue*, et al.* 1992).

Calculation value	20°C	4°C
Buffer density (ρ) g ml^{-1}	1.02085	1.02266
Buffer viscosity (η) Poise	0.010458	0.016355
Partial specific volume ($\bar{v}$) ml g^{-1}	0.7828	0.722
Partial specific volume with N-Acetylglucosamine ($\bar{v}$) ml g^{-1}	0.718	0.725
Water density (ρ) g ml^{-1}	0.9982	
Water viscosity (η) Poise	0.01002	
*Frictional coefficient $s_{20,w} = s_{T,B}\left(\frac{\eta_{T,B}}{\eta_{20,w}}\right)\frac{(1-\bar{v}\rho)_{20,w}}{(1-\bar{v}\rho)_{T,B}}$	= 1.596	

*The equation of frictional coefficient

$s_{20,w}$ = Sedimentation coefficient at 20 ^{o}C in water (units Svedberg S = 10^{-13} s)

$s_{T,B}$ = Sedimentation coefficient at 4^{o}C in buffer (units Svedberg S = 10^{-13} s)

$\eta_{20,w}$ = Water viscosity at 20^{o}C (Poise)

$\eta_{T,B}$ = Buffer viscosity at 4^{o}C (Poise)

$\bar{v}$ = Partial specific volume of molecule (ml g^{-1})

ρ = Density of solvent (g ml^{-1})

2.2.9.2 Sedimentation velocity ultracentrifugation (SV)

Sedimentation velocity ultracentrifugation was performed using IgM prepared by Hitrap purification with *P. hypophthalmus* IgM at 0.74 mg ml^{-1} and *P. borcoti* at 3.3 mg ml^{-1}. The Beckman Coulter Optima XL-I Analytical Ultracentrifuge (Palo Alto, USA) with an An-50 Ti 8 hole rotor was operated at 4^{o}C. Samples were diluted with phosphate buffered saline (PBS; 0.02 M sodium phosphate, 0.15 M sodium chloride, pH 7.2) to 1.0, 0.7, 0.4, 0.2 mg ml^{-1}. Samples were loaded into one side of the double sector (12 mm path length) centrepiece, made of charcoal-filled Epon, and the other side was loaded with PBS. The cells were assembled and weighed to ensure even balancing. The rotor with the cells was then placed into the centrifuge and cooled under vacuum for several hours until the experimental temperature reached 4^{o}C. The samples were then centrifuged at 45,000 rpm (NB rpm is normally used for AUC rather than g force) for 17 h 44 min. Both absorbance and interference optics were used to record scans over a radial range of 6.0-7.25 cm. Each cell was scanned every 8 min and a total of 200 scans was taken.

Data were analysed using SEDFIT version 13 (Schuck 2000; Schuck*, et al.* 2002; Schuck 2003) to determine the number of species in the samples and to obtain their sedimentation coefficients using the continuous distributions c(s) Lamm equation

model (Cole*, et al.* 2008). The non-interacting discrete species model (Schuck 2005) was used to obtain the relationship between sedimentation coefficients, molar mass and frictional coefficient; this model uses finite element analysis.

$$S = \frac{M(1-\bar{\nu}\rho)}{N_A f}$$

S = Sedimentation coefficient (units Svedberg S = 10^{-13} s)

M = Molar mass (g mol^{-1})

$\bar{\nu}$ = Partial specific volume of molecule (ml g^{-1})

ρ = Density of solvent (g ml^{-1})

N_A = Avogadro's number (number of molecules in a mole)

f = frictional coefficient (g s^{-1})

The sedimentation coefficients (at 4°C) were then standardised to 20°C ($s_{20,w}$), using the frictional coefficient equation (Table 2.3.), and a graph plotted against protein concentration to obtain the sedimentation coefficient at infinite dilution ($s^{0}_{20,w}$) from the y intercept. The following equations were used to calculate the sedimentation coefficient and standard value concentration at 20°C.

2.2.9.3 Homology modelling of immunoglobulin M (IgM)

The protein structure and function prediction used was SOMO modelling (Byron 2008; Brookes*, et al.* 2010) or hydrodynamic bead modelling using SOMO software (https://www.ultrascan.uthscsa.edu/SOMO). The L and H chain amino acid sequences from Human (*Homo Sapia*) were used to generate the model of IgM with tetrameric structure.

2.3 Results

2.3.1 Purification of immunoglobulin M (IgM) from family Pangasiidae and other fish species

Serum (0.5 ml) from fish was purified using Na_2SO_4 or affinity column chromatography (HiTrap). The yield of protein is presented in Table 2.4.

2.3.2 Characterisation of immunoglobulin M (IgM) from family Pangasiidae

The protein profiles of *P. hypophthalmus* IgM, purified by different methods, were examined by SDS-PAGE to determine the molecular weight of the H and L chains of the IgM molecule. The banding profiles obtained by SDS-PAGE for the 14% and 16% Na_2SO_4 precipitated IgM were very similar, but the intensity and clarity of the banding profile obtained for the former was better than either the 16% Na_2SO_4 or 20% Na_2SO_4 precipitated serum as there appeared to be less background staining (Figures 2.1; Lanes 6, 7 and 8). The IgM purified using the HiTrap affinity column (Figures 2.1; Lane 1, 2, 3, 4 and 5) showed the least background staining and the best clarity on the gels. It was noted all bands present in the HiTrap purified IgM could be found in the Na_2SO_4 precipitated IgM.

There were difference banding profiles for the IgM protein purified from the various pangasiidae family members as well as and the other fish species under reduced condition in SDS-PAGE, as shown when stained with Coomassie blue and silver staining following Na_2SO_4 purification (Figure 2.2(A)) and affinity column chromatography (Figures 2.2(B and C). For example, as shown for *P. sanitwongsei* IgM (lanes 5 of Figure 2.2), where two bands around 25 kDa were identified by silver staining but only one was apparent with Coomassie blue staining.

Table 2.4. Protein concentration of immunoglobulin M from Pangasius and other fish species following purification by ammonium sulphate precipitation and affinity column chromatography

Fish species	Concentration (µg/ml)	
	14% Na_2SO_4	**HiTrap**
P.hypophthalmus	2,792	1,541
P.hypophthalmus	3,386*	-
P.hypophthalmus	7,067#	-
P. hypophthalmus x *P.gigas*	5,229	383
P. gigas	2,091	464
P. larnaudii	2,479	652
P. sanitwongsei	2,823	267
P.borcoti	4,596	1,450
Hemibragus filamentus	4,200	322
Hamibragus wyckioides	1,023	291
Clarias bactracus	4,829	687
Clarias Macrocephalus	953	81
Cyprinus carpio	2,304	2,467

***16% Na_2SO_4; #20% Na_2SO_4**

The estimated molecular weight of the H chain of IgM from *P. hypophthalmus* was 70-72 kDa and L chain was 25-26 kDa. The L chains of IgM from the other fish species were similar to *P. hypophthalmus* (Table 2.5), while the IgM H chains differed with *P. hypophthalmus* x *P. gigas* 75 kDa, *P. gigas* and *P. larnaudii* 76 kDa, *P. sanitwongsei* 69 kDa, *P. borcoti* 75 kDa, *Hamibragus wyckioides* 75 kDa, *Hemibragus filamentus* 73 kDa, *Clarias bactracus* 74 kDa, *Clarias Macrocephalus* 73 kDa, *Cyprinus carpio* 70kDa.

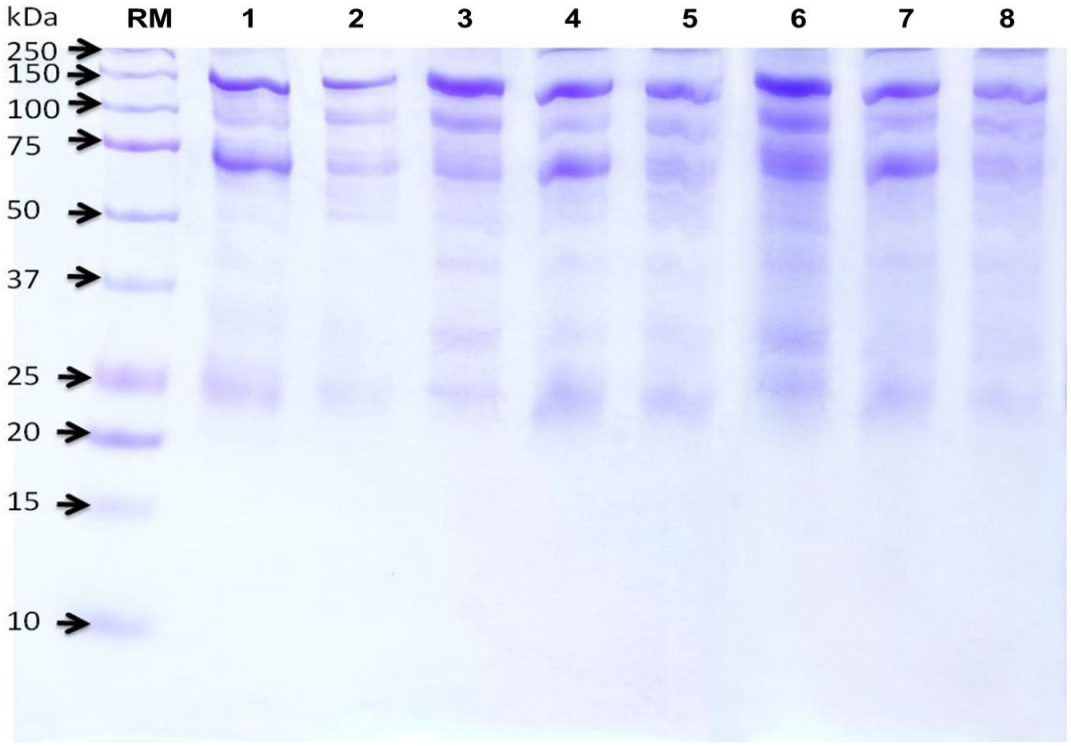


Figure 2.1. SDS-PAGE (12.5%) of *Pangasianodon hypophthalmus* IgM under reducing conditions. The IgM was purified from serum using affinity column chromatography and Na_2SO_4 precipitation (Staining with Coomassie blue). RM=Rainbow marker 10-250 kDa, 1-5=five serum samples of *P. hypophthalmus* IgM purified by affinity chromatography (HiTrap), 6=20% Na_2SO_4 precipitation of serum, 7=16% Na_2SO_4 precipitation of serum and 8=14% Na_2SO_4 precipitation of serum

Table 2.5. Molecular weight of IgM heavy chain and light chain from Family Pangasiidae and other fish species, estimated by SDS-polyacrylamide gel electrophoresis

Species	Molecular Mass (kDa)	
	Heavy chain	Light chain
P. hypophthalmus (Thailand)	70	25
P. hypophthalmus (Vietnam)	72	26
P. hypophthalmus x *P.gigas*	75	26
P. gigas	76	26
P. larnaudii	76	27
P. sanitwongsei	69	25
P. borcoti	75	23
Hamibragus wyckioides	75	23
Hemibragus filamentus	73	25
Clarias bratracus	74	27
Clarias macrocephalus	73	24
Cyprinus carpio	70	25

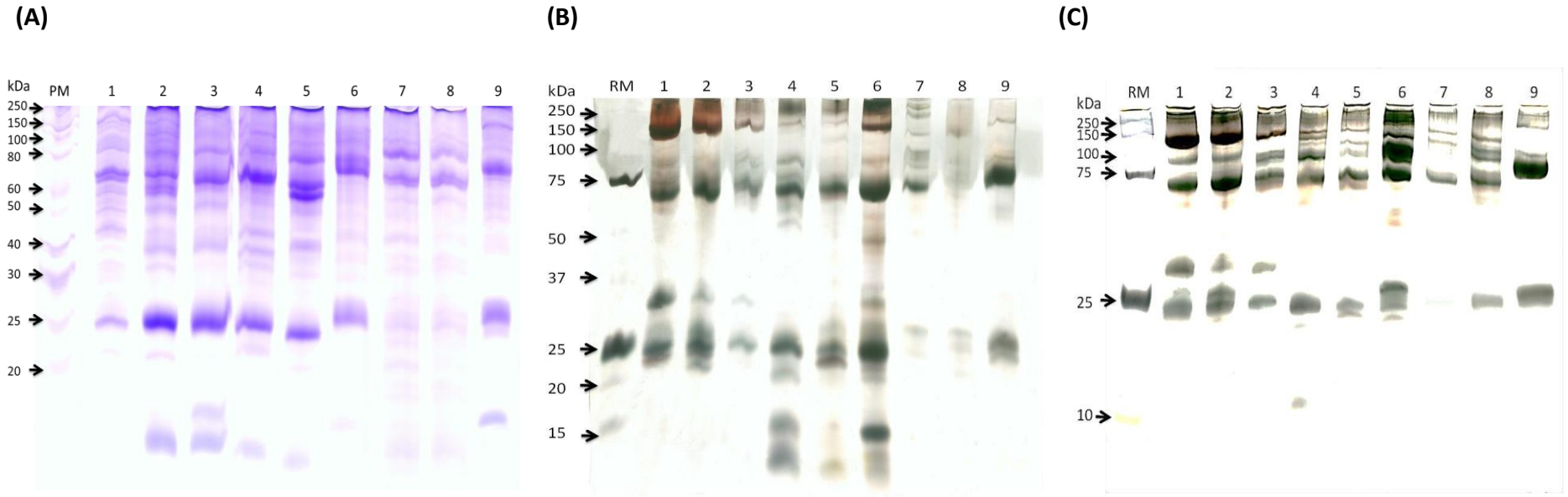


Figure 2.2(A-C). SDS-PAGE (12.5%) of IgM purified by 14% Na_2SO_4 precipitation of serum with Coomasie blue stain (A) and Hitrap purified IgM from the serum with silver stain (B and C) of Family Pangasiidae and other fish species under reducing conditions. Staining with silver stain.

(A) PM=Protein marker 10-250 kDa, 1=*P. hypophthaplus (Vietnam),* 2=*P. gigas,* 3=*P. hypophthalmus (Thailand),* 4=*P. larnaudii,* 5=*P. sanitwongsei,* 6=*Hemibragus filamentus,* 7=*Clarias bartracus,* 8=*Clarias macrocephalus,* 9=*Cyprinus carpio*

(B) RM=Rainbow marker 10-250 kDa, 1=*P. hypophthalmus* (Thailand), 2=*P. hypophthalmus x P. gigas,* 3=*P. gigas,* 4=*P. larnaudii,* 5=*P. sanitwongsei,* 6=*P. borcoti,* 7=*Hamibragus wyckioides,* 8=*Clarias bartracus,* 9=*Cyprinus carpio*

(C) RM=Rainbow marker 10-250 kDa, 1=*P. hypophthalmus* (Thailand), 2=*P. hypophthalmus x P. gigas,* 3=*P. gigas,* 4=*P. larnaudii, 5=P. sanitwongsei,* 6=*Hamibragus wyckioides,* 7=*Clarias bartracus,* 8=*Clarias macrocephalus,* 9=*Cyprinus carpio*

2.3.3 Characterisation of anti- *P. hypophthalmus* IgM monoclonal antibodies

The supernatant of the different hybridoma cell lines provide by the Aquatic Vaccine Unit producing anti-*P. hypophthalmus* mAbs were screened by indirect ELISA to determine specificity. The cells were cloned three times to ensure that the antibodies being produced were monoclonal. This established that hybridoma cells 1, 2, 7, 18, 23 and 28 produced positive results against *P. hypophthalmus* IgM after cloning (Table 2.6). Thus, six anti- *P. hypophthalmus* IgM mAbs were produced. These were affinity purified and then isotyped and the results indicated that mAbs 1, 2, 7, 18 and 23 were IgG1, while mAb 28 was isotype IgG2. In addition, all the mAbs had kappa light chains (Table 2.7)

Table 2.6. Screening of supernatants by indirect ELISA following cloning of hybridoma cells producing anti- *P. hypophthalmus* IgM antibodies

Anti- *P. hypophthalmus* IgM monoclonal antibodies	1st Cloning	2nd Cloning	3rd Cloning
1	√	√	√
2	√	√	√
3	√	√	-
5	√	-	-
6	√	√	-
7	√	√	√
15	√	-	-
16	√	√	-
18	√	√	√
23	√	√	√
26	√	-	-
28	√	√	√

Table 2.7. Isotyping of anti- *P. hypophthalmus* IgM monoclonal antibodies

Anti- *P. hypophthalmus* IgM monoclonal antibodies	Heavy chain type	Light chain type
1	Ig G1	Kappa
2	Ig G1	Kappa
7	Ig G1	Kappa
18	Ig G1	Kappa
23	Ig G1	Kappa
28	Ig G2a	Kappa

2.3.4 Characterisation of immunoglobulin M (IgM) from family Pangasiidae and other fish species using anti-P. *hypophthalmus* IgM monoclonal antibodies

2.3.4.1 ELISA

Six anti-*P. hypophthalmus* IgM mAbs (1, 2, 7, 18, 23 and 28) were found to react with *P. hypophthalmus* IgM by ELISA (Table 2.8 and 2.9). All of the mAbs also cross-reacted with the IgM from the other members of the Pangasiidae family and with the IgM from the other fish species tested (*P. hypophthalmus*, *P. hypophthalmus* cross breeding *P. gigas*, *P. larnaudii, P. Sanitwongsei*, *Hamibragus filamentus, Hamibragus wyckioides, Clarias bactracus*, *Clarias Macrocephalus* and *Cyprinus carpio*). The antibodies reacted with both sodium sulphate precipitated IgM and HiTrap purified IgM.

Table 2.8. Screening of anti-*Pangasianodon hypophthalmus* IgM monoclonal antibodies by ELISA against Na_2SO_4 purified IgM from different fish species (*P. hypophthalmus*, *P. hypophthalmus* cross breeding *P. gigas*, *P. larnaudii, P. sanitwongsei*, *Hamibragus filamentus, Hamibragus wyckioides, Clarias bactracus*, *Clarias Macrocephalus* and *Cyprinus carpio*).

Fish Species IgM (14% Na_2SO_4 precipitated)	mAb 1	mAb 2	mAb 7	mAb 18	mAb 23	mAb 28
P. hypophthalmus	1.616 ± 0.004	1.573 ± 0.037	1.211 ± 0.081	1.656 ± 0.009	1.719 ± 0.004	1.680 ± 0.006
P. hypophthalmus (16% $Na_2SO_{4)}$	1.705 ± 0.086	1.646 ± 0.039	1.378 ± 0.035	1.756 ± 0.022	1.817 ± 0.030	1.818 ± 0.064
P. hypophthalmus (20% Na_2SO_4)	1.644 ± 0.017	1.585 ± 0.015	1.184 ± 0.018	1.694 ± 0.016	1.725 ± 0.006	1.788 ± 0.004
P. hypophthalmus x *P. gigas*	1.875 ± 0.011	1.889 ± 0.007	1.635 ± 0.076	1.51 ± 0.061	1.832 ± 0.055	1.776 ± 0.023
P. gigas	1.556 ± 0.035	1.526 ± 0.029	1.099 ± 0.021	1.528 ± 0.024	1.621 ± 0.001	1.506 ± 0.042
P. larnaudii	1.897 ± 0.008	1.919 ± 0.042	1.419 ± 0.622	1.565 ± 0.025	1.731 ± 0.045	1.907 ± 0.021
P. Sanitwongsei	2.182 ± 0.083	2.146 ± 0.087	2.112 ± 0.142	1.783 ± 0.132	2.073 ± 0.083	2.156 ± 0.177
P. borcoti	1.985 ± 0.074	1.859 ± 0.084	1.821 ± 0.063	1.654 ± 0.085	1.995 ± 0.063	1.987 ± 0.097
Hemibragus filamentus	1.324 ± 0.074	1.304 ± 0.045	1.304 ± 0.056	0.869 ± 0.021	1.814 ± 0.139	1.746 ± 0.007
Hamibragus wyckioides	1.564 ± 0.085	1.685 ± 0.069	1.589 ± 0.087	1.258 ± 0.074	1.789 ± 0.083	1.657 ± 0.036
Clarias bactracus	1.922 ± 0.047	1.917 ± 0.006	1.920 ± 0.051	1.713 ± 0.012	1.825 ± 0.014	1.782 ± 0.093
Clarias Macrocephalus	2.314 ± 0.450	2.326 ± 0.159	2.280 ± 0.531	2.053 ± 0.580	2.231 ± 0.342	2.203 ± 0.556
Cyprinus carpio	1.472 ± 0.005	1.745 ± 0.148	1.679 ± 0.029	0.699 ± 0.016	0.900 ± 0.035	0.535 ± 0.572

* Results are expressed as absorbance at 450 nm (Mean ± SD, n=3)

Table 2.9. Screening of anti-*Pangasianodon hypophthalmus* IgM monoclonal antibodies by ELISA against HiTrap affinity column purified IgM from different fish species (*P. hypophthalmus*, *P. hypophthalmus* cross breeding *P. gigas*, *P. larnaudii, P. sanitwongsei*, *Hamibragus filamentus, Hamibragus wyckioides, Clarias bactracus*, *Clarias Macrocephalus* and *Cyprinus carpio*).

Fish Species IgM	mAb 1	mAb 2	mAb 7	mAb 18	mAb 23	mAb 28
HiTrap *P. hypophthalmus*	1.952 ± 0.034	2.006 ± 0.014	1.903 ± 0.131	2.064 ± 0.307	1.957 ± 0.021	2.104 ± 0.195
HiTrap *P. hypophthalmus* cross breeding	1.968 ± 0.049	2.050 ± 0.088	2.034 ± 0.250	2.083 ± 0.060	1.745 ± 0.006	1.944 ± 0.074
HiTrap *P. gigas*	1.873 ± 0.198	2.044 ± 0.076	2.161 ± 0.112	2.003 ± 0.120	1.887 ± 0.182	1.934 ± 0.089
HiTrap *P. larnaudii*	1.999 ± 0.197	2.07 ± 0.154	2.164 ± 0.296	2.23 ± 0.262	2.114 ± 0.003	2.118 ± 0.054
HiTrap *P. sanitwongsei*	1.991 ± 0.164	2.043 ± 0.027	2.012 ± 0.093	2.145 ± 0.049	1.865 ± 0.104	1.872 ± 0.035
HiTrap *P. borcoti*	1.998 ± 0.098	1.987 ± 0.065	1.895 ± 0.069	1.965 ± 0.085	1.999 ± 0.086	1.985 ± 0.098
HiTrap *Hamibragus filamentus*	1.034 ± 0.047	1.025 ± 0.088	1.882 ± 0.028	1.281 ± 0.189	2.004 ± 0.081	1.983 ± 0.113
HiTrap *Hamibragus wyckioides*	1.859 ± 0.098	1.758 ± 0.086	1.897 ± 0.064	1.652 ± 0.075	1.985 ± 0.063	1.906 ± 0.038
HiTrap *Clarias bactracus*	1.988 ± 0.099	1.984 ± 0.274	2.015 ± 0.062	1.952 ± 0.012	1.849 ± 0.149	1.685 ± 0.251
HiTrap *Clarias Macrocephalus*	1.837 ± 0.048	1.518 ± 0.029	1.796 ± 0.087	1.865 ± 0.061	1.543 ± 0.026	1.803 ± 0.023
HiTrap *Cyprinus carpio*	1.654 ± 0.048	1.593 ± 0.018	1.624 ± 0.014	1.736 ± 0.117	1.038 ± 0.024	1.761 ± 0.134

* Results are expressed as absorbance at 450 nm (Mean ± SD, n=3)

2.3.4.2 Western blot analysis

The mAbs were also tested in western blot to determine if they recognised the H or L chains of the IgM molecules. The reaction of the various anti-*P. hypophthalmus* IgM mAbs with the different preparations of *P. hypophthalmus* IgM (14%, 16%, 20% Na_2SO_4 precipitation and HiTrap affinity column chromatography purification) confirmed that all six mAbs recognized *P. hypophthalmus* IgM (Figures 2.3 (A and B)). Western blot analysis also confirmed cross reaction of the mAbs the other Pangasiidae species (*P. gigas*, *P. larnaudii*, *P. sanitwongsei* and *P. borcoti*) tested and other fish species IgM (*Hemibragus filamentus*, *Clarias bactracus*, *Clarias Macrocephalus* and *Cyprinus carpio*) (Figure 2.4). Four of the mAbs (1, 2, 7 and 18) reacted with L chain of IgM, with the exception of mAbs 2 and 7, which did not react with the *C. carpio* L chains (Table 2.10). On the other hands, mAbs 23 and 28 reacted with the H chains of IgM from the different fish species (Table 2.10). A commercial product from Aquatic Diagnostics Ltd (MAb 12) was used as a positive control.

Table 2.10. Reaction of anti-*P. hypophthalmus* IgM monoclonal antibodies (mAbs) with Family Pangasiidae and other species H and L chains

Species	Anti- *P. hypophthalmus* Ig M monoclonal antibodies													
	1		2		7		12		18		23		28	
P. hypophthalmus (Vietnam)	L	H	L	H	L	H	L	H	L	H	H	-	H	-
P. hypophthalmus x *P. gigas*	L	H	L	H	L	H	L	H	L	H	H	-	H	-
P. gigas	L	H	L	-	L	-	L	-	-	H	H	-	H	-
P. hypophthalmus (Thai)	L	H	L	H	L	H	L	H	-	H	H	-	H	-
P. larmaudii	L	H	L	H	L	H	L	H	-	H	H	-	H	-
P. sanitwongsei	L	H	L	H	L	H	L	-	-	H	H	-	H	-
P. borcoti	L	H	L	H	L	H	L	-	-	H	H	-	H	-
Hemibragus filamentus	L	H	L	H	L	H	L	-	-	H	H	-	H	-
Hamibragus wyckioides	L	H	L	H	L	H	L	-	-	H	H	-	H	-
Clarias bratracus	L	H	L	H	L	H	L	H	-	H	H	-	H	-
Clarias macrocephalus	L	H	L	H	L	H	L	H	L	H	H	-	H	-
Cyprinus carpio	L	H	-	H	-	-	L	H	-	H	H	-	H	-

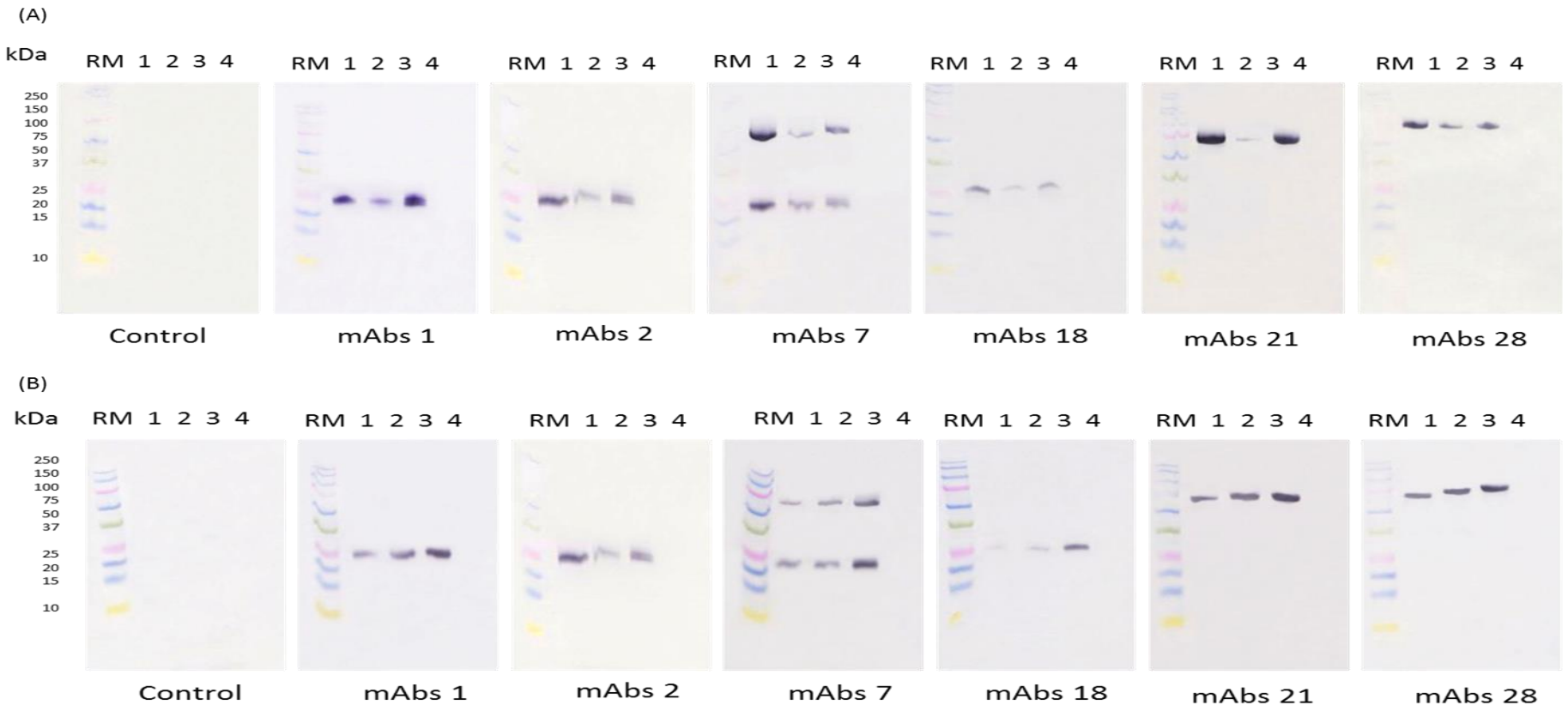


Figure 2.3(A-B). Western blotting of *P. hypophthalmus* IgM (A) purified using HiTrap affinity chromatography with anti- *P. hypophthalmus* IgM monoclonal antibodies. RM= Rainbow marker 10-250 kDa, 1=*P. hypophthalmus* No.1, 2=*P. hypophthalmus* No.2, 3=*P. hypophthalmus* No.3 and 4=Trout (HiTrap affinity chromatography) **(B) 14%, 16% and 20% Na_2SO_4 precipitated *P. hypophthalmus* IgM with anti- *P. hypophthalmus* IgM monoclonal antibodies.** RM=Rainbow marker 10-250 kDa, 1=14% Na_2SO_4 precipitation *P. hypophthalmus,* 2=16% Na_2SO_4 precipitation *P. hypophthalmus*, 3=20% Na_2SO_4 precipitation *P. hypophthalmus* and 4 =14% Na_2SO_4 precipitation Trout

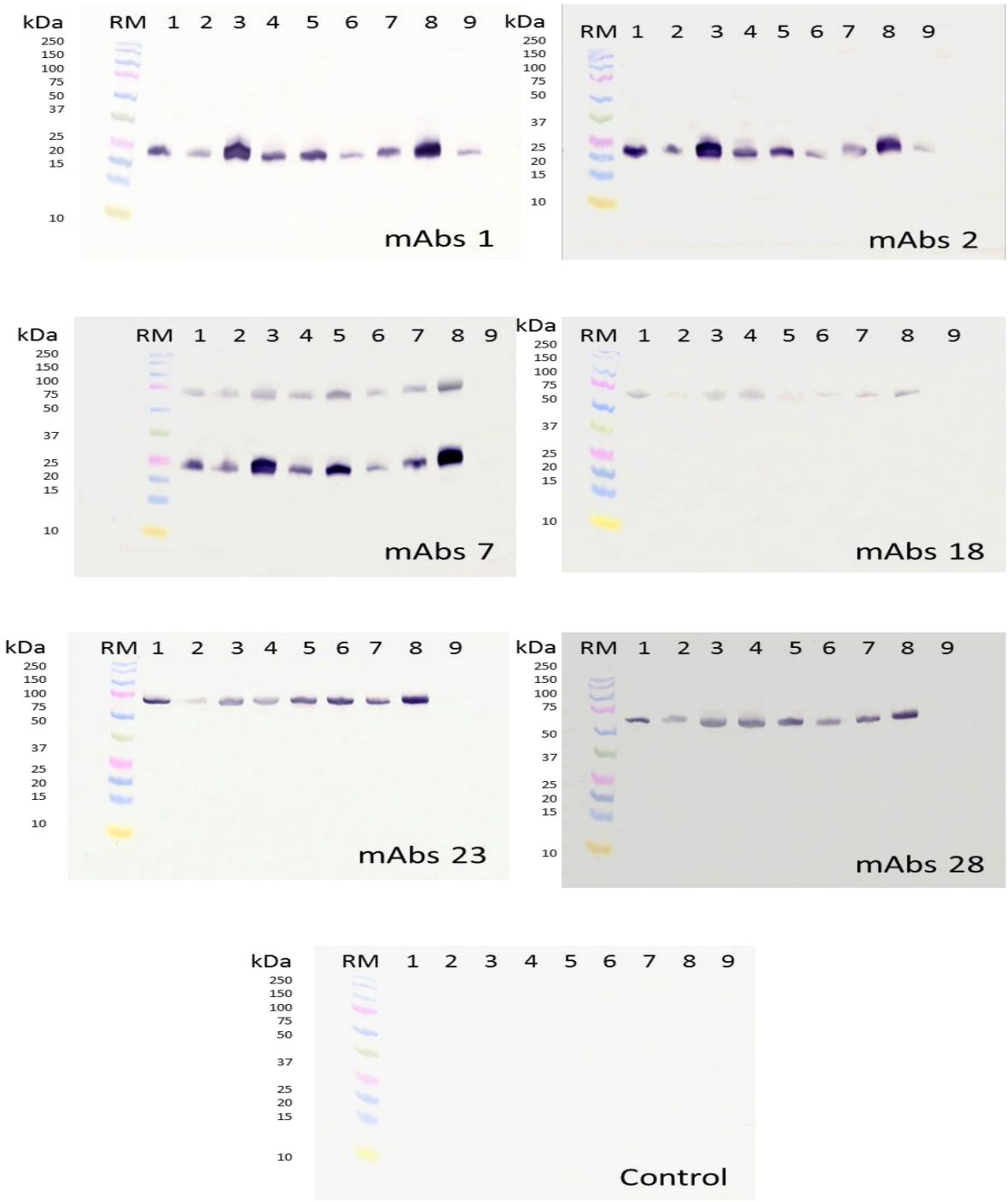


Figure 2.4. Western blotting of purified using HiTrap affinity chromatography IgM from Family Pangasiidae and other fish species with anti-*P. hypophthalmus* IgM monoclonal antibodies. RM = Rainbow marker 10-250 kDa, 1 = *P. hypophthalmus (Vietnam),* 2=*P. gigas,* 3=*P. hypophthalmus (Thailand),* 4=*P. larnaudii,* 5=*P. sanitwongsei,* 6=*Hemibragus filamentus,* 7=*Clarias bartracus,* 8=*Clarias macrocephalus,* 9=*Cyprinus carpio*

2.3.5 Analytical ultrancentrifugation: sedimentation velocity

2.3.5.1 Sedimentation coefficient of IgM

A concentration range of 0.2-0.7 mg ml^{-1} *P. hypophthalmus* IgM and 0.2-1.0 mg ml^{-1} *P. borcoti* IgM were analysed by SV ultracentrifugation. The reference buffer was PBS, and the density and viscosity values used for c(s) distribution analysis using SEDFIT (Schuck 2000) were $\rho_{4,TB}$ =1.02266 g ml^{-1} and $\eta_{4,TB}$=0.016355 Poise, respectively. The partial specific volumes ($\bar{v}$) of *P. hypophthalmus* IgM and *P. borcoti* IgM were 0.783 ml g^{-1} at 20°C and 0.722 ml g^{-1} at 4°C, respectively. The resultant sedimentation coefficients for *P. hypophthalmus* IgM are shown in Table 2.11 and for *P. borcoti* IgM in Table 2.12.

The *P. hypophthalmus* IgM seemed to consist of possible species with $s_{20,w}$ in the region of 15.5 ±0.1 S as shown in Figure 2.5(A). The *P. borcoti* IgM consisted of possible species with $s_{20,w}$ in the region of 15.9 ±0.2 S, as shown in Figure 2.5(B).

2.3.5.2 Homology modeling of IgM

The results from the hydrodynamic calculation from the protein structure of human IgM determined using SOMO modelling was computed to be s=13.4 S and the mass of the tetramer model was 680 kDa, as shown in Table 2.13. The protein structure of IgM is shown in Figure 2.6.

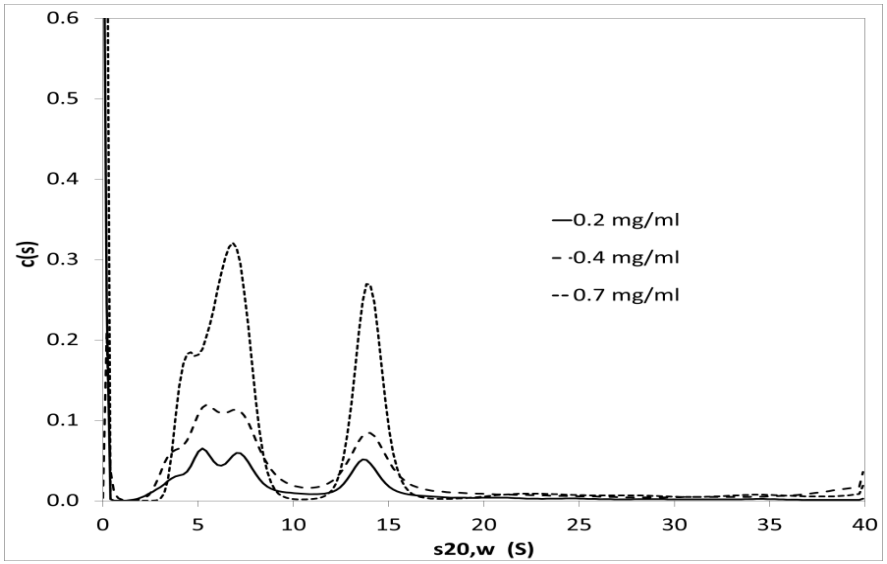
Table 2.11. SEDFIT (c(s)) analysis of *Pangasianodon hypophthalmus* IgM at different concentrations (0.2-0.7 mg ml^{-1}). Scans 1-50 of the absorbance dataset (acquired at 280 nm) were analysed with a sedimentation coefficient range of 0 ≤ s ≤ 40 S and a resolution of 200.

Concentration	0.7 mg ml^{-1}				
	integral (from-to)	Loading concentration	Weight average	Std.deviation	$s_{4,w}$ transformed to $s_{20,w}$ conditions
Peak 5	6.9-12.1	0.32	8.9	0.8	15.5
Concentration	0.4 mg ml^{-1}				
Peak 5	7.0-12.0	0.17	9.1	1.0	15.6
Concentration	0.2 mg ml^{-1}				
Peak 5	6.9-12.9	0.09	9.2	1.1	15.5

Table 2.12. SEDFIT (c(s)) analysis of *Pangasianodon borcoti* IgM at different concentration (0.2-1 mg ml^{-1}). Scans 1-50 of the absorbance dataset (acquired at 280 nm) were analysed with a sedimentation coefficient range of 0 ≤ s ≤ 40 S and a resolution of 200.

Concentration	1 mg ml^{-1}				
	Integral (from-to)	Loading concentration	Weight average	Std.deviation	$s_{4,w}$ transformed to $s_{20,w}$ conditions
Peak 6	7.9-10.6	0.47	9.1	0.3	15.8
Concentration	0.7 mg ml^{-1}				
Peak 5	8.1-10.9	0.35	9.3	0.4	16.2
Concentration	0.4 mg ml^{-1}				
Peak 4	6.8-11.4	0.22	9.1	0.6	15.9
Concentration	0.2 mg ml^{-1}				
Peak 4	6.9-11.6	0.10	9.0	0.8	15.8

(A)



(B)

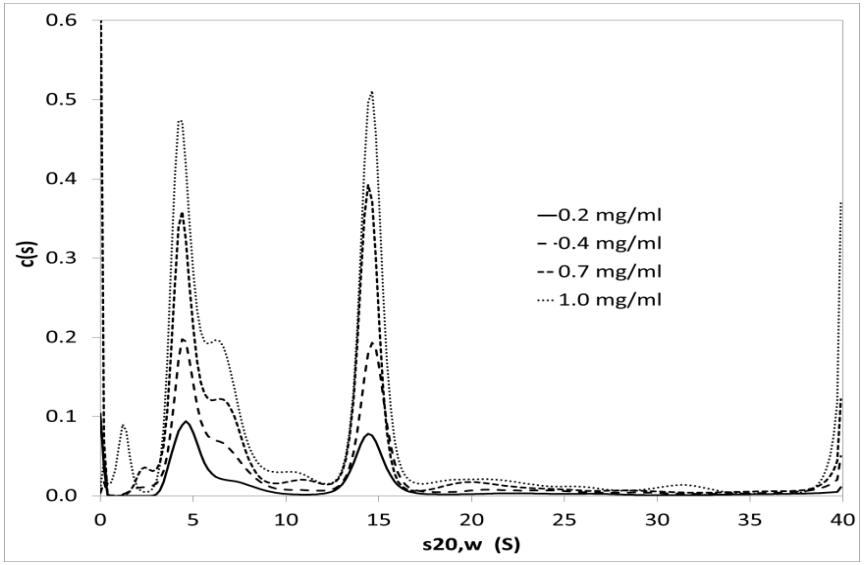


Figure 2.5(A-B). Distribution analysis for a concentration range of *P. hypophthalmus* and *P. borcoti* IgM generated by using the SEDFIT (c(s)) data analysis tool.

(A) *P. hypophthalmus* IgM analysed at concentrations in the range 0.2-0.7 mg ml^{-1} showing the presence of species with an average $s_{20,w}$ of 15.5 S (B) *P. borcoti* IgM analysed at concentrations in the range 0.2-1.0 mg ml^{-1} showing the presence of species with an average $s_{20,w}$ of 15.9 S .

Table 2.13. SOMO Hydrodynamic bead modelling of Human IgM

Somo Hydrodynamic Bead Modelling of Human IgM*	
Model	**IgM tetramer-a2b**
Total Beads in Model	1555
Used Beads in Model	1549
Molecular Mass	6.7991e+0.5 Da
Part Specific Volume	0.724 cm^3/g
Sedimentation Coefficient s	1.34e+01 S
Tr. Diffusion Coefficient D	1.73e-07 cm/sec^2
Stokes Radius	1.24e+01 nm
Frictional Ratio	2.14
Radius of Gyration	1.45e+01 nm
Relaxation Time	3.06e+0.3 ns
Intrinsic viscosity	1.96e+01 cm^3/g

***Water at 20°C, Density 1.00194 cP, Viscosity 0.998234 g ml^{-1}**

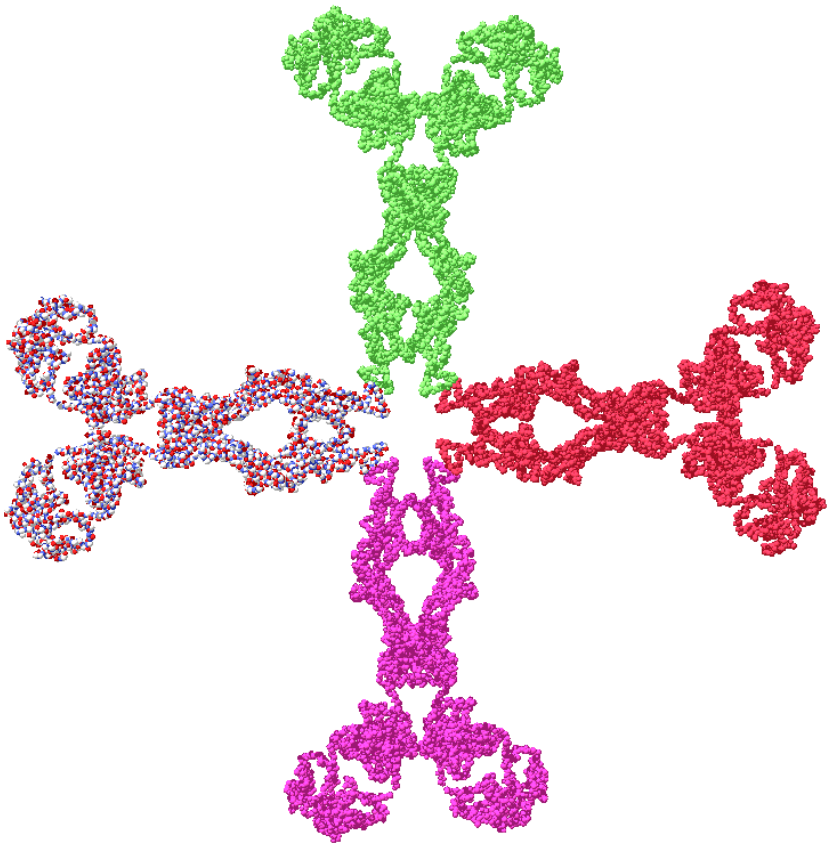


Figure 2.6. Model of the protein structure of IgM from human where the mass of the tetrametic molecule was estimated at 680 kDa.

2.4 Discussion

The IgM from *P. hypophthalmus* and other species of family pangasiidae was purified using affinity column chromatography (HiTrap) and Na_2SO_4 precipitation, and its biochemical structure was then examined by gel electrophoresis and SV ultracentrifugation. The molecule was further characterised using anti- *P. hypophthalmus* mAbs in Western blotting.

The percentage of Na_2SO_4 used to purify the IgM from *P. hypophthalmus* and other Pangasiidae members influenced the concentration of protein obtained; the higher the percentage of Na_2SO_4 used, the higher the concentration of protein obtained. It is likely that proteins other than IgM were precipitated using Na_2SO_4, while the purity obtained with affinity column chromatography should be higher because IgM would bind to the column and no other proteins. The results confirm this, showing that mainly IgM is extracted from serum samples by HiTrap affinity column chromatography, while Na_2SO_4 precipitation resulted in many other proteins being present, as reported by others. For example, Hrubec and Smith (1999) found albumin and globulin in both serum and plasma samples purified in this way. This was evident from the multiple protein bands observed in the protein profiles obtained in SDS-PAGE with Na_2SO_4 precipitated samples compared with the HiTrap affinity column. However, the HiTrap affinity column was not completely successful at purifying only IgM. The column is designed to purify human IgM and is pre-packed with a thiophilic adsorption medium, 2-mercaptopyridine coupled to sepharose. An additional band between 25-75 kDa (*i.e.* not IgM) was found (Figure 2.1) with the HiTrap IgM, which suggests something other than IgM was also carried over in the eluting buffer from the column.

The results suggest that this column may not be entirely suitable for the purification of IgM from fish.

The molecular weights of IgM from *P. hypophthalmus* and other family pangasiidae members, and its H and L subunits were determined using electrophoresis, and found to be comparable with the molecular weights found for other teleost fish species (See Table 2.1). These included species such as Atlantic salmon, which were reported to have H and L chains of 71.5 and 26.5 kDa, respectively, halibut 72 and 26.5 kDa, haddock 72 and 26.5 kDa and cod 72.5 and 26.5 kDa (Magnadóttir 1998). The results of this study indicated that the H and L of IgM from *P. hypophthalmus* were 70-72 kDa and 25-26 kDa, respectively. The L chains of IgM from the other fish species tested were similar to *P. hypophthalmus*, while the H chains varied (*i.e*. *P. gigas* and *P. larnaudii* 76 kDa, *P. sanitwongsei* 69 kDa, *H. filamentus* 73 kDa, *P.borcoti* and *H.wyckioides* 75 kDa, *C. bactracus* 74 kDa, *C. macrocephalus* 73 kDa and *C. carpio* 70 kDa), as did the native IgM molecules.

The molecular weight of the single subunit IgM (two H chains and two L chains) are shown in parenthesis: *P. hypophthalmus* (193 kDa), *P.hypophthalmus* cross breeding *P. gigas* (202 kDa), *P.gigas* (204 kDa), *P.larnaudii* (208 kDa), *P.Sanitwongsei* (188 kDa), *P. borcoti* (196 kDa), *Hamibragus filamentus* (196 kDa), *Hamibragus wyckioides* (196 kDa), *Clarias bactracus* (202 kDa), *Clarias Macrocephalus* (194 kDa) and *Cyprinus carpio* (190 kDa). Kaattari *et. al*. (1998) showed structural heterogeneity to trout IgM, with the mono-, di-, tri- and tetrameric forms observed by electrophoresis and these have also been found in toadfish (*Spheroides glaber*), Atlantic salmon and Channel catfish.

Monoclonal antibodies (mAbs) and polyclonal antibodies were used in Western blotting to characterise immunoglobulins (IgM) for a variety of fish species, including Asian sea bass (Choudhury and Prasad 2011), Carp (Choi*, et al.* 2002), Striped trumpeter (*Latris lineata*)(Covello*, et al.* 2009), Channel catfish (Hall*, et al.* 1973), Tilapia (Al-Harbi*, et al.* 2000), Chum salmon (*Oncorhynchus keta*) (Fuda*, et al.* 1992), Atlantic salmon, Halibut, Haddock and Cod (Magnadóttir 1998). These studies revealed that the molecular weight range of fish IgM molecules range between 610-890 kDa, while the H chain is between 51 and 86 kDa and the L chain between 22 and 28 kDa. A variety of different methods of purification and charactersation were used in these studies, however. There is only one report relating to IgM from *P. hypophthalmus,* where the H chain was reported to be 72 kDa and up to three small L chains reported at 24, 26 and/or 28-29 kDa by using SDS-PAGE and Western blotting with mouse monoclonal antibodies against *P. hypophthalmus* IgM (Aquatic Diagnostic Ltd.) (Huong Giang*, et al.* 2012). This is in agreement with the results from the present study where *P. hypophthalmus* IgM H chains were determined to be 70-72 kDa and L chains were 25-26 kDa. However, to date there are no reports of the comparison of IgM from *P. hypophthalmus* and related species using electrophoresis or western blot, as is presented here. A number of the mAbs produced against *P. hypohthalmus* IgM were found to cross-react with the family pangasiidae IgM, and were used to confirm the molecular weight of H and L chains. The mAbs were also shown to cross-react with IgM form other fish species, including *Hamibragus filamentus, Hamibragus wyckioides, Clarias bactracus*, *Clarias Macr ocephalus* and *Cyprinus carpio*.

Sedimentation velocity ultracentrifugation was used to determine the molecular weight of whole IgM molecule from *P. hypophthalmus* as an alternative to the more commonly used native gels, run under non-denaturing conditions. The latter was attempted here but proved complex. The molecular weight of teleost IgM determined using SV ultracentrifuagtion has been previously reported in fish , including channel catfish 14 S (610 kDa), gar (*Lepisosteus osseus*) 13.9 S (610 kDa), paddle fish (*Polyodon sputhula*) 14.2 S (630 kDa) where these were reported as tetramers, and as a dimer inskate (*Raja kenojer*) 8.9 S (320 kDa) (Acton, *et al.* 1971; Acton, *et al.* 1971; Kunihiko, *et al.* 1984). The present study showed that the sedimentation coefficient of *P. hypophthalmus* was 15.5 S while, *P. borcoti* was 15.9 S. Hydrodynamic bead modelling (SOMO modelling) was performed using the amino acid sequence of human IgM (NB without carbohydrate), and the data obtained by SV determined the mass of the tetrameric molecule to be 680 kDa (13.4S). The calculation for the molecular weight for pangasius IgM, using the human "tetrameric" model, was calculated to be 787 kDa (15.5 S) for *P. hypophthalmus* and 807 kDa (15.9 S) for *P. borcoti*. However, this molecular weight was calculated without including the carbohydrate content of the molecule in the model, and as fish IgM contains a lot of carbohydrate, the molecular weight presented here represents an under estimate of the true mass of the molecule. Magnatdóttir (1998) found that the carbohydrate composition of fish IgM, in terms of the oligosaccharide associated with the H chain, varied from 7.8-11.4% in four different fish species (Atlantic salmon, halibut, haddock and cod). If a similar carbohydrate contribution is assumed for *P. hypophthalmus* IgM and *P. borcoti* IgM the molecular weight would be expected to range between 848-876 kDa and 870-899 kDa,

respectively. Huong Giang, *et al.* (2012) used gel filtration to find the molecular mass of *P. hypophthalmus* and determined it to be 900 kDa. However from diethylaminoethyl cellulose based ion exchange chromatography and non-reducing gradient polyacrylamide gel electrophoresis, the molecular weight of *P. hypophthalmus* IgM was indicated to be 798 kDa (Sudhagar*, et al.* 2013). When the molecular weight was calculated using H and L chains with 7.8-11.4% carbohydrate in the present study, the molecular weight for whole IgM: 832-860 kDa (*P. hypophthalmus*), 845-873 kDa (*P. borcoti, Hamibragus filamentus* and *Hamibragus filamentus*), 871-900 kDa (*P. hypophthalmus* cross breeding *P. gigas* and *Clarias bactracus*), 880-909 kDa (*P.gigas*), 940-971 kDa (*P.larnaudii*), 811-838 kDa (*P. sanitwongsei*), 837-865 kDa (*Clarias macrocephalus*) and 819-847 kDa (*Cyprinus carpio*). Calculation of the molecular weights of whole IgM by SV ultracentrifugation appeared to give higher molecular weights than those calculating using the molecular weights of the H and L chains, but lower than that obtained using gel filtration. Magnadóttir (1998) determined the molecular weight of IgM for four fish species (Atlantic salmon, halibut, haddock and cod) using gel filtration and reported that these were higher than when estimated using H chain and L chain molecular weights.

Anti-*Pangasianodon hypophthalmus* IgM mAbs were shown to cross-react with family pangasiidae and other species, *Hamibragus filamentus, Hamibragus wyckioides, Clarias bactracus*, *Clarias Macrocephalus* and *Cyprinus c arpio* IgM in both ELISA and Western blotting. As *P. hypophthalmus, P. gigas*, *P.larnaudii* and *P. sanitwongsei* are pangasiidae, and most likely have similar IgM molecules, the mAbs which recognised IgM from one species are more likely to recognize those of a related species. The fact

that the mAbs specific for *P.hypophthalmus* IgM crossed reacted with other species is interesting. Based on mitochondria DNA of pangasiidae, there is a close phylogenetic relationship to *Hamibragus filamentus, Hamibragus wyckioides, Clarias bactracus*, *Clarias Macrocephalus* and *Cyprinus carpio* (Wong*, et al.* 2011; Sriphairoj*, et al.* 2010; Jondeung*, et al.* 2007). The anti- *P. hypophthalmus* IgM mAbs that cross-reacted with the other species recognized the H chain (mAbs 23 and 28) and the L (mAbs 1, 2 and 18) of the IgM molecule, and only mAb 7 reacted with both the H and L chains. However, the anti- *P. hypophthalmus* IgM mAbs recognized the heavy chain of the molecule (mAbs 23) better than the light chain (mAbs 2 and 18). Higher backgrounds were observed in ELISA with Na_2SO_4 precipitated IgM compared to affinity column purified IgM due to the non-specific material binding to the mAbs.

2.5 Conclusions

The results indicated that affinity column chromatography and Na_2SO_4 precipitation can both be used for IgM purification from the serum of *P. hypophthalmus*. Affinity chromatography resulted in purer IgM, but Na_2SO_4 precipitation (14%) reduces the cost and time of the purification process. The H and L chains of IgM from *P. hypophthalmus* were estimated to be 70-72 kDa and 25-26 kDa, respectively, using SDS-PAGE. The L chains of IgM from other Asian fish species were similar in molecular weight to *P. hypophthalmus*, while the H chains varied between species. The molecular weight of whole IgM (the tetramer) form *P. hypophthalmus* was determined to be 787 kDa using SV ultracentrifugation. This is a protein-only estimation, and when 7.8-11.4% carbohydrate composition was included the molecular weight was estimated to be 848-876 kDa. This is similar to the estimate made using H and L chains (832-860 kDa)

and between the molecular weights calculated using gel filtration (900 kDa) (Huong Giang*, et al.* 2012) and non-reducing gradient polyacrylamide gel electrophoresis (798 kDa) (Sudhagar*, et al.* 2013). The six anti- *P. hypophthalmus* IgM mAbs used reacted with other Asian fish species IgM, some reacting with the L chain and others with the H chain of the molecule, indicating common epitopes between the L and H chains of IgM of family Pangasiidae and other species. These mAbs provide useful tools for detecting and quantifying the immune response in these fish species and are used in subsequent chapters of this thesis.

Chapter 3 Standardisation of functional assays to evaluate immune response of *Pangasinodon hypophthalmus* and application of these to compare response to live and heat killed *Aeromonas hydrophila*

3.1 Introduction

Pangasianodon hypophthalmus is the most important fish species currently cultured in Vietnam. Aquaculture production of pangasius is also developing in other Asian countries such as Bangladesh, India, Indonesia, Malaysia, Myanmar, the Philippines and Thailand, however Vietnam still remains the largest supplier of pangasius catfish globally (Globefish 2012).

Outbreaks of bacterial diseases in *P. hypophthalmus* culture systems have a significant impact on production. Two of the main problems reported are bacillary necrosis of *Pangasianodon* (BNP) (Ferguson*, et al.* 2001) caused by *Edwardsiella ictaluri,* and motile aeromonas septicaemia (MAS)(Subagja*, et al.* 1999) used by *Aeromonas hydrophila*. The latter is a Gram negative, rod-shaped, motile, facultative anaerobe that is widely distributed in the aquatic environment (Banerjee*, et al.* 2012). The clinical signs of MAS include gastroenteritis, endocarditis, septicaemia and red sores (Roberts 2012).

Vaccination is, on economic, environmental and ethical grounds, the most appropriate method for controlling disease outbreaks caused by these pathogens. The concept behind vaccination is to stimulate the immune response of fish to develop a long-lasting immunological memory against the immunising pathogen. The ideal vaccine must be immunogenic and be able to stimulate components of the humoral and/or the cell-mediated immune response, but which is not pathogenic to the fish. There are many types of vaccines available or under development to protect fish against disease. Killed or inactivated vaccines made from the whole organism and inactivated through heat or chemical treatment such as formalin, are very effective in

stimulating a humoral antibody response and have been shown to be safe for this application, but are less effective at inducing a strong cell mediated immune response compared to live attenuated vaccines. Unlike live vaccines, which replicate in their host, killed vaccines often require multiple administration and/or adjuvants, and are often not affective against intracellular pathogens (Thompson and Adams 2004; Mak and Saunders 2005; Toranzo*, et al.* 2009; Tafalla*, et al.* 2013). On the other hand, a live attenuated vaccine is a live pathogen that has been rendered non-pathogenic or non-virulent by physical, chemical, or genetic modification (Shoemaker*, et al.* 2009), but still retains the ability to survive and replicate in its host. A single dose can produce long-lasting immunity, and is effective at inducing a strong cellular immune response against intracellular organisms. Unlike dead vaccines, in many countries live vaccines are not approved for use in aquaculture because of the risk of reversion to virulence by the attenuated pathogen (Tafalla*, et al.* 2013).

The ability of many different types of vaccine preparations to protect fish against infection by *A. hydrophila* have been tested, based on dead pathogens, killed using formalin or heat (*i.e.* whole bacterial cells) or isolated non-replicating pathogen subunits such as outer membrane proteins, lipopolysaccharide (LPS), extra-cellular proteins, recombinant S-layer protein, biofilms and or live attenuated vaccines (Ruangpan*, et al.* 1986; Azad*, et al.* 1999; Nayak*, et al.* 2004; Poobalane*, et al.* 2010; Viji*, et al.* 2013).

The potential of these different *A. hydrophila* vaccines has been investigated in a variety of fish species. Azad et al. (1999), for example, assessed an *A. hydrophila* biofilm vaccine in three different carp species, *i.e.* common carp (*Cyprinus carpio* Lin.),

Indian carp (*Catla catla Ham.*) and rohu (*Labeo rohita Ham.*), while Nayak, et al. (2004) later tested the efficacy of an oral biofilm *A. hydrophila* vaccine in Asian catfish (*Clarias batrachus* L.). They found that the serum agglutinating antibody titre and the relative percentage survival of the vaccinated fish challenged with *A. hydrophila,* was significantly higher than fish immunised orally with free bacterial cells. More recently a study was performed with goldfish (*Carassius auratus*), in which fish were immunised with either a whole cell preparation, extracellular products (ECPs), outer membrane protein or a biofilm vaccine prepared from a virulent isolate of *A. hydrophila* (Viji*, et al.* 2013b). Nile tilapia (*Oreochromis niloticus* L.) (Ruangpan*, et al.* 1986) and channel catfish (*Ictalurus punctatus*)(Schachte 1978) have also been used as model fish species to evaluate *A. hydrophila* vaccines. A more recent study was performed by Dehghani *et al*., (2012) in which they used a bivalent vaccine against *A. hydrophila* and *A. veronei* bv. *sobria* consisting of formalin-killed, heat-killed and LPS preparations of the bacteria, tested in rainbow trout (*Oncorhynchus mykiss*). Relative percentage survival in the fish vaccinated with the heat-killed vaccine was significantly higher than seen with the other two vaccine preparations.

Early in the 1990s, live bacterial vaccines were trialled against *Vibrio anguillarum* (Norqvist*, et al.* 1994), *A. salmonicida* (Thornton*, et al.* 1991; Thornton*, et al.* 1994), then later against *Edwardsiella ictaluri* (Wise and Terhune 2001), *E. tarda* (Takano*, et al.* 2010), *Nocardia seriolae* (Itano*, et al.* 2006) and *Streptococcus iniae* (Locke*, et al.* 2010). Pridgeon and Klesius (2011), found significantly different levels of protection and antibody titres between Nile tilapia and channel catfish vaccinated intra-peritoneally with *A. hydrophila* mutants, based on the resistance of *A. hydrophila*

to novobiocin and rifampicin. A number of other live vaccines have been developed for other fish pathogens based on antibiotic resistance genes. LaFentz, *et al*. (2008), for example, selected a rifampicin resistant *F. psychrophilum* isolate that was successfully used as a live attenuated vaccine for the prevention of bacterial cold water disease (BCWD). A modified live *Flavobacterium columnare* vaccine was developed by repeatedly passaging a virulent strain of the bacterium with increasing concentrations of rifampicin. Administration of the vaccine to early life-stage channel catfish (*i.e*. 10 days post-hatch) or largemouth bass (*Micropterus salmoides*) proved safe and reduced levels of mortalities were obtained when fish were experimentally infected with *F. columnare* (Shoemaker*, et al.* 2011), and similarly a live attenuated *esrB* mutant of *E. tarda* gave significant protection against the wild-type strain of *E. tarda* in turbot (*Scophthamus maximus*) (Lan*, et al.* 2007). Shoemaker *et al*., (1999) and Klesius and Shoemaker (1999) found the modified live *E. ictaluri* RE-33 vaccine stimulated protective immunity against enteric septicaemia in channel catfish.

Several studies examining resistance to infection following administration of either live or dead *A. hydrophila* have been carried out in various fish species *e.g*. rainbow trout (Loghothetis and Austin 1994; Dehghani*, et al.* 2012), roho (Vasudeva Rao*, et al.* 2006), indian carp *i.e*. *Catla catla, Labeo rohita* and *Cirrhunas mrigala* (Karunasagar*, et al.* 1991), Asian catfish (Kumari and Sahoo 2006a), zebrafish (*Danio rerio*) (Rodriguez*, et al.* 2008), carp (Selvaraj*, et al.* 2005) and European eel (*Anguilla anguilla*) (Song-Lin*, et al.* 2012), but few of these studies have compared the effect of live and dead *A. hydrophila* on the immune response of fish, and none have used striped catfish as their model fish species. Bich Hang, et al. (2012) showed that LPS

from outer membrane protein of Gram negative bacteria stimulated a variety of immune parameters in striped catfish (*i.e.* haematology parameters, lysozyme activity, the alternative complement pathway and total plasma immunoglobulin levels).

As *P. hypophthalmus* is a relatively new aquaculture species, there are few reports evaluating their immune response to pathogens, either dead or alive. The aim of this study was to standardise functional assays to evaluate both innate and adaptive immune responses of *P. hypophthalmus* and to use these assays to compare their immune response to live and dead *A. hydrophila.*

3.2 Materials and methods

3.2.1 Experimental animals

Pangasianodon hypophthalmus were purchased from a local fish farm in Nakornsawan Province, Thailand and transported to the Aquatic Animal Laboratory, Faculty of Veterinary Sciences, Mahidol University, Bangkok. The fish were quarantined and acclimated to laboratory conditions for two weeks prior to use, and were fed with a commercial diet (Inteqc Feed Company, Thailand) at 3 % body weight per day. The weight of the fish at the start of the experiment was 53.2 ±14.8 g.

3.2.2 Experimental design

The experimental design consisted of four treatment groups, and three replicate tanks (40 fish/replicate) were used for each treatment. Fish were maintained in 500 L recirculation tanks with a water temperature of 28 ±2^{O}C and a photoperiod consisting of a 12:12 h artificial light regime. Water quality parameters *i.e*. dissolved oxygen, NH_3, pH and temperature were checked daily, and 20-30% water exchanges were also made each day. *A. hydrophila,* isolate T4 (provided by the Institute of Aquaculture, University

of Stirling), was cultured in tryptone soy agar (TSA, Oxoid England) for 18-24 h. Three to five colonies of *A. hydrophila* were picked from the plate and placed into 100 ml of sterile tryptic soya broth (TSB). The bacteria were grown to late logarithmic growth in a shaking incubator (200 rpm, 28^{o}C) (Incu-shaker^{TM} 10L; Benchmark, USA) for 18-24 h. The bacterial concentration was determined using spectrophotometer $OD_{600\ nm}$ and confirmed using a plate counting method. The fish were injected intraperitoneally (0.1 ml per fish) with either tryptic soya broth (TSB; Merk-Datmstadt) as negative control group, adjuvant (Montanide ISA 760 VG), or heat-killed *A. hydrophila* 1 x10^{9} cfu ml^{-1} mixed with the adjuvant. Another group was injected with a subclinical dose of live *A. hydrophila* (2.7 x10^{5} cfu ml^{-1}). The fish were maintained for 21 days before assessing the effect of these treatments on various immune parameters of the fish. Many of these methods were not previously described for Pangasius, therefore the assays were optimised and standardised for this species prior to use.

3.2.3 Experimental plan

Two fish per tank (6 fish per group) were sampled to assess their immune response on Day 0, 1, 3, 7, 14 and 21 post-treatment. Blood and head kidney samples were collected from these fish for the various haematological and immunological analyses outlined in Figure 3.1.

3.2.4 Haematological and immunological analysis

Blood samples (1 ml) were taken from the caudal vein of fish using disposable syringes (3 ml) flushed with heparin (Sigma, UK). These were divided into two aliquots, one sample for white and red blood cell (RBC) counts and different white blood cell (WBC) counts, and the other for plasma collection. For the plasma collection, blood was

centrifuged at 3,000 x g for 5 min and once separated stored at -70^{o}C for further analysis (*i.e*. lysozyme activity, total plasma IgM, complement activity, plasma peroxidase activity and specific antibody titre against *A. hydrophila*).

3.2.4.1 Haematological analysis

3.2.4.1.1 Haematocrit values

Haematocrit values or red blood cell packed volume were determined according to the method of Wells and Weber (2006). Capillary tubes were filled with blood and sealed at one end with clay. The filled tubes were then centrifuged at 10,000 to 15,000 x g for 5 min in a micro-haematocrit centrifuge. The haematocrit values were expressed as a percentage of the packed cell volume (Briggs and Bain, 2011), while the Mean Corpuscular volumes (MCV) were determined using Equation 3.1.

Equation 3.1 MCV (fl)

$$MCV = Hct\ (\%)\ x\ \frac{10}{RBC\ count\ (10^6/mm^3)}$$

3.2.4.1.2 White blood cell and red blood cell counts

White blood cell and RBC counts were measured as described by Natt and Herrick (1952). Blood (20 µl) was added to 4 ml Natt-Herricks's solution and mixed thoroughly. A haemocytometer was filled with the blood suspension (10 µl), which was allowed to settle for 2-3 min before counting the RBCs as shown in Figures 3.2(A and B) and the WBC in Figures 3.3(A and B). The RBC numbers were determined using Equation 3.2, while WBC numbers were determined using Equation 3.3.

3.2.4.1.3 Differential WBCs counts

Differential WBC counts were made according to Nussey, *et al*. (1995). Blood smears were prepared and allowed to air dry, before fixing with methanol for 3-5 min. The

slides were then stained with Giemsa (IVD, UK) 5 % (v/v) Giemsa's azure eosin methylene blue solution in buffer solution (see Appendix 1) for 30 min (this time giving the best staining of blood smears), rinsed two times with buffer solution for 1 min, before air drying the slides and mounting them with Pertex® (Cellpath, UK). The cells were examined under a light microscope (x100) and the number of different WBCs present in 200 cells was counted (Figure 3.4(A-F)). Cells morphology was distinguished using the key shown in Table 3.1.

Table 3.1. Red and white blood cell morphology with Giemsa stain

Cell type	Giemsa stain
Nuclei	red to violet
Lymphocytes	plasma blue
Monocytes	plasma dove-blue
Neutrophilic granulocytes	granules light violet
Eosinophilic granulocytes	granules red to grey-blue
Basophilic granulocytes	granules dark-violet
Thrombocytes	violet
Erythrocytes	reddish

3.2.4.2 Kidney macrophage activity

3.2.4.2.1 Isolation of *P. hypophthalmus* head kidney macrophages

Macrophages were isolated using the method of Secombes (1990). Fish were overdosed using anesthetic (200 mg/l Benzocaine; Sigma, UK) and fish bled to reduce red blood cell contamination. The head kidney was isolated aseptically and placed on to a piece of sterile 100 µm nylon mesh placed over a small Petri dish containing 3 ml of L-15 medium (Leibovitz; Sigma, UK) with 10 µl heparin (Sigma, UK). The head kidney was teased through the mesh with the blunt end of the syringe plunger to form a cell suspension. The mesh was washed with a further 2-3 ml of L-15 medium. The cell suspension was placed into a bjoux and kept on ice until used in the phagocytosis activity and respiratory burst activity assays outlined below.

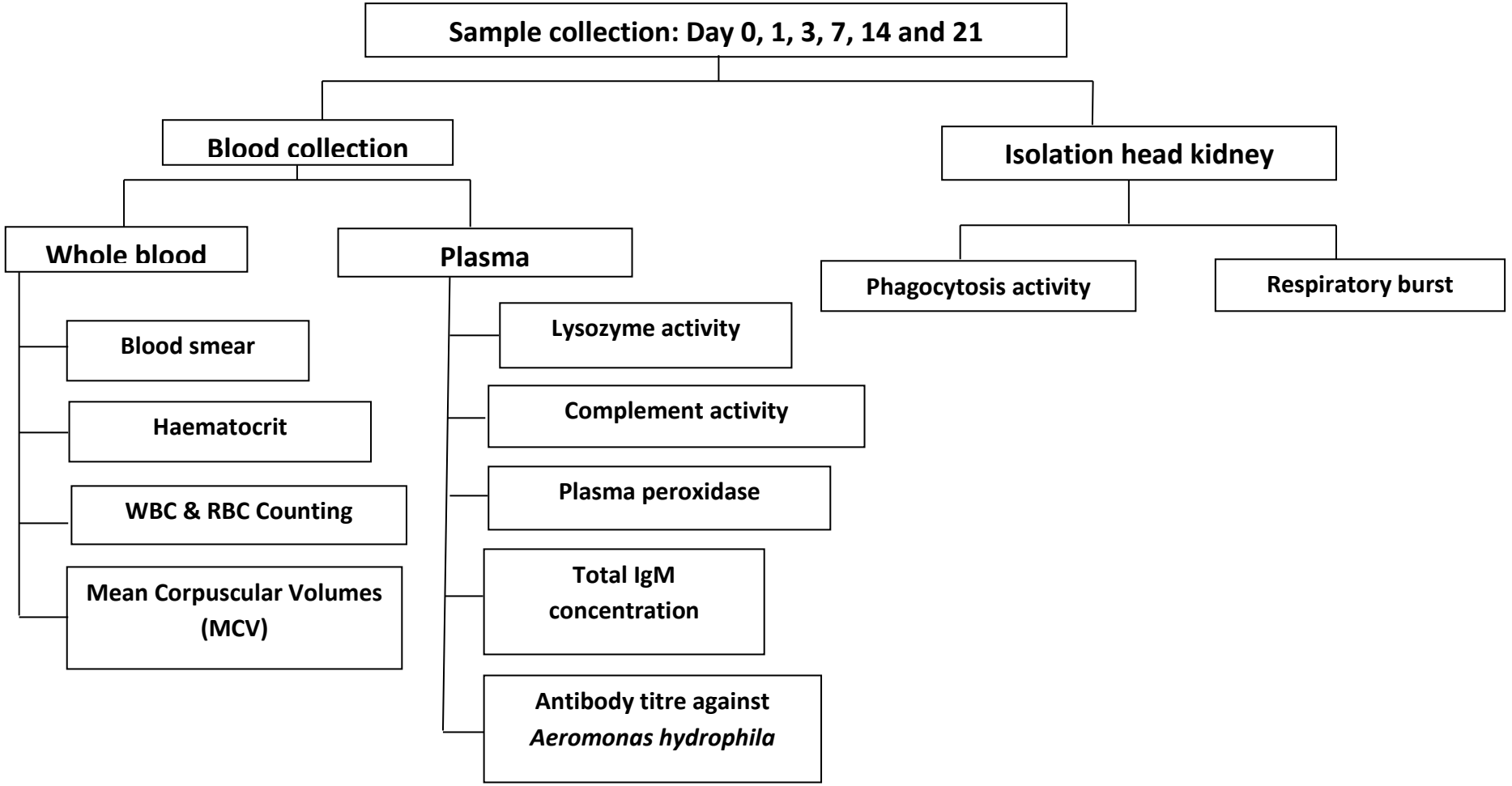


Figure 3.1. The sampling regime and immunological analysis performed with blood and head kidney from *Pangasius hypophthalmus*

(A) (B)

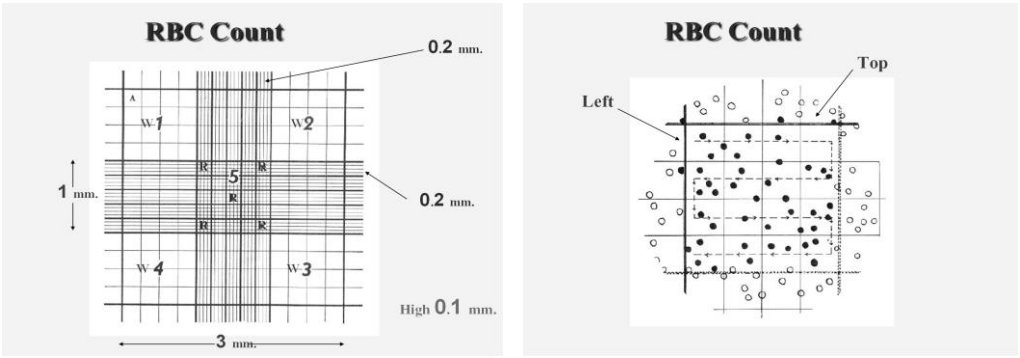


Figure 3.2(A-B). (A) The RBC counted area (5 squares) in haemocytometer under microscope (x4) and (B) the RBC counted area (1 square) in haemocytometer under microscope (x40).

Equation 3.2 Red blood cell count

$$RBC\ count$$
$$= No.of\ cells\ counted\ (N) x Volume\ factor\ (= 50) x\ Dilution\ factor (= 200\ mm^3)$$
$$= N\ x\ 10{,}000$$

(A) (B)

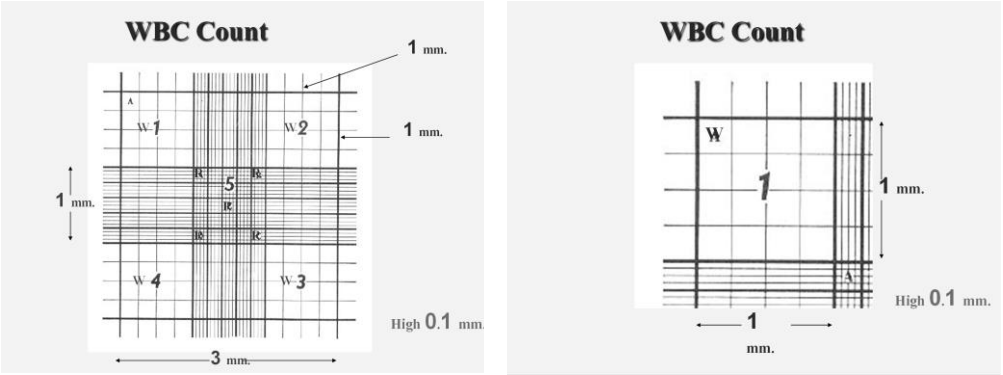


Figure 3.3(A-B). (A) The WBC counted area (4 squares) in haemocytometer under microscope (x4) and (B) the WBC counted area (1 square) in haemocytometer under microscope (x10).

Equation 3.3 White blood cell count

$$WBC\ count$$
$$= No.of\ cells\ counted\ (N) x Volume\ factor\ (= 2.5) x\ Dilution\ factor (= 200\ mm^3)$$
$$= N\ x\ 500$$

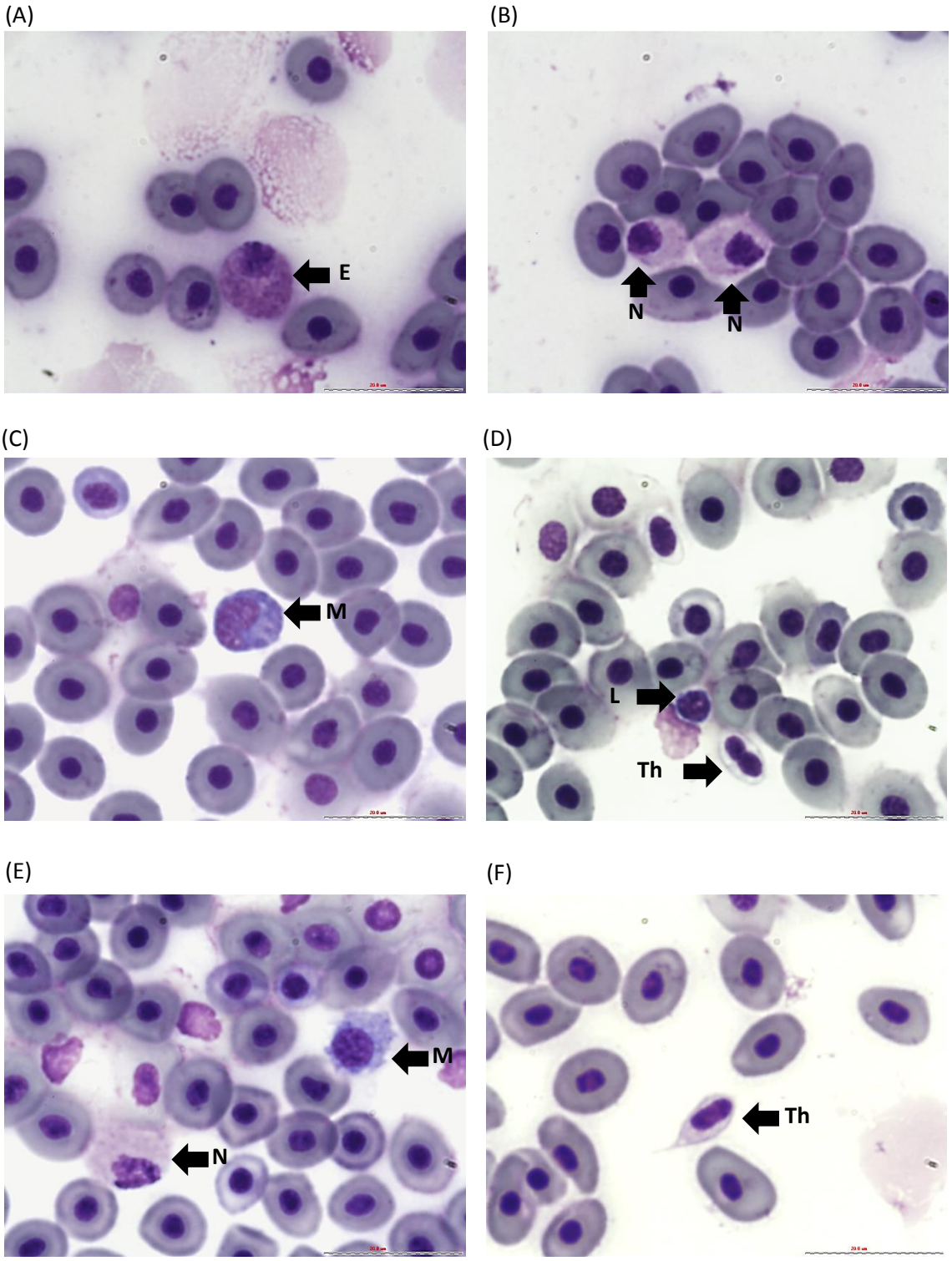


Figure 3.4(A-F). The morphology of differential WBCs counts (x100); (A) E = Eosinophil; (B) N = Neutrophil; (C) M = Monocyte; (D) L = Lymphocyte, Th = Thrombocyte; (E) M = Monocyte, N = Neutrophil; (F) Th = Thrombocyte.

3.2.4.2.2 Phagocytosis by *P. hypophthalmus* head kidney macrophages

Phagocytosis by head kidney macrophages was performed using the method of Thompson (1996). The slides were cleaned with absolute ethanol and two large circles drawn on the slide using a Pap Pen (Vector, CA). The cell suspension (100 µl) was placed into each circle and the slide placed in a moist incubation chamber for 1 h at room temperature (28-30^{o}C) to enable macrophages to attach to the slide. Slides were then washed with L-15 to remove non-adherent cells. The yeast was re-suspended in L-15 medium at 0.5 % (w/v), made up immediately prior to use, and 100 µl placed onto one circle of attached cells and L-15 (100 µl) placed onto the second circle. The slide was incubated for 1 h at room temperature to allow phagocytosis to take place and then washed with L-15 medium. The cells were fixed with 100 % methanol (100 µl) for 5 min then washed five times with 70 % methanol. The cells were finally stained with Giemsa stain, air dried and mounted with Pertex. The cells were examining under oil immersion (×100) and the number of macrophage cells containing yeast out of 200 macrophages in total counted. The phagocytic activity (PA), phagocytic index (PI) and phagocytic capacity (PC) were determined according to (Findlay and Munday 2000), using the equations outlined below.

Equation 3.4 Phagocytic Activity (PA)

$$PA = \frac{Mean\ average\ of\ yeast\ cells\ engulfed\ by\ active\ macrophage}{Total\ number\ of\ macrophages\ with\ engulfed}\ x\ 100$$

Equation 3.5 Phagocytic Index (PI)

$$PI = \frac{Total\ number\ yeast\ cells\ engulfed}{Number\ of\ macrophage\ counted}\ x\ 100$$

Equation 3.6 Phagocytic Capacity (PC)

$$PC = \frac{Total\ number\ of\ macrophages\ containing\ a\ given\ number\ of\ yeast\ cells}{Total\ number\ of\ macrophages\ with\ engulfed\ yeast}\ x\ 100$$

3.2.4.2.3 Respiratory burst of head kidney macrophage assessed using Nitroblue tetrazolium (NBT)

Respiratory burst by head kidney macrophages was performed using the method of Secombes (1990). The macrophage suspension prepared from head kidney was added to a 96 well tissue culture plate (100 µl per well), using at least 8-10 wells per fish. The macrophage monolayer was incubated for 2 h at room temperature. During this time nitroblue tetrazolium (NBT 1mg ml^{-1}; Sigma, UK) solution with and without phorbol myristate acetate (PMA 1µg ml^{-1}; Sigma, UK) were prepared. The non-adherent cells were removed by washing the plate three times in L-15 medium, before adding 100 µl of NBT solution to three replicate wells and 100 µl of NBT solution containing PMA to another three replicate wells, and 100 µl lysis buffer to two remaining wells to determine the number of attached cells. The plates were incubated for 1 h at 22^{o}C. Meanwhile the number of adherent cells was determined by counting the number of released nuclei with a haemocytometer (using the wells containing the lysis buffer), incubating with the buffer for at least 2 min. The respiratory burst reaction was stopped after 1 h by fixing the cells with 100 % (v/v) methanol. The plates were washed three times with 70 % (v/v) methanol and wells allowed to air dry. The insoluble formazan in the wells was dissolved by adding 120 µl 2 M KOH (BDH, UK) and 140 µl DMSO (Sigma, UK). The content of each well was mixed carefully and air bubbles removed with a needle. The absorbance of the wells was measured at 610 nm

using a micro-plate reader (Synergy HT; Bio Tek Instruments, Winooski, VT, USA) and the results expressed as an absorbance at 610 nm for 10^5 cells. The reactive oxygen species (ROS) stimulation index was also determined according to Vowells, et al. (1995) and Skouras (2002) using Equation 3.7.

Equation 3.7 Stimulation index (SI)

$$SI = \frac{Mean\ PMA\ triggered\ ROS\ [OD]}{Mean\ unstimulated\ ROS}$$

3.2.4.3 Analysis of immunological plasma parameters

3.2.4.3.1 Lysozyme activity

Plasma samples collected from the fish were used to measure lysozyme activity using the method of Morgan, *et al.* (2008). Sodium phosphate buffer, pH 5.4 (0.04 M $NaH_2PO_4.2H_2O$ (Sigma, UK) and 0.04 M $Na_2HPO_4.2H_2O$ (Sigma, UK) was warmed to 30°C for 30 min. A suspension of 0.2% (w/v) *M. lysodeikticus* (Sigma, UK) was prepared in the sodium phosphate buffer. Sodium phosphate buffer (without bacteria) was added to columns 11 and 12 of a microtitre plate (200 µl) as a negative background control. Plasma samples (10 µl) were added to four replicate wells and 190 µl of sodium phosphate buffer then to the wells. The reduction in absorbance at 540 nm was measured at 1 min and 5 min. One unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance at 0.001/min.

3.2.4.3.2 Total plasma immunoglobulin M assay

The total immunoglobulin M was measured in plasma using the method of Magnadóttir and Gudmundsdottir (1992). The concentration of plasma IgM was measured using ELISA. A standard curve of IgM (purified IgM 1.54 mg ml^{-1}) was diluted

making two-fold series dilutions (0-0.32 mg ml^{-1}) with coating buffer (Sigma, UK) and 100 µl of each dilution added to two replicate wells of a 96 well Immulon™ ELISA plate (Thermo Scientific, Maine, USA). Three dilutions of the plasma samples were prepared in coating buffer (*i.e*. 1/6,400, 1/12,800 and 1/25,600) and 100 µl of each dilution added to duplicate wells. The ELISA plates were incubated overnight at 4°C, then washed five times with low salt water buffer (LSWB; 0.02M Trizma base, 0.38 M NaCl, 0.05% (v/v) Tween 20, pH 7.2). Plates were post-coated (to block non-specific binding sites) with 250 µl $well^{-1}$ 1% (w/v) bovine serum albumin (BSA; Sigma, UK) added and incubated for 1-2 h at 25°C or overnight at 4°C. The blocker was discarded from the wells by tapping of the plates onto paper tissue. Anti-*P. hypophthalmus* IgM monoclonal (mAb 23) supernatant (as described in Chapter 2) was added to the plate at 100 µl $well^{-1}$ and incubated for 1 h at room temperature (25°C). The plates were washed five times with high salt water buffer (HSWB; 0.02 M Trizma base, 0.5 M NaCl, 0.01% (v/v) Tween 20, pH 7.4) incubating for five min on the last wash, before adding 100 µl $well^{-1}$ goat anti-mouse IgG-HRP diluted to 2 µg ml^{-1} in conjugate buffer. The plates were incubated for 60 min at 25°C and washed 5 times with high salt buffer, incubating for 5 min on the last wash. The reaction was developed with 100 µl $well^{-1}$ 42 µM 3'3'5'5'-tetramethylbenzidine dihydrochloride (TMB; Sigma, UK) in substrate buffer containing 0.1 % hydrogen peroxide (Sigma, UK) for 10 min at 25°C. The assay was stopped by the addition of 50 µl 2 M sulphuric acid (H_2SO_4; BDH, UK) per well. The optical density (OD) was read at 450 nm using a microplate reader (Synergy HT; Bio Tek Instruments, Winooski, VT, USA), and the unknown IgM concentrations in the plasma

samples extrapolated from the straight portion of the standard curve graph of IgM against $OD_{450\ nm}$.

3.2.4.3.3 Alternative complement activity assay

The alternative complement activity based on spontaneous haemolytic activity was a modification of the method outlined by Sakai, (1992) and Langston, *et al*. (2001). Briefly, de-fibrinated sheep red blood cells (SRBC, Oxoid®, UK) were used as target cells at 0.5 % (a final concentration of 2.5 $x10^8$ cells ml^{-1}). Plasma samples were diluted two fold in 0.1 % gelatine-complement fixation buffer (0.1 % G-CFB) and 25 µl of each dilution was added to duplicate wells of a non-absorbent U-well micro-plate (Sterillin®). The SRBC suspension (10 µl) was then added to each well. To the control (100 % lysis) was added 0.1 % (v/v) anhydrous Na_2CO_3 (Sigma, UK) instead of plasma, for the 0 % lysis control; G-CFB was added in place of the plasma sample. Reference wells were set up in duplicate wells with the plasma dilutions and G-CFB but no SRBC suspension. Then micro-plates were incubated at 28^oC for 90 min with constant shaking and the reaction terminated by the addition of 140 µl G-CFB with 20 mM EDTA and centrifuged to pellet the remaining SRBC. Supernatant from the wells (100 µl) was transferred to a new flat-bottomed 96-well non-absorbent micro-titre plate (Sterillin®) and read at 450 nm by using a micro-plate reader (Synergy HT; Bio Tek Instruments, Winooski, VT, USA). The percentage lysis of SRBCs was calculated. The absorbance values of samples were corrected by subtracting the absorbance of the sample blank control (0 % haemolysis). A graph of log x (x = concentration of plasma) (ordinate axis) vs log y/ (1-y) (y = % SRBC haemolysis) (abscissa axis) was drawn and after estimating the volume of plasma giving 50 % haemolysis

($SH_{50\%}$), and the $SH_{50\%}$/ml of plasma calculated by dividing the dilution factor of plasma with the estimated plasma volume causing 50 % SRBCs lysis expressed per ml.

3.2.4.3.4 Plasma peroxidase

The plasma peroxidase was measured using a modification of the method outlined by Quade and Roth (1997) and Sitjà-Bobadilla, *et al.* (2005) The plasma samples were added (15 µl $well^{-1}$) to wells of a flat bottomed 96 well plate together with 135 µl $well^{-1}$ HBSS without Ca^{2+} and Mg^{2+} (Sigma, UK) before adding 50 µl $well^{-1}$ of 20 mM TMB in substrate buffer (40 mM acetic acid) containing 0.1 % hydrogen peroxide (Sigma, UK). The reaction was stopped with 50 µl $well^{-1}$ of 2 M H_2SO_4 and read with an ELISA reader at 450 nm.

3.2.4.3.5 Antibody titre against *Aeromonas hydrophila*

The specific antibody response of *P. hypophthalmus* to *A. hydrophila* was measured using a modification of the method by Adams *et al*. (1995). Briefly, a 96-well ELISA plate (Thermo Scientific, Maine, USA) was coated with 50 µl $well^{-1}$ 0.05 % (w/v) poly-L-lysine (Sigma, UK) in coating buffer (Sigma, UK) for 60 min at 25^{o}C. The plates were then washed twice with low salt wash buffer. A bacterial suspension of *A. hydrophila* at a concentration of 1 x10^{8} bacteria ml^{-1} in PBS was added to the wells at 100 µl $well^{-1}$. The plates were incubated overnight at 4^{o}C, after which 50 µl $well^{-1}$ of 0.05% (v/v) gluteraldehyde (Sigma, UK) in PBS was added to the plate and incubated for 20 min at 22^{o}C. The plates were washed 3 times with low salt water buffer and post-coated with 1 % (w/v) BSA in water by adding 250 µl $well^{-1}$ and incubating for 2 h at 22^{o}C. The plates were then washed 3 times with LSWB. Doubling-dilutions of plasma samples were prepared in PBS from 1/32 to 1/16,384. Both PBS and pre-immune plasma samples from non-vaccinated fish were used as a negative control. Samples were

added to wells (100 µl well^{-1}) and incubated overnight at 4^{o}C, and then the plates were washed 5 times with HSWB, incubating for 5 min on the last wash. The presence of *P. hypophthalmus* IgM was detected by adding 100 µl well^{-1} of anti-*P. hypophthalmus* IgM mAb supernatant (mAb 23) and incubating for 60 min at 22^{o}C and the plate was washed again as described previously. Goat anti-mouse IgG-HRP (Sigma, UK), diluted 1/4000 diluted in conjugate buffer was added to the plate at 100 µl well^{-1} for 60 min at 22^{o}C. The plates were washed with HSWB as above. The assays were developed with 100 µl well^{-1} of chromogen in substrate buffer as described in Section 3.2.4.3.2. After incubating for 10 min at 22^{o}C, the reaction was stopped by the addition of 50 µl well^{-1} of 2 M H_2SO_4 and the absorbance measured at 450 nm on a micro-plate reader (Synergy HT; Bio Tek Instruments, Winooski, VT, USA). The antibody titre was defined as the reciprocal of the highest dilution (1/x dilution) showing an absorbance at least two times greater than the negative control.

3.2.5 Statistical analysis

Data were examined using a one-way analysis of variance (ANOVA), general linear model, and pairwise comparison (Turkey) of means. All statistical tests were performed using Minitab software (version 16 © University of Stirling, 2013). Differences were considered statistically significant at $P<0.05$. Statistical differences were examined between groups at each sampling point and within groups over time.

3.3 Results

Functional assays were optimised and standardised for *Pangasianodon hypophthalmus* and then these were used to measure innate and adaptive immune responses of *P. hypophthalmus* following injection with live and killed *A. hydrophila.* The haematological

parameters examined in the blood of these fish included haematocrit values, RBC and WBC counts, differential WBC counts and MCV values, while plasma was used to assess lysozyme, complement and plasma peroxidase activity, total IgM concentration and specific antibody titres against *A. hydrophila*. Phagocytic and respiratory burst activities were determined for head kidney macrophages.

3.3.1 Haematological parameters

Of the haematological parameters measured, no significant differences were seen in haematocrit values, total RBC counts or MCV values for any of the time points analysed (Figure 3.5(A-C)), while significant differences were seen in total WBC counts between fish infected with live *A. hydrophila* and non-infected fish at 3, 7 and 21 days post infection (d.p.i.) ($P<0.05$) (Figure 3.5(D)). In general, the fish injected with live *A. hydrophila* had the highest WBC counts.

The differential WBC counts for the four experimental groups are shown in Figure 3.6. Significant differences in the percentage of monocytes were seen on 0 and 1 d.p.i. with the control group having the highest values (Figure 3.6(A)). The levels of neutrophils seen at 3 d.p.i. in fish injected with adjuvant were significantly higher than fish injected with killed *A. hydrophila*. At 14 d.p.i. fish injected with live *A. hydrophila* showed the highest level between groups and at 21 d.p.i. fish injected with either adjuvant, killed or live *A. hydrophila* were all higher than the control group (Figure 3.6(B)). At 1 d.p.i. the percentages of lymphocytes in the groups injected with adjuvant and both killed and live *A. hydrophila,* were higher than the control fish. On 3 d.p.i. the groups injected with killed and live *A. hydrophila* were higher than fish injected with adjuvant, while on 14 d.p.i the control fish had higher levels of lymphocytes than fish injected with live *A. hydrophila* and

by 21 d.p.i. the control group had the highest levels compared to the other three groups (Figure 3.6(C)). The percentage of thrombocytes in the blood of fish injected with killed *A. hydrophila* and control fish were higher than the adjuvant group at 1 and 7 d.p.i (Figure 3.6(D)).

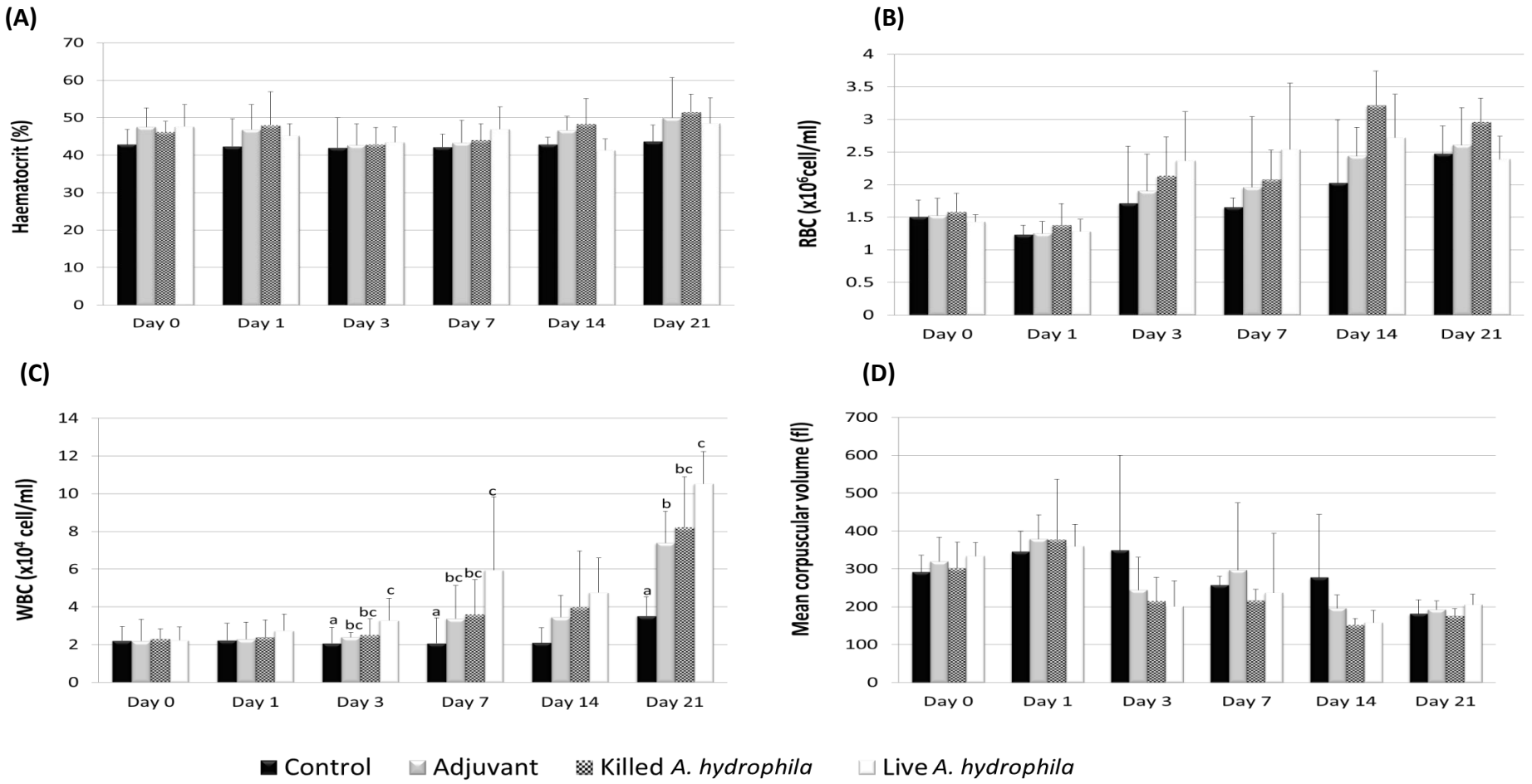


Figure 3.5(A-D). Comparison of (A) blood haematocrit value (%); (B) mean corpuscular volume (MCV) (fl); (C) total red blood cell counts (x10^{6}cell ml^{-1}); and (D) total white blood cell (x10^4 cell ml^{-1}) between experimental groups. Different letters indicate significant differences (P<0.05) between groups for a given time point (Mean±SD, n=12).

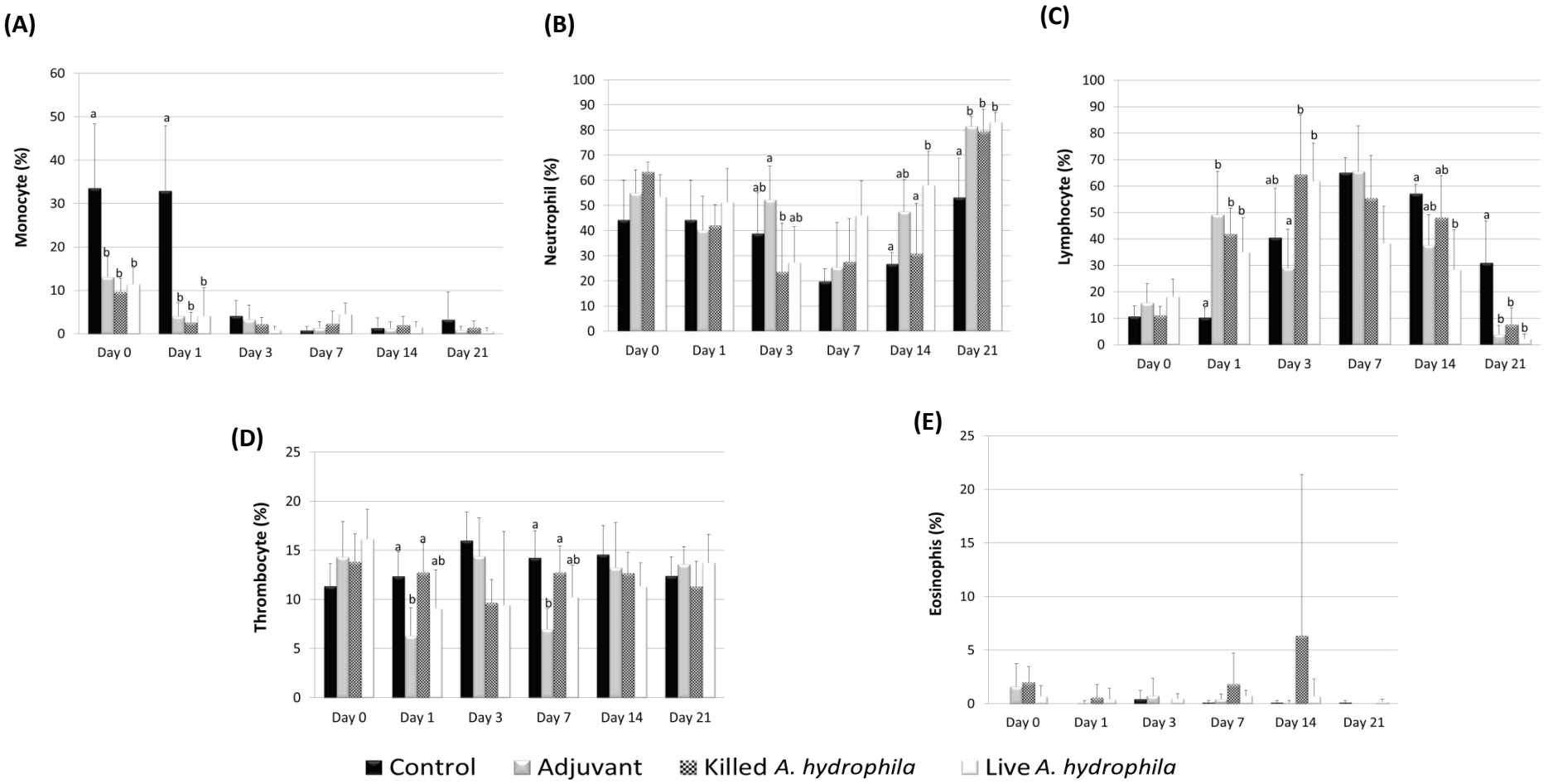


Figure 3.6(A-E). Comparison of different white blood cell counts (%) between experimental group (A) monocytes, (B) neutrophils, (C) lymphocytes, (D) thrombocytes and (E) eosinophils. Different letters indicate significant differences (P<0.05) between groups (Mean±SD, n=12).

3.3.2 Head kidney macrophage activities

3.3.2.1 Phagocytic activity of head kidney macrophages

The phagocytic activity of head kidney macrophages was significantly different between the control group and fish infected with live *A. hydrophila* on 7 and 14 d.p.i. ($P<0.05$) (Figure 3.7(A)). By 21 d.p.i. the phagocytic index of this group was also higher than the other groups ($P<0.05$) as shown in Figure 3.7(B). Also, the phagocytic capacity (containing of 6 yeast cells and 6+ yeast cells) of live *A. hydrophila* on 21 d.p.i. was significantly different between the control groups ($P<0.05$) as shown in Figure 3.7(H).

3.3.2.2 Respiratory burst activity of macrophages

The respiratory burst activity of macrophages collected from the group of fish infected with live *A. hydrophila* was significantly different ($P<0.05$) to the control group on 7 d.p.i. (Figure 3.8(A)). When PMA was included in the assay the respiratory burst activity with PMA was significantly different ($P<0.05$) between the groups on 3, 7, 14 and 21 d.p.i. from the onset of injection (Figure 3.8(B)). The macrophages from fish infected with live *A. hydrophila* had a higher level of respiratory burst activity in the presence of PMA than control groups on 3, 7 and 14 d.p.i. However, on 21 d.p.i. the macrophages from fish injected with killed *A. hydrophila* had the highest level of respiratory burst activity with PMA. Generally, live *A. hydrophila* induced the highest level of respiratory burst activity both with and without PMA within 2 weeks of injection, followed by the group injected with killed *A. hydrophila.* The reactive oxygen species (ROS) stimulation index displayed by macrophages from fish injected with killed and live *A. hydrophila* were higher than the control and the adjuvanted group on 7 d.p.i.

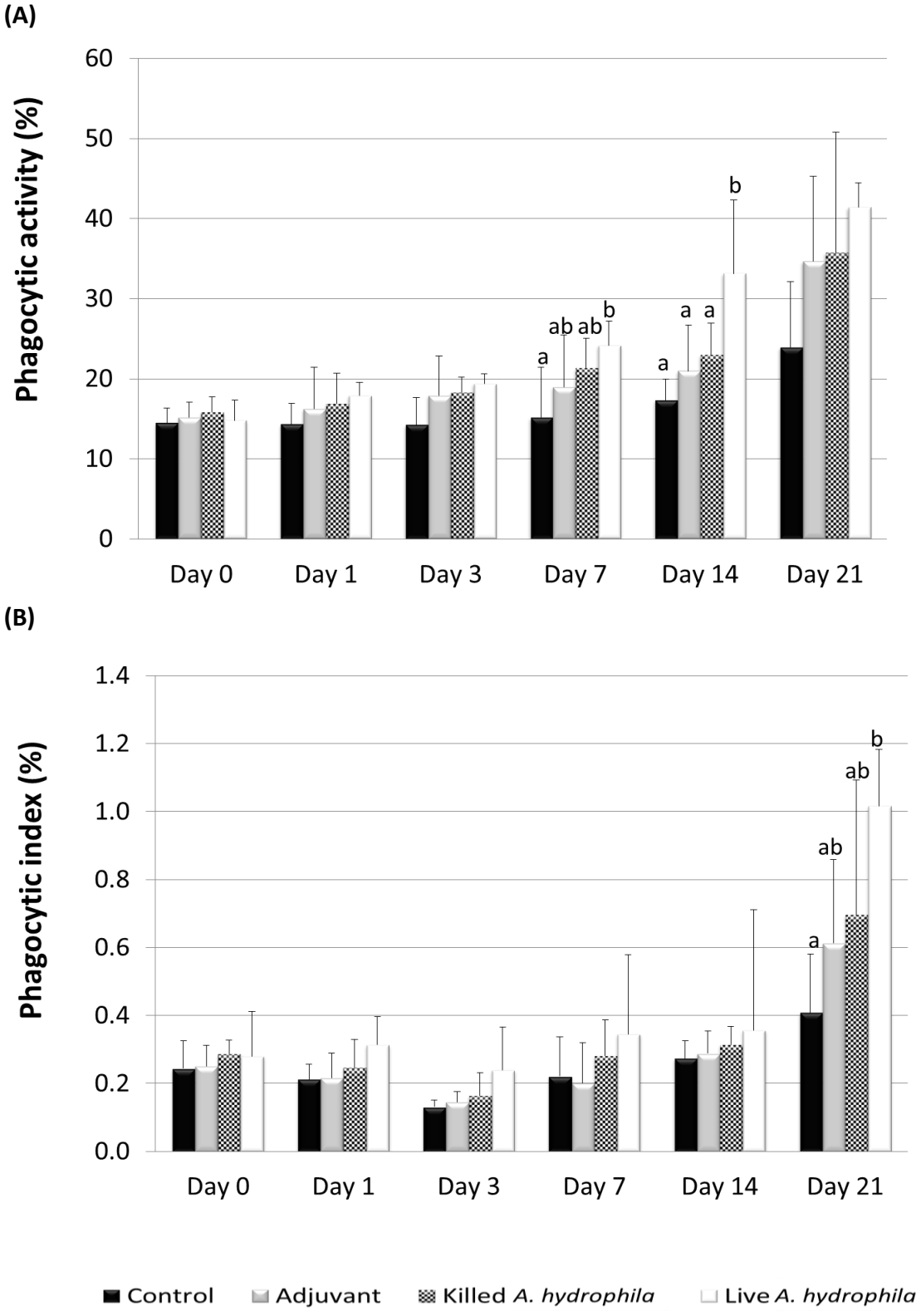


Figure 3.7(A-B). Comparison of phagocytic activity (A), phagocytic index (B) between experimental groups. Different letters indicate significant differences (P<0.05) between groups for a given time point (Mean±SD, n=12).

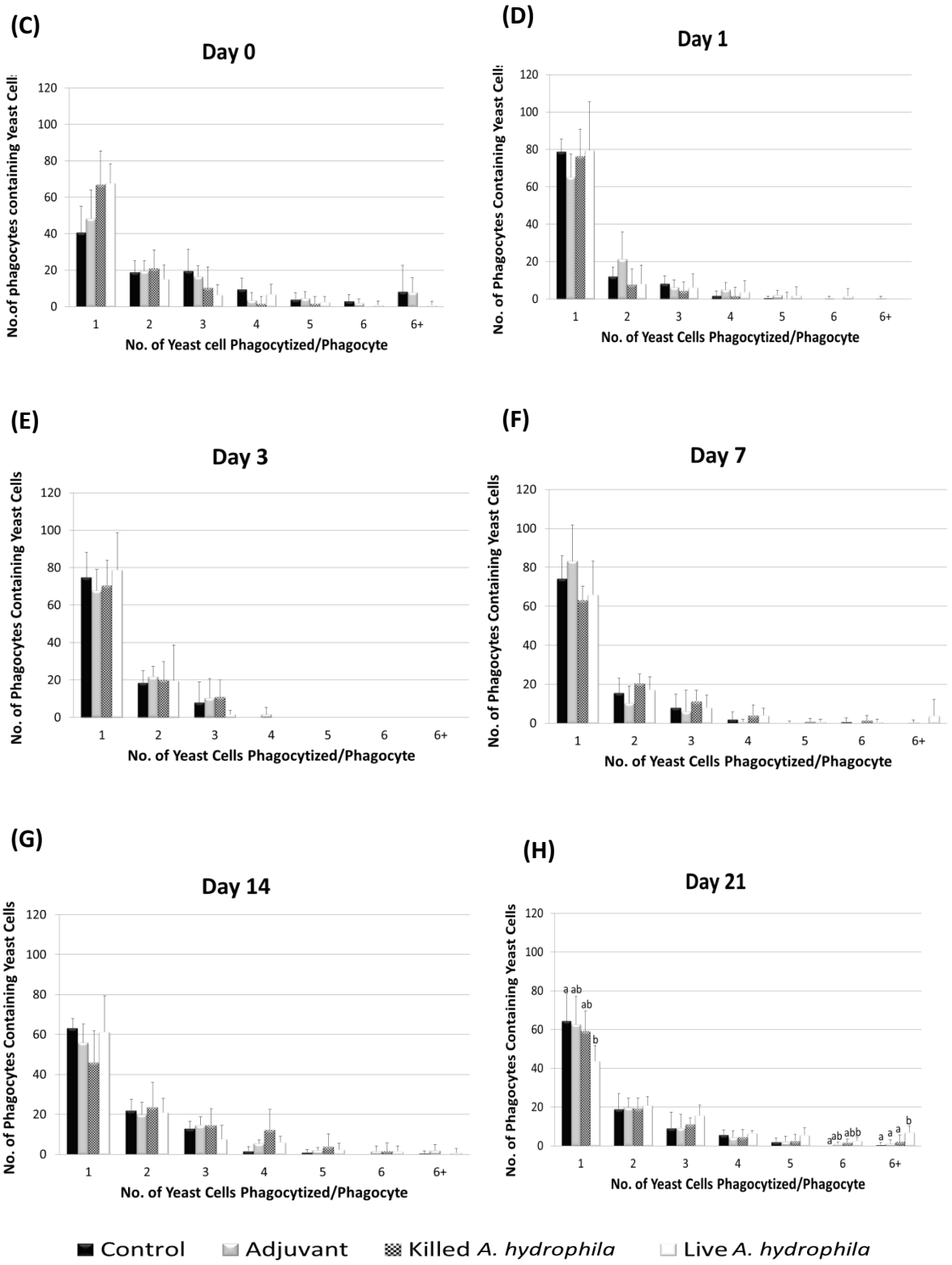


Figure 3.7(C-H; cont.). Comparison of phagocytic capacity between experimental groups. Different letters indicate significant differences (P<0.05) between groups for a given time point (Mean±SD, n=12).

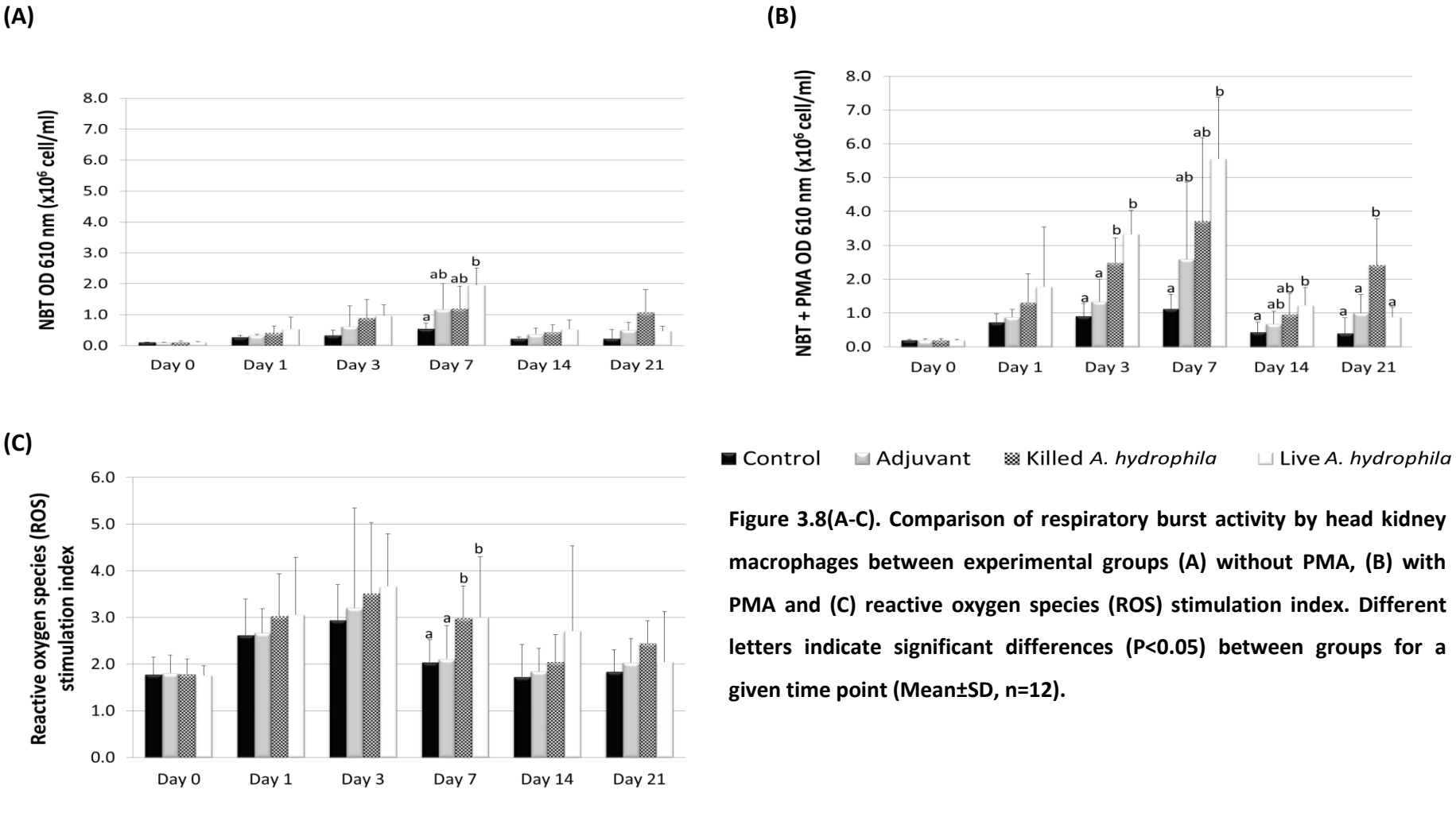


Figure 3.8(A-C). Comparison of respiratory burst activity by head kidney macrophages between experimental groups (A) without PMA, (B) with PMA and (C) reactive oxygen species (ROS) stimulation index. Different letters indicate significant differences (P<0.05) between groups for a given time point (Mean±SD, n=12).

3.3.3 Plasma protein activities

3.3.3.1 Plasma lysozyme activity

The plasma lysozyme activity was significantly different (P<0.05) between groups on 1 d.p.i. The values obtained with plasma from fish infected with live *A. hydrophila* was higher than control group at 3, 7, 14 d.p.i. Values obtained with live *A. hydrophila*, killed *A. hydrophila* and adjuvant were higher than the control group and at 21 d.p.i. Values of plasma lysozyme activity in fish injected with killed *A. hydrophila* were higher than fish injected with live *A. hydrophila*, adjuvant or control fish as shown in Figure 3.9(A).

3.3.3.2 Plasma complement activity

The only time-points where there was a statistical difference between groups was at 7 d.p.i. when the plasma complement activity was significantly higher (P<0.05) in the groups of fish injected with killed or live *A. hydrophila* (Figure 3.9(B)).

3.3.3.3 Plasma peroxidase activity

The plasma peroxidase activity was significantly different (P<0.05) at 3 d.p.i., with fish injected with live *A. hydrophila* having a higher activity than control fish, and at 7, 14 and 21 d.p.i. for fish injected with either live or killed *A. hydrophila* having higher levels than the group injected with adjuvant or the control group, as shown in Figure 3.9(C).

3.3.3.4 Total plasma immunoglobulin M

The total plasma immunoglobulin M levels in fish injected with live or killed *A. hydrophila* or adjuvant were statistically higher to the control group at 1, 3 d.p.i. and at 21 d.p.i. for the groups injected with live or killed *A. hydrophila* (Figure 3.9(D)).

3.3.3.5 Specific antibody titre against *Aeromonas hydrophila*

The antibody (IgM) titre between groups of fish was significantly different (P>0.05). At 3 and 14 d.p.i. for fish injected with live *A. hydrophila* compared to the control group), at 7 and 21 d.p.i. for fish injected with either live or killed *A. hydrophila* compared to the control and adjuvanted groups, (Figure 3.9(E)).

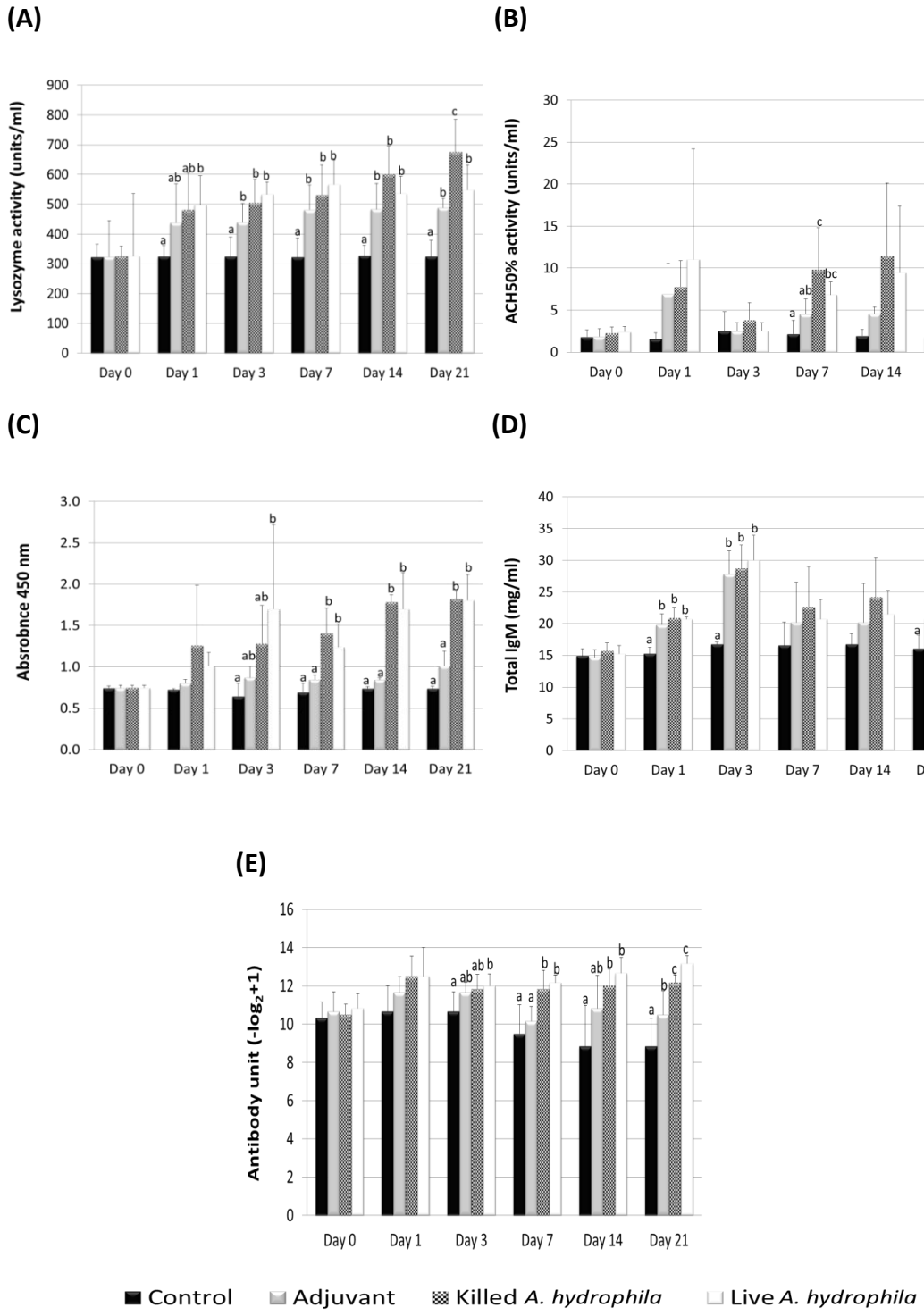


Figure 3.9(A-E). Comparison of (A) plasma lysozyme activity (units ml^{-1}), (B) plasma complement activity (units ml^{-1}), (C) plasma peroxidase activity, (D) total plasma immunoglobulin M (mg ml^{-1}) and (E) antibody (IgM) titre against *A. hydrophila* ($-\log_2+1$) between the experimental groups. Different letters indicate significant differences ($P<0.05$) between groups for a given time point (Mean±SD, n=12).

3.4 Discussion

The aim of this Chapter was to standardise functional assays to evaluate both innate and adaptive immune responses of *P. hypophthalmus* and to use these assays to compare immune response to live and heat killed *A. hydrophila.* These were used as models of infection and vaccination, respectively.

In the present study, the pre-challenge data (0 d.p.i.) indicated that the normal range of blood parameters for *P. hypophthalmus* were RBC (1.28-1.74 $x10^6$ cell ml^{-1}), WBC (1.48-3.0 $x10^4$ cell ml^{-1}), Hct (41.32-50.76%), MCV (257.68-368.27 fl) monocyte (4-29%), neutrophil (42-66%), lymphocyte (8-20%), eosinophil (0-3%) and thrombocyte (10-17%). The blood parameter results (MCV, Hct, and neutrophil counts) were higher than those stated by Breazile, *et al*. (1982) for channel catfish, while the levels of RBCs, Hct, lymphocytes and thrombocytes were lower. Prasad and Charles (2010) found similar Hct, RBC and WBC in yellow catfish (*Horabagrus brachysoma*), although MCV levels were higher. When the levels of various blood cells were examined in the experimental fish, a significant increase in WBC levels was seen at 3, 7 and 21 d.p.i, with both live and killed *A. hydrophila* stimulating an increase in this response by 3 d.p.i. However, the level of RBCs and haematocrit values were not up-regulated compared to the control group. The level of lymphocytes was significantly increased between the vaccinated group and control group on 1 d.p.i.

Granulocytes, mononuclear and macrophages represent the main cellular components of innate immunity in fish (Ellis 2001). In the present study, head kidney macrophage function was assessed using phagocytic activity and oxidative burst activity as indicators of their activity. These activities were significantly higher in fish

injected with live *A. hydrophila* by 7 d.p.i. compared to the control group. The phagocytic cells are involved in eliciting an inflammatory response, phagocytosis and bactericidal activity (producing bactericidal reactive oxygen species (ROS) and nitric oxide (NO)) for killing pathogens (Ellis 2001). Higher levels of superoxide anion (O_2^-) and hydrogen peroxide produced during respiratory burst are associated with pathogen clearance and tissue healing (Novoa*, et al.* 1996). The level of macrophage activity depends on species of fish, pathogen and type of external stimulant, as seen between the responses to live or dead bacteria, and techniques used to measure this activity.

Ainsworth and Dexiang (1990) found the higher phagocytic activity in channel catfish neutrophils against *Micrococcus luteus* than with *A. hydrophila, E. ictaluri* or *E. tarda*. The incubation time and temperature for optimal macrophage expression also differs between fish species. The optimal time to allow the macrophages of striped catfish to adhere to the plates, prior to assessing their respiratory burst and phagocytic activity was shown to be 2 h. In contrast, the optimal adherence time for Nile tilapia was 60 min for peak macrophage phagocytic activity (El-Boshy*, et al.* 2010).

The normal range for the phagocytic activity of head kidney macrophages from striped catfish was found to be 13-17%, the phagocytic index was 0.18-0.35, and respiratory burst ($OD_{610\ nm}$ 1 $x10^6$ cell ml^{-1}) was 0.10-0.12 (in the presence of NBT) 0.16-0.23 (in the presence of NBT and PMA). These results are lower than those reported for channel catfish (Ainsworth and Dexiang 1990), Atlantic salmon (*Salmo salar* L.) (Thompson*, et al.* 1996), Asian catfish (Kumari and Sahoo 2006a), Indian carp and Rohu (Sahoo*, et al.* 2005), using similar methods of detection.

In the present study, significantly higher levels of phagocytic activity and respiratory burst activity (in the presence of PMA), were seen in fish injected with live *A. hydrophila* compared with the other groups at 14 d.p.i. and the control group, respectively. In contrast, respiratory burst activity (in the presence of PMA) in fish injected with killed *A. hydrophila* were significantly higher compared with the other groups by 21 d.p.i. The innate cellular response of the striped catfish appeared to be more rapid against live *A. hydrophila* than the heat killed bacterium. Kusuda and Hamaguchi (1988) similarly found higher phagocytic activity in yellowtail immersed in a live attenuated preparation of *Pasteurella piscicida* (Synonym to *Photobacterium damselae* subsp. *piscicida*) compared with formalin-killed and heat-killed bacterin.

The plasma protein activities determined in this study for striped catfish were lysozyme, alterative complement, peroxidase and total IgM concentration. Lysozyme activity is an important part of innate immune response of fish and is present in mucus, plasma, lymphoid organs and other body fluids of fish. The main function of this enzyme is its lytic activity against bacteria, but it also opsonises bacteria to enhance phagocytosis and activates the complement system (Saurabh and Sahoo 2008). The lysozyme activity obtained depends on a variety of factors such as the concentration of the enzyme in the blood, the organ being analysed, the type of pathogens involved in the infection and the concentration of the phosphate buffer used in the assay (Fänge*, et al.* 1976; Grinde 1989). The pH of the buffer used in the lysozyme assay is also important to obtain optimal activity and this value appears to differ between fish species. For examples the pH used for Nile tilapia (Sarder*, et al.* 2001b) and Atlantic salmon (Paulsen*, et al.* 2003) was pH 5.75, for European sea bass

pH 5.8 (Mourente*, et al.* 2007), for turbot pH 6.24 (Santarem*, et al.* 1997), for channel catfish pH 6.0 (Welker*, et al.* 2007; Lim*, et al.* 2009), Jian carp (*Cyprinus carpio* var. *Jian*) pH 6.4 (Jian and Wu 2004) and rainbow trout pH 6.2 (Kunttu*, et al.* 2009). The optimum pH for pangasius catfish lysozyme activity was determined to be pH 5.4 using a turbidometric microplate assay, measuring the activity after 5 min. A similar value was used to measure the lysozyme activity in plaice (*Pleuronectes platessa* L.) serum (Fletcher and White 1976). Bich Hang, *et al*. (2012) determined the lysozyme activity in the spleen and plasma of *P. hypophthalmus* using a pH of 6.2. The normal range for plasma lysozyme activity in striped catfish was found to be 207-440 units ml^{-1}.Great interspecies variation has been found for lysozyme levels in 12 different fish species with 5-10 fold variation in activity reported (Grinde, *et al*. 1988).

Lysozyme activity in fish injected with live *A. hydrophila* was significantly higher compared to control fish on 1 d.p.i., while this activity was higher in all three experimental groups relative to the control fish on 3, 7 and 14 d.p.i. There was no difference in the level of lysozyme activity between fish injected with adjuvant, and live or killed *A. hydrophila*, except at 21 d.p.i when this activity was significantly higher in fish injected with killed *A. hydrophila* compared to those injected with live *A. hydrophila.*

Complement is another important component of the innate immune system of fish, destroying invading bacteria by lysing their cell wall or acting as an opsonin to enhance bactieral uptake by phagocytic cells (Holland and Lambris 2002). The alternative complement pathway is triggered by a variety of substances, including bacterial endotoxins, fungal components, polysaccharides, plant factors and animal

venom (D. Sakai 1992). In the present study the optimal assay temperature and incubation time to assess alternative complement activity ($ACH_{50\%}$) was determined to be 28^{o}C for 90 min. Similar assay conditions were used by Bich Hang, *et al*. (2012), for *P. hypophthalmus,* using 28^{o}C for 100 min. The normal range of complement activity ($ACH_{50\%}$) was found to be 1.35-2.89 units ml^{-1} and this was lower level than Asian catfish (Kumari and Sahoo 2006a), Indian carp and Rohu (Sahoo*, et al.* 2005). Stimulation with killed and live *A. hydrophila* significantly increased complement activity compared with control fish 7 d.p.i. However, there was no significant difference in activity between live and killed *A. hydrohila.* The results presented here suggest that the alternative complement pathway can be stimulated by both live and dead bacteria in striped catfish.

Myeloperoxidase (MPO) utilizes oxidative radicals to produce hypochlorous acid (HOCl) to kill pathogens during oxidative respiratory burst and is mainly released by the azurophilic granules of neutrophils (Mak and Saunders 2005). The normal range for plasma peroxidase activity in striped catfish was 0.72-0.78 at $OD_{450\ nm}$, similar to the range measured in Asian catfish (Kumari and Sahoo 2006a), but higher than Indian carp (Sahoo*, et al.* 2005). Significantly higher levels of plasma peroxidase activity were found in fish injected with live *A. hydrophila* on 3 d.p.i. compared with control fish, and this activity was significantly higher both in fish injected with live or killed *A. hydrophila* compared to the control fish on 7, 14 and 21 d.p.i. However, there was no significant difference in the ability of fish injected with live or killed *A. hydrophila* to stimulate MPO activity. Interestingly, there was a correlation between plasma peroxidase level and reactive oxygen species from respiratory burst activity. On 7 and 14 d.p.i., the

respiratory burst activity of macrophages obtained from fish injected with live *A. hydrophila* was significantly different from the control group. In addition, the plasma peroxidase levels of both live and killed *A. hydrophila* were significantly different from the control and adjuvant groups.

Important plasma proteins involved in the innate immune system include for example immunoglobulins, transferrin, and precipitins or agglutinins (Magnadóttir 2006). In the present study the normal range of total IgM in *P. hypophthalmus* was found to be 14-16 mg ml^{-1}, *i.e*. higher than in Atlantic salmon (Magnadóttir and Gudmundsdottir 1992) and seabream (Hanif*, et al.* 2004). Levels of total plasma IgM were shown to be significantly induced in fish injected with live or killed *A. hydrophila* compared with control fish on 1, 3 and 21 d.p.i. The specific antibody titre against *A. hydrophila* was also shown to differ significantly compared between fish injected with live or killed *A. hydrphila* and control fish at 3, 7, 14 and 21 d.p.i. Furthermore, levels in fish injected with adjuvant alone were also significantly different to the control fish at 21 d.p.i. Specifically-induced antibodies do not always related to protection *e.g*. Cossarini-Dunier (1986) reported circulating antibodies against *Yersinia ruckeri* were not protective; suggesting that protection against Enteric Red Mouth may be because of cell-mediated immunity rather than antibodies.

This present study showed that although intraperitoneal injection with either live or killed *A. hydrophila* significantly stimulated both innate and specific antibody responses in *P. hypophthalmus*, there were some differences in immune response between live and killed bacteria. In the first week following injection, the fish injected with live *A. hydrophila* had significantly higher white blood cell count, head kidney

phagocytic function (respiratory burst activity and reactive oxygen species stimulation index), plasma lysozyme and peroxidase activities, and specific antibody titre against *A. hydrophila* compared with the control fish. It was not until three weeks post-injection that the group injected with killed *A. hydrophila* showed any significant differences in their immune response compared to the control group *i.e.* lysozyme activity, total IgM and respiratory burst activity. Live bacteria can stimulate the immune response of fish in response to the bacteria's virulence, increasing T lymphocyte response I *i.e.* both T-helper cells (CD4) associated with MHC class II and T-cytotoxic cells (CD8) associated with MHC class I, while dead bacteria or inactivated bacteria stimulate mainly T-helper cells (Brostoff*, et al.* 1994; Wong and Fish 2003; Fischer*, et al.* 2006). Marsden, *et al*. (1996) found an increase in the proliferative response of leucocytes isolated from rainbow trout vaccinated intraperitoneally with live or formalin-killed *A. salmonicida* (Brivax II), and re-stimulated with antigen *in vitro* provides good evidence that a live vaccine is a better immunostimulant than a dead vaccine.

Vaccination with whole cells, ECPs, outer membrane proteins or biofilms of *A. hydrophila* has been shown to stimulate a variety of innate cellular and humoral parameters in carp (Kozinska and Guz 2004), and goldfish (Viji*, et al.* 2013), and differences in response were shown between resistant and susceptible families (Sahoo*, et al.* 2008; Z. Jeney*, et al.* 2009; Ardó*, et al.* 2010; G. Jeney*, et al.* 2011). Moreover, intraperitoneal injection of viable *A. hydrophila* and its ECPs resulted in very high mortalities after a few hours of infection and caused cell death in zebra fish (Rodriguez*, et al.* 2008), common carp (Ardó*, et al.* 2010) and Olive barb (*Puntius sarana)* (Das*, et al.* 2011) due to the cytotoxic, haemolytic activity and release of ROS

and nitrogen (NO) reactive free radicals. However, the level of innate and adaptive immune parameters depended on the routes of administration, concentration of bacteria, adjuvant and environment factors. For example, bath or immersion vaccination stimulated immune protection against *A. hydrophila* more than oral administration in common carp (Selvaraj*, et al.* 2009).

The concentration of the bacteria is also an important factor for stimulating the fish's immune system. Song-Lin, *et al.* (2012) found bathing eels with low concentrations of *A. hydrophila* (*i.e*. 1.0 x10^7 cfu ml^{-1}) enhanced the proliferation of different types of blood cells and the serum titres of anti-*A. hydrophila* antibody. In this present study, the concentration of heat killed *A. hydrophila* injected into fish was 1 x10^9 cfu ml^{-1} and live *A. hydrophila* was 2.7 x10^5 cfu ml^{-1}. Both of these doses were shown to enhance the innate and adaptive immune responses of *P. hypoophthalmus*. Previous studies have shown that live and killed *A. hydrophila* administered by injection, immersion or orally can stimulate antibody levels in the serum, bile, skin and gut mucus, and skin and muscle extracts of rainbow trout (Loghothetis and Austin 1994). In the present study, intraperitoneal administration of both live and killed *A. hydrophila* stimulated the production of total and specific IgM in the plasma of experimental fish.

An adjuvant is usually mixed and injected with antigen to enhance the immune response and elevate immune activity (Anderson 1992). In the present study, the adjuvant used was Montanide ISA 760 VG. It was seen to enhance some innate immune parameters within 3 days of injection *i.e*. lysozyme activity, total IgM, white blood cell count were all significantly different to the control group at this time.

3.5 Conclusions

In summary, standardised functional assays for *P. hypophthalmus* have been developed and basic information on the immune response in this species has been elucidated following stimulation with live and killed *A. hydrophila* as models to investigate immune response of this fish species. It can be concluded that administration of live (at a low dose) and formalin killed *A. hydrophila* by intraperitoneal administration stimulates both the innate and the adaptive immune responses of striped catfish. However, live *A. hydrophila* stimulated the immune response earlier than killed *A. hydrophila*. It appears the most appropriate immunoassays for monitoring the health of this species are those examining phagocytosis, respiratory burst, complement, lysozyme (pH 5.4), total immunoglobulin M, plasma peroxidase and specific antibody (IgM) titre against *A. hydrophila.*

Chapter 4 The effects of feeding immunostimulant β-glucan on the immune response of *Pangasianodon hypophthalmus*

4.1 Introduction

Immunostimulants have proven to be a very useful food additive for the aquaculture industry, since they can be easily fed to fish to enhance their immune response at times of stress and to improve their resistance to disease (Sakai 1999; Bairwa*, et al.* 2012; Meena*, et al.* 2012). Derived from either natural or synthetic sources, they are known to enhance the non-specific defence mechanisms of fish (Siwicki*, et al.* 1994; Dong*, et al.* 2013). One of their modes of action is through enhanced macrophage function, including increased phagocytosis and bactericidal activities against invading pathogens (Ranjan*, et al.* 2012). Some immunostimulants have been shown to increase complement and lysozyme activity and enhance antibody responses in immuno-stimulated fish (Sakai 1999; Dong*, et al.* 2013). The most common types of immunostimulants used in aquaculture are β-glucans. These are a heterogeneous group of glucose polymers, consisting of a backbone of β (1,3)-linked β-D-glucopyranosyl units with β-(1,6)-linked side chains of varying distribution and length, depending on their source. They tend to be derived from the cell wall of different plants (including wheat, rye, barley and oats), yeast (*Saccharomyces* genus), and species of the genus *Echinacea* (Tokunaka*, et al.* 2000). Other sources of β-glucan include seaweed (*e.g*. *Laminaria* sp.), various species of mushrooms such as Shiitake (*Lentinus edodes*), Maitake (*Grifola frondosa*), Reishi (*Ganoderma lucidum*) (Wasser and Weis 1999), Schizophylan (*Schizophyllum commune*)(Borchers*, et al.* 1999), other fungi (*e.g*. *Agaricus subrufesuns*; *Pneumocystis carini*; *Cryptococcus neoformans*) (Lebron*, et al.* 2003; Reese*, et al.* 2007) and some bacteria (*e.g*. Rhizobiaceae family)(Breedveld and Miller 1994). The most common source of β-glucan currently

used in aquaculture is derived from the cell wall of baker's yeast *Saccharomyces cerevisiae,* and is one of the most important sources of β-1,3 and 1,6 glucans. Oats and barley β-glucans have linear β (1,4) and (1,3) linkages, while mushroom β-glucans have short β (1,6)-linked branches with a β (1,3) backbone. The yeast β-glucans have β (1,3) glycosidic linked D-glucose subunits with irregular β (1,6) linked side chains of various lengths (Meena*, et al.* 2012; Auinger*, et al.* 2013). The β-glucans having β (1,6) and β (1,3) linkages in their structure are more immuno-stimulatory than those with only β (1,3) linkages (Bohn and BeMiller 1995). Structural differences can affect the extraction process of some β-glucans, and in turn, this can affect their immuno-stimulatory activity. Larger molecular weight glucans can activate leukocytes, stimulating their phagocytic, cytotoxic and antimicrobial activities, and increase their production of reactive oxygen species (ROS), while very short, low molecular weight glucans are considered to be inactive (Akramiene*, et al.* 2007). Glucans derived from either *Schizophyllum commune*, *Sclerotium glucanicum* or *Lentinus edodes* have been shown to enhance the disease resistance of common carp (*Cyprinus carpio*) to *Edwardsiella tarda* infections (Yano*, et al.* 1989), while Robertsen *et al*. (1990) used M-glucan isolated from the cell walls of *S. cerevisiae* to enhance resistance of Atlantic salmon (*Salmo salar*) to *Yersinia ruckeri, Vibrio anguillarum* and *Aeromonas salmonicida.* Chen and Ainsworth (1992) also found that β-1,3 glucan extracted from the cell wall of bakers' yeast, injected intraperitoneally into channel catfish (*Ictalurus punctatus*) reduced the level of mortality in fish experimental infected with *E. ictaluri*, while Whittington *et al* (2005) showed that it helped to enhance the innate immune response of Nile tilapia (*Oreochromis niloticus*), enhancing the fish's response to a

Streptococcus iniae vaccine and improving their level of protection against *S. iniae* infection. Duncan and Klesius (1996), on the other hand, found feeding blue green algae (*Spirulina platensis*) to channel catfish enhanced their innate immune responses, but did not improve their resistance to *E. ictaluri*.

The aims of Chapter 4 were to evaluate the effects of feeding different levels of fungal derived β-glucan on the immune response of *P. hypophthalmus* and compare this response to that obtained with commercial β-glucan derived from yeast. The effect of feeding these immunostimulants on the disease resistance of *P. hypophthalmus* to *E. ictaluri* was also examined. Ultimately, the purpose of this study was to formulate and test β-glucan-containing diets for use by the Pangasius aquaculture industry.

4.2 Materials and methods

4.2.1 Experimental animals

The feeding trial was performed at the Novus Aqua Research Center (NARC), Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam. *Pangasianodon hypophthalmus* were purchased from a local fish farm and transported to NARC, where the fish were quarantined and acclimated to laboratory conditions for at least 2 weeks prior to starting the experiment. During this time they were fed with the basal control diet (Diet A) shown in Table 4.1 at 3% body weight per day. The initial body weight of the animals was 60.3 ±11.7 g.

4.2.2 Formulation of experimental diets

A basal control diet was formulated without β-glucan supplementation (Table 4.1). The experimental diets consisted of the basal diet supplemented with 0.05%, 0.1%, 0.2%

fungal-derived β-glucan (Supplied by Novus®, USA) or 0.1% commercial yeast-derived β-glucan (MacroGard®, USA) as a positive control. To prepare the diets, the macro and micro-ingredients (including vitamin and mineral premixes and additives) were mixed together for 30 min in a Hobart mixer, then oil was slowly added and the feed mixed for a further 15 min. Boiling distilled water was then slowly added to the diets and mixed for another 15 min to form a soft dough. The diets were pelleted using a Hobart chopper to form pellets with a 3 mm diameter. The pellets were dried at 60^{o}C for 18 h using a feed dryer, with the duration of drying depending on the moisture content of the pellets, which was checked every 6 h. The dried pellets were stored at 5^{o}C until used.

4.2.3 Experimental design and feeding regimes

The experiment consisted of five treatment groups with four replicate tanks per group (using 42 fish per replicate during the feeding period and 15 fish per replicate for the challenge) as shown in Table 4.2. The concentration of β-glucan added to the diet of the various groups is shown in Table 4.1. Fish were fed twice daily to satiation, except for the 24 h prior to handling or challenging the fish when fish were starved. Fish were maintained in square 340 L tanks with re-circulated water at a temperature of 28 ±2^{o}C and a photoperiod regime consisting of 12:12 h artificial light. The fish were maintained on the experimental diets for 4 weeks before performing the experimental infection, which lasted for three weeks. Two fish were sampled per tank to assess their immune response on Days 0, 1, 3, 7, 14, 21, 28 post-feeding (*i.e*. from the onset of feeding the immunostimulant diet) (p.f.) and 14 days post-infection (d.p.i). Blood and

head kidney samples were collected from these fish for the various haematological and immunological analyses outline in Figure 4.1

Table 4.1. Feed formulations

Ingredients	**(%) of total weight**	**weight of ingredients (g)**				
		Diet A	**Diet B**	**Diet C**	**Diet D**	**Diet E**
Fungal derived β-glucan	-	0	2.5	5.0	10.0	0
Commercial yeast drived β-glucan	-	0	0	0	0	5.0
Cassava	13.7	685.0	685.0	680.0	675.0	680.0
Soybean meal (48%)	46.7	2,335.0				
Rice bran solvent extract	12.2	610.0				
Canola meal	8.0	400.0				
Wheat flour	5.9	295.0				
Fish meal	3.0	150.0				
Soy protein concentrate	2.73	136.51				
Fish oil	2.54	127.0				
Soybean oil	2.53	126.5				
Dicalcium phosphate (DCP)	1.2	60.0				
Mineral premix	0.5	25.0				
Vitamin premix	0.5	25.0				
Methionine Hydoxy Analogue (84%)	0.3	15.0				
Ascorbic phosphate (25%) (Stay C)	0.1	5.0				
Choline Chloride (50%)	0.1	5.0				
Total	**100**	**5,000**				

Table 4.2. Feeding regime and fish numbers for the dietary groups fed with the experimental diets

Treatment (Diets)	**A**	**B**	**C**	**D**	**E**
Fungal derived β-glucan (g/kg)	0	0.05	0.1	0.2	0
Commercial yeast derived β-glucan (g/kg)	0	0	0	0	0.1
Phase 1 – Feeding	Continuous 28 days				
Tanks	4				
Total fish/tank	42				
Sample collection/time/tank	2				
Day collection sample	0, 1, 3, 7, 14, 21,28				
Total fish	840				
Phase 2- Challenge with *E. ictaluri*	14 days for infection period				
Tanks	4				
Total fish/tank	15				
Sample collection/time/tank	2				
Total fish	300				

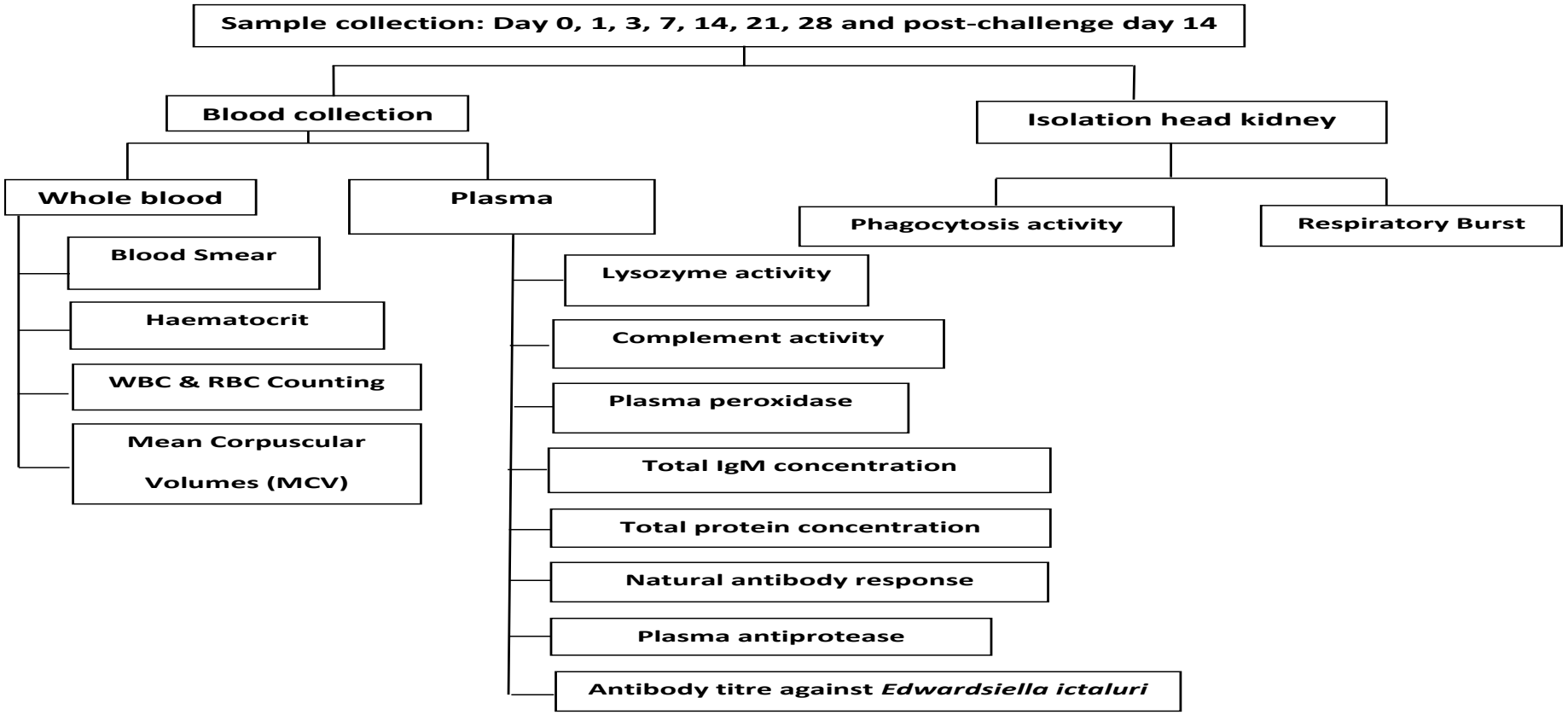


Figure 4.1. Sampling regime and immunological analysis performed with blood and head kidney from *Pangasianodon hypophthalmus*

4.2.4 Haematological and Immunological analysis

4.2.4.1 Haematological analysis

Blood samples were collected for white blood cell (WBC) and red blood cell (RBC) counts by placing the blood in Natt-Herricks's stain and counting cells using a haemocytometer. Blood samples were also used to determine haematocrit and mean corpuscular volume (MCV) values, and blood smears were prepared and stained for differential white blood cell counts. These analyses were performed using the protocols outlined previously in Section 3.2.4.1.

4.2.4.2 Macrophage activity of head kidney

Head kidney was collected from the experimental fish and head kidney macrophages were extracted to assess phagocytic activity and respiratory burst activity (RBA), using the protocols described in Section 3.2.4.2.

4.2.4.3 Analysis of immunological plasma parameters

Plasma was prepared by centrifuging the sampled blood at 3,000 x g for 5 min, and this was stored at -70^{o}C for further analysis. Plasma lysozyme activity was measured using a turbidimetric assay, which plasma complement activity used gelatine-complement fixation buffer, and plasma peroxidase activity and total immunoglobulin M (IgM) were determined using a direct enzyme linked immunosorbent assay (ELISA). These analyses were performed using the protocols described earlier in Section 3.2.4.3. Additional methods used to analyse the various plasma parameters are outlined below.

4.2.4.3.1 Natural antibody response

The natural antibody response was measured in plasma using modified method of Sinyakov, *et al.* (2002) and Ardó, *et al.*(2010). An ELISA was used to measure the levels

of natural antibody in the plasma of experimental fish. A 96-well ELISA plate was coated with 1 % (w/v) bovine serum albumin (BSA; Sigma, UK) in 0.02 M phosphate buffer saline (PBS) pH 7.3 by added 250 µl $well^{-1}$ and incubating for 1-2 hours at room ($28^{o}C$) or overnight at 4°C. The plates were then washed with 3 washes of low salt water buffer (Appendix 1). Doubling-dilutions of fish serum were prepared in PBS starting at a dilution of 1/32 dilution to 1/16,384, adding 100 µl $well^{-1}$ and incubated for 3 h at $22^{o}C$ or overnight at $4^{o}C$. Pre-immune serum or serum from non-vaccinated fish and PBS were used as negative controls. The plates were washed 5 times with high salt wash buffer, incubated for 5 min on the last wash, before adding 100 µl per well of anti-*P. hypophthalmus* IgM mAb 23 (see Chapter 2 for details on production of monoclonal antibody) and incubated for 60 min at room temperature. The plates were washed with 5 washes of high salt wash buffer (Appendix 1), incubated for 5 min on the last wash and then 100 µl $well^{-1}$ conjugate (goat anti-mouse IgG-HRP (Sigma, UK) diluted 1/4000 in conjugate buffer (1% BSA in low salt wash buffer) was added. Plates were incubated for 60 min at room temperature before washing with 5 washes of high salt wash buffer, incubating for 5 min on last wash. Finally, 100 µl $well^{-1}$ chromogen/substrate (Appendix 1) was added and the reaction incubated for 10 min at room temperature before stopping with 2M H_2SO_4.The plates were read at 450 nm in an ELISA reader.

4.2.4.3.2 Total plasma protein

Total plasma protein was measured in plasma and analysed using a Bradford assay by method of Sharifuzzaman and Austin (2009). A protein standard series (from 0 to 2 mg ml^{-1}) was prepared by diluting 6 mg bovine serum albumin (BSA; Sigma, UK) in PBS.

Twenty µl of sample or standard were added to the wells of a 96 well plate, to which was then added 200 µl of Bradford solution (Sigma, UK) to each well and mixed thoroughly on a plate shaker for 30 sec. After 5 min the plates were read at an absorbance of 595 nm in a microplate reader. A standard curve of protein concentration against absorbance at 595 nm was plotted and the best fit of the data to a straight line was determined from the following equation 4.1 "y = ax+b" (y = absorbance at 595 nm, x = protein concentration, b = solve from equation total volume = 0.22 ml, volume of sample or standard = 0.02 ml).

Equation 4.1 Protein concentration

$$Protein\ concentration\ (\mu g/ml) = \frac{(y-b)}{a} x \frac{(0.22\ ml)}{0.02\ ml}$$

4.2.4.3.3 Plasma anti-protease

Total anti-protease activity was determined from Sharifuzzaman and Austin (2009) the ability of plasma to inhibit trypsin activity. Twenty µl plasma was added to 20 µl of trypsin solution in an eppendorf tube and incubated at 22^{o}C for 10 min, then 200 µl of PBS and 250 µl azocasein (2% w/v; Azocasein (Sigma, UK)) were added and incubated for 1 h at 30^{o}C. The positive control consisted of PBS with trypsin and the negative control consisted of PBS without serum or plasma and trypsin. The reaction was stopped by adding 500 µl of 10% (v/v) trichloroacetic acid (Sigma, UK) incubated for 30 min at 30^{o}C then centrifuged at 17,000 x g for 5 min. One hundred µl well^{-1} 1 N sodium hydroxide was added. Supernatant from the eppendorfs was added to the microtitre plate (100 µl well^{-1}) and the plate was then read with an ELISA reader at 450 nm. The percentage of trypsin inhibition was determined using Equation 4.2.

Equation 4.2 Trypsin inhibition (%)

$$Trypsin\ inhibition\ (\%)\ = \frac{(Trypsin\ OD - Sample\ OD)}{Trypsin\ OD} x\ 100$$

4.2.5 Bacterial challenge with *E. ictaluri*

Edwardsiella ictaluri, isolate NLF33 (supplied and previously characterised by Nong Lam University, Vietnam), was cultured in tryptone soya agar (TSA, Oxoid England) for 48 h. Three to five colonies of *E. ictaluri* were picked from the plate and placed into 100 ml of sterile brain heart infusion broth. The bacteria were grown to late logarithmic growth in a shaking incubator (200 rpm, 28^{o}C) (DaiHan LAB TECH, Korea) for 6 h then transferred to 300 ml of fresh TSB medium and incubated in the shaker at 28^{o}C, 200 rpm for a further 16-18 h. The bacterial concentration was determined using a Neubauer's haemocytometer and cfu confirmed using a plate counting method. Fish were immersed in static, well aerated water at a final bacterial concentration of 8 x10^{4} cfu ml^{-1} for 60 min before returning the fish to their experimental tanks. Fish were observed at least twice daily for two weeks. Moribund or dead fish were removed and examined for gross external and internal clinical signs of disease and the kidney sampled for bacterial culture to confirm specific mortalities.

4.2.5.1 Antibody titre against *E. ictaluri*

Plasma samples were collected from moribund fish and fish surviving the experimental infection with *E. ictaluri,* and analysed using a direct ELISA for detection of *E. ictaluri* specific antibodies, using the method described in Section 3.2.4.3.5.

4.2.6 Statistical analysis

Data were examined using a one-way analysis of variance (ANOVA), general linear model, and pairwise comparison (Tukey) of means. All statistical tests were performed using Minitab software version 16 and survival analysis by Cox Regression model using

SPSS software Version 19 under licence to the University of Stirling, 2013. Differences were considered statistically significant when *P<0.05.* Statistical differences were examined between groups at each sampling point and examined within groups over time.

4.3 Results

4.3.1 Haematological parameters

A variety of haematological parameters were measured in experimental fish including % haematocrit values, total red blood cells counts (RBC), total white blood cells counts (WBC) and mean corpuscular volumes (MCV). The haematocrit values over the course of the trial are shown in Figure 4.2(A). No change in haematocrit values were observed between the experimental groups on Day 1, 3 or 28 p.f., however there were statistically significant differences between some groups on 7, 14, 21 p.f. and following bacterial challenge on 14 d.p.i (*P<0.05*). Generally, the fish fed 0.05% β-glucan had the lowest haematocrit values over the course of the feeding trial and % haematocrit values were seen to decrease in all groups by 14 d.p.i., indicative of anaemia as a result of the infection. Significant differences in haematocrit values were seen between groups over time (*P<0.05*).

No significant differences were observed in total red blood cells counts between any of the dietary groups at any of the sampling points, including the sampling points post-infection (Figure 4.2(B)). The total white blood cell counts were very variable over the course of the trial with the lowest levels seen at Day 0 before the onset of feeding with the immunostimulant diets (Figure 4.2(C)). Generally, the WBC levels were then seen to increase in the immuno-stimulated groups compared to

the control group, with significant differences seen among the experimental groups on Day 1, 3, and 7 (*P<0.05*) p.f., while a decrease in total WBC counts was observed at 14 d.p.i. compared to the previous time point at Day 28 p.f., just prior to the challenge (*P<0.05*). The group fed 0.2% β glucan had the highest levels of WBC counts post-infection. However, there were no statistical differences in the mean corpuscular volume (MCV) between experimental groups at any of the time points examined (Figure 4.2(D)).

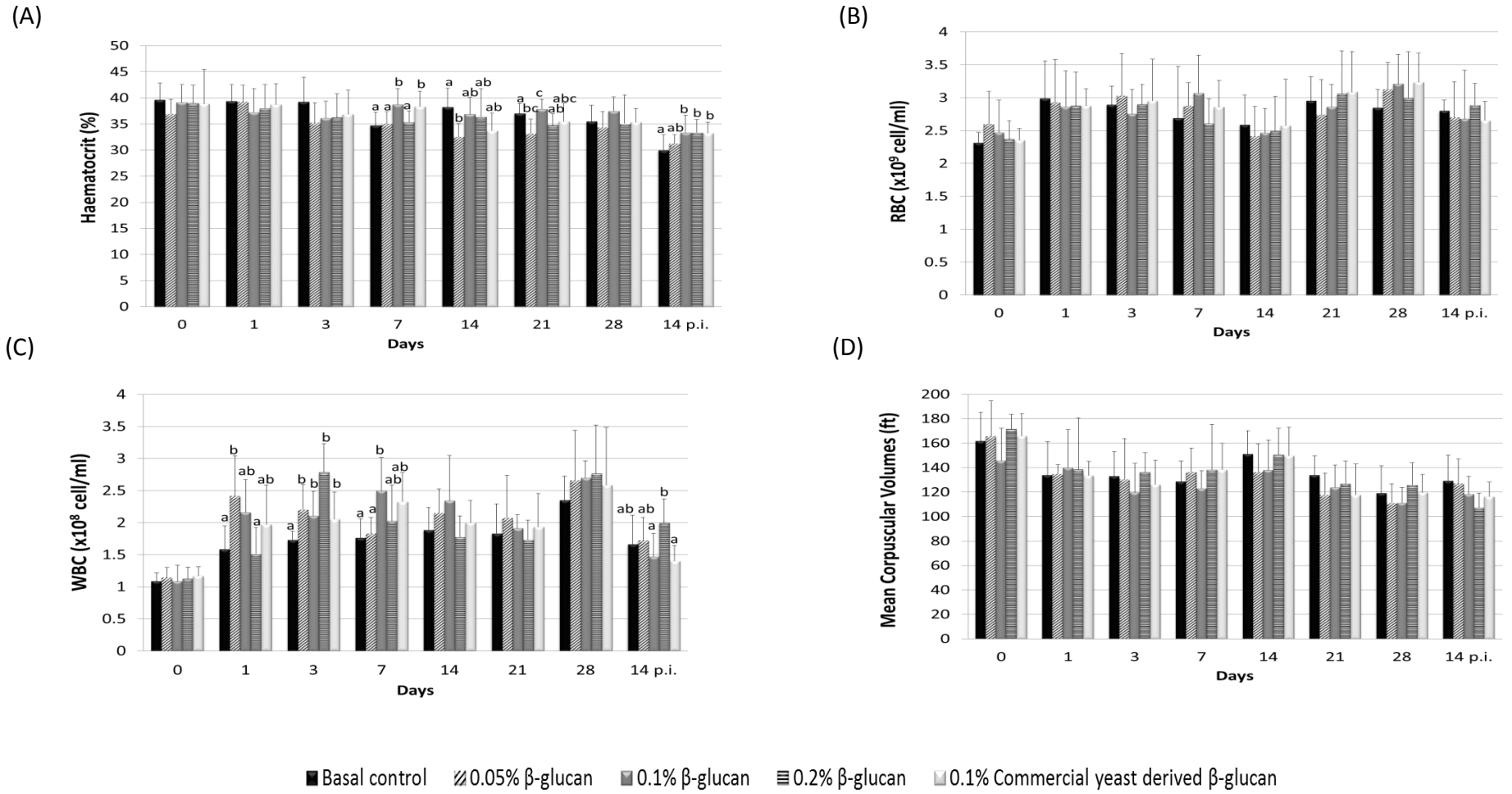


Figure 4.2(A-D). Comparison of (A) haematocrit values (%); (B) total RBC counts (x10^9 cell ml^{-1}) (C) total WBC counts (x10^8 cell ml^{-1}); (D) mean corpuscular volumes (ft) between groups fed different diets for upto 28 days, after which all groups were challenged with *Edwardsiella ictaluri* for 14 days (Mean±SD, n=8). Different letters indicate significant differences (P<0.05) between groups at a given time point.

The proportion of different WBC counts, including monocytes (M), lymphocytes (L), neutrophils (N) and thrombocytes (Th) in the blood of fish from the different dietary groups was determined (Figure 4.3(A-D)). There were no significant differences in the proportions of lymphocytes, neutrophils or thrombocytes between the groups at any of the time points examined (data not shown), but differences in the proportion of monocytes were observed. At Day 1 p.f. the basal control diet and the group fed 0.2% β-glucan had the highest proportion of monocytes, while the group fed 0.1% β-glucan had the lowest proportion. At 14 d.p.i. the fish fed 0.05% β-glucan had the highest proportion of monocytes, while fish fed and 0.1 % β-glucan had the lowest proportion ($P<0.05$). A summary of the differences found with the various haematological parameters between groups is shown in Table 4.3. There was also a significant difference in the differential WBC counts between the groups over time ($P<0.001$) (see Table 4.8. below in Section 4.3.5).

Table 4.3. Summary of significantly different haematocrit and WBC values between days and treatments in fish fed different levels of β-glucan and challenged with *E. ictaluri*

Parameters	Day	Treatment
Haematocrit values	7 p.f.	A=B=D<C=E
	14 p.f.	A>B,C=D=E
	21 p.f.	A=C>B, E=D
	14 p.i.	A<C=D=E,B
White blood cell (WBC) counts	1 p.f.	B>A=D,C=E
	3 p.f.	A<E,C,B,D
	7 p.f.	C>A=B, D=E
	14 p.i.	D>B=E, A=B
Different WBC counts		
Monocyte	0 p.f.	C<A=D=E, B
	1 p.f.	C<A=D=E, B
	14 p.i.	C<B, E=A=D
Lymphocyte	-	-
Neutrophil	-	-
Thrombocyte	-	-

*p.f.=post feeding, p.i.=post infection, A=Basal control diet, B=0.05% β-glucan, C=0.1% β-glucan, D=0.2% β-glucan and E=0.1% Commercial yeast derived β-glucan

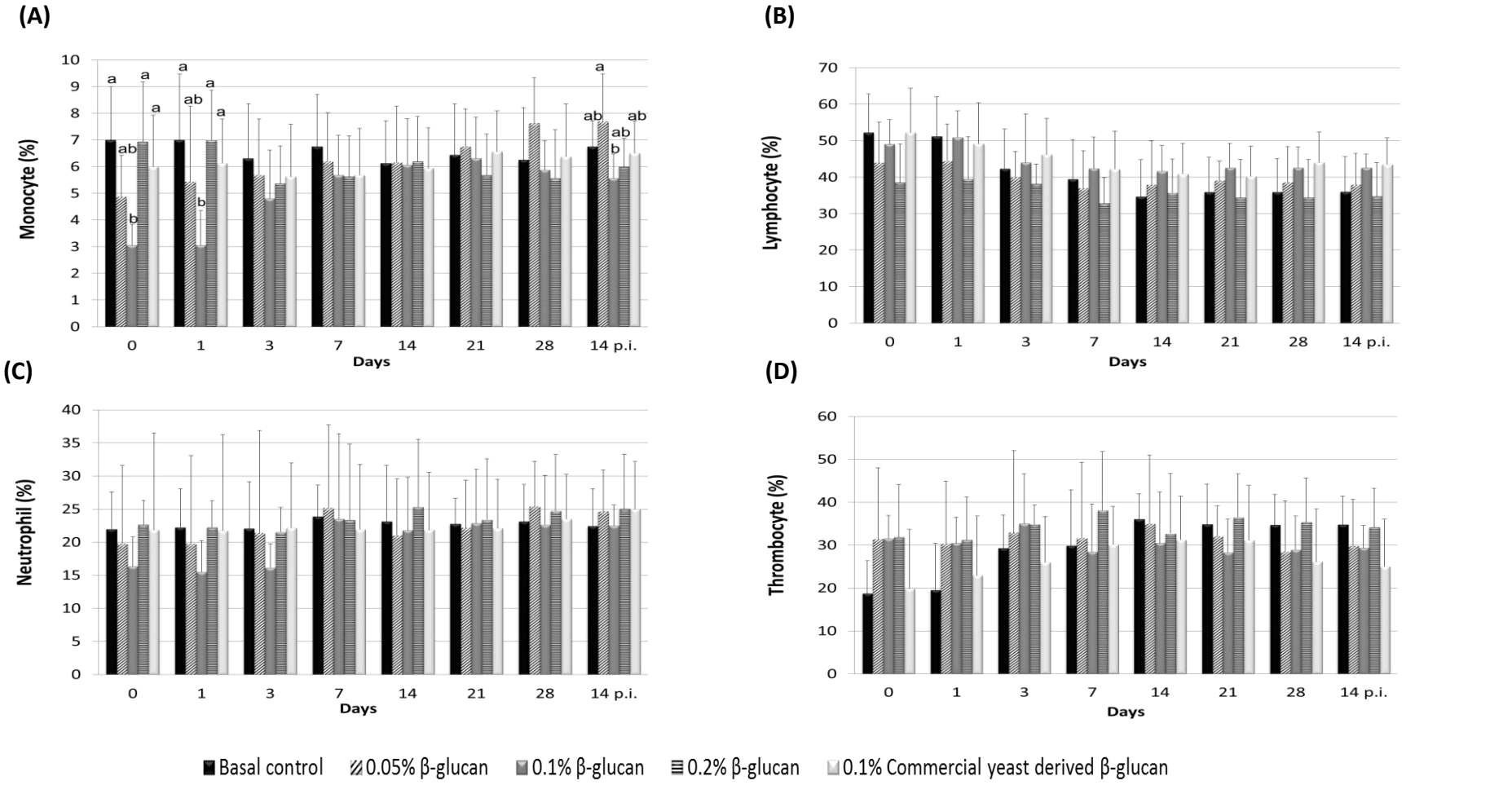


Figure 4.3(A-D). Comparison of differential white blood cell counts (%) between groups fed different diets for upto 28 days, after which all groups were challenged with *Edwardsiella ictaluri* for 14 days (Mean±SD, n=8). Different letters indicate significant differences (P<0.05) between groups for a given time point) (A) monocytes; (B) lymphocytes; (C) neutrophils and (D) thrombocytes

4.3.2 Head kidney macrophage activities

4.3.2.1 Respiratory burst activity

The respiratory burst activity of head kidney macrophages in the 0.2% β-glucan group was significantly higher than the level observed for the basal control group on Day 1 and Day 7 p.f., and the 0.05% β-glucan fed group on Day 7 p.f. (*P<0.05*) (Figure 4.4(A)). However, when PMA was added to the assay, significant differences in activity were observed between groups on Days 1, 3, 21 and 28 p.f. (Table 4.4 and Figure 4.4(B)) (*P<0.05*), with fish fed with 0.2% β-glucan showing higher level than the basal control group. Generally, the fish fed 0.2% β-glucan had the highest level of respiratory burst activity both with and without PMA compared with the basal control group. This group of fish also had the highest reactive oxygen species (ROS) stimulation index compared to the other groups on Day 3 p.f. (*P<0.05*) (Table 4.4 and Figure 4.4(C)). However, there were no significant differences in the level of respiratory burst activity with and without PMA between the groups of fish fed with 0.05%, 0.1% 0.2% β-glucan and 0.1% commercial yeast derived β-glucan.

4.3.2.2 Phagocytic activity of head kidney macrophages

The phagocytic activity and index of head kidney macrophages was significantly greater for all four immuno-stimulated groups compared to the control group on Days 21 and 28 p.f. (and also 14 d.p.i. for the phagocytic index) from the onset of feeding the experimental diets (*P<0.05*) (Table 4.4 and Figure 4.5(A and B)). The ability of macrophages to phagocytise different amounts of yeast are shown in Table 4.4 and Figure 4.5(C-J).

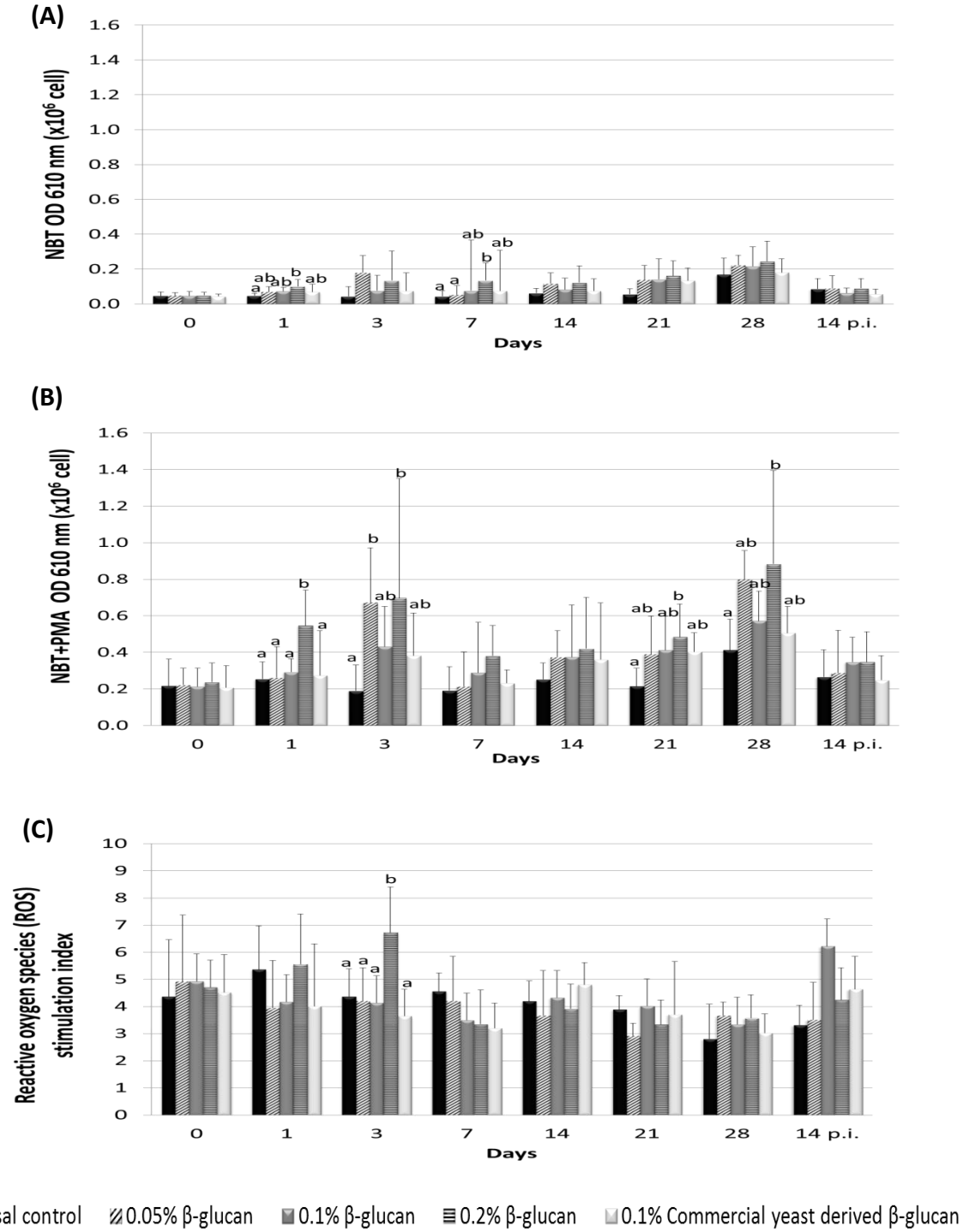


Figure 4.4(A-C). Respiratory burst activity by head kidney macrophages between groups fed different diets for upto 28 days, after which all groups were challenged with *Edwardsiella ictaluri* for 14 days (Mean±SD, n=8). (A) Without PMA (B) With PMA (C) Reactive oxygen species (ROS) stimulation index

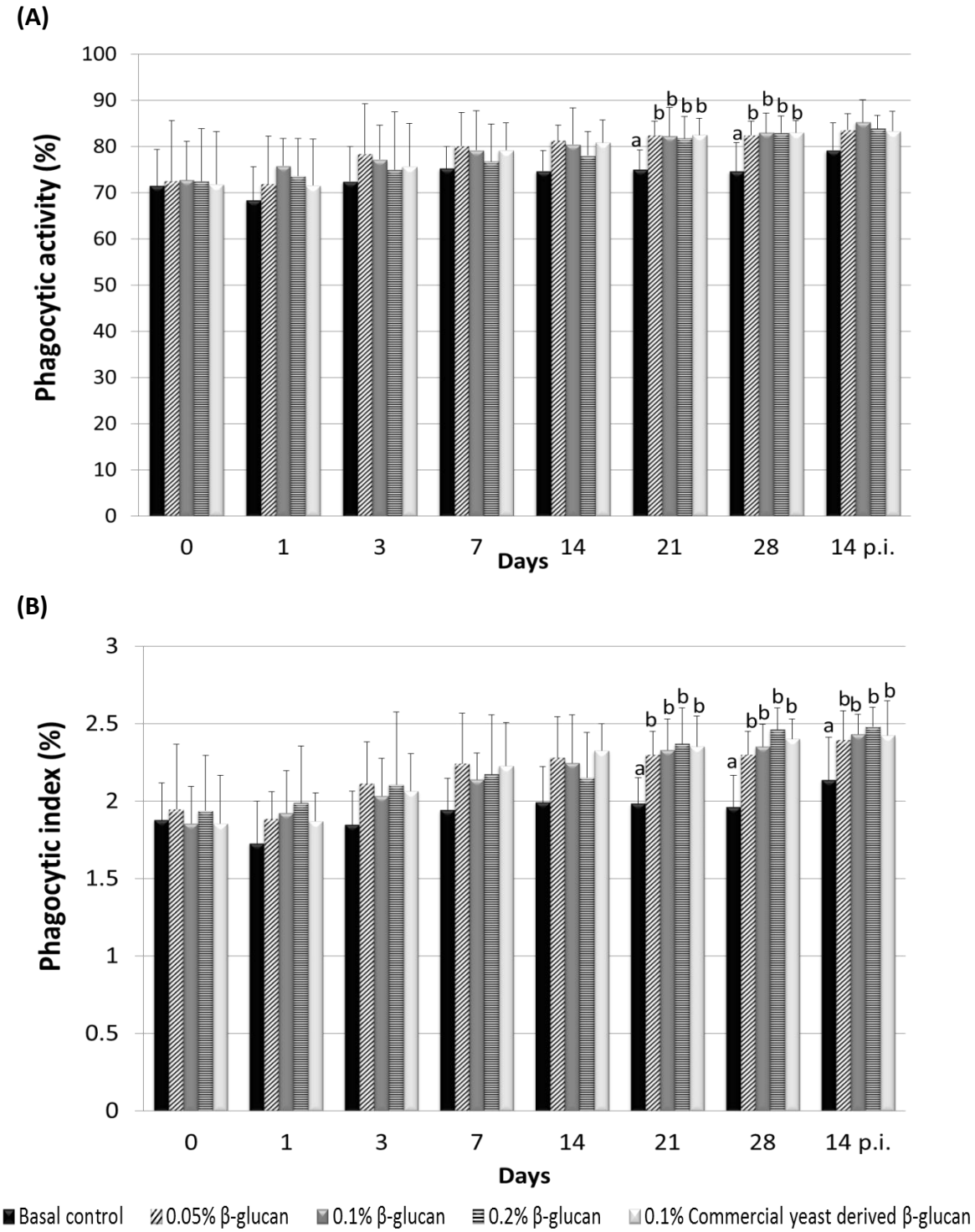


Figure 4.5(A-B). Comparison of (A) percentage phagocytic activity and (B) percentage of phagocytic index of head kidney macrophages between groups fed different diets for upto 28 days, after which all groups were challenged with *Edwardsiella ictaluri* for 14 days (Mean±SD, n=8). Different letters indicate significant differences (P<0.05) between groups for a given time point.

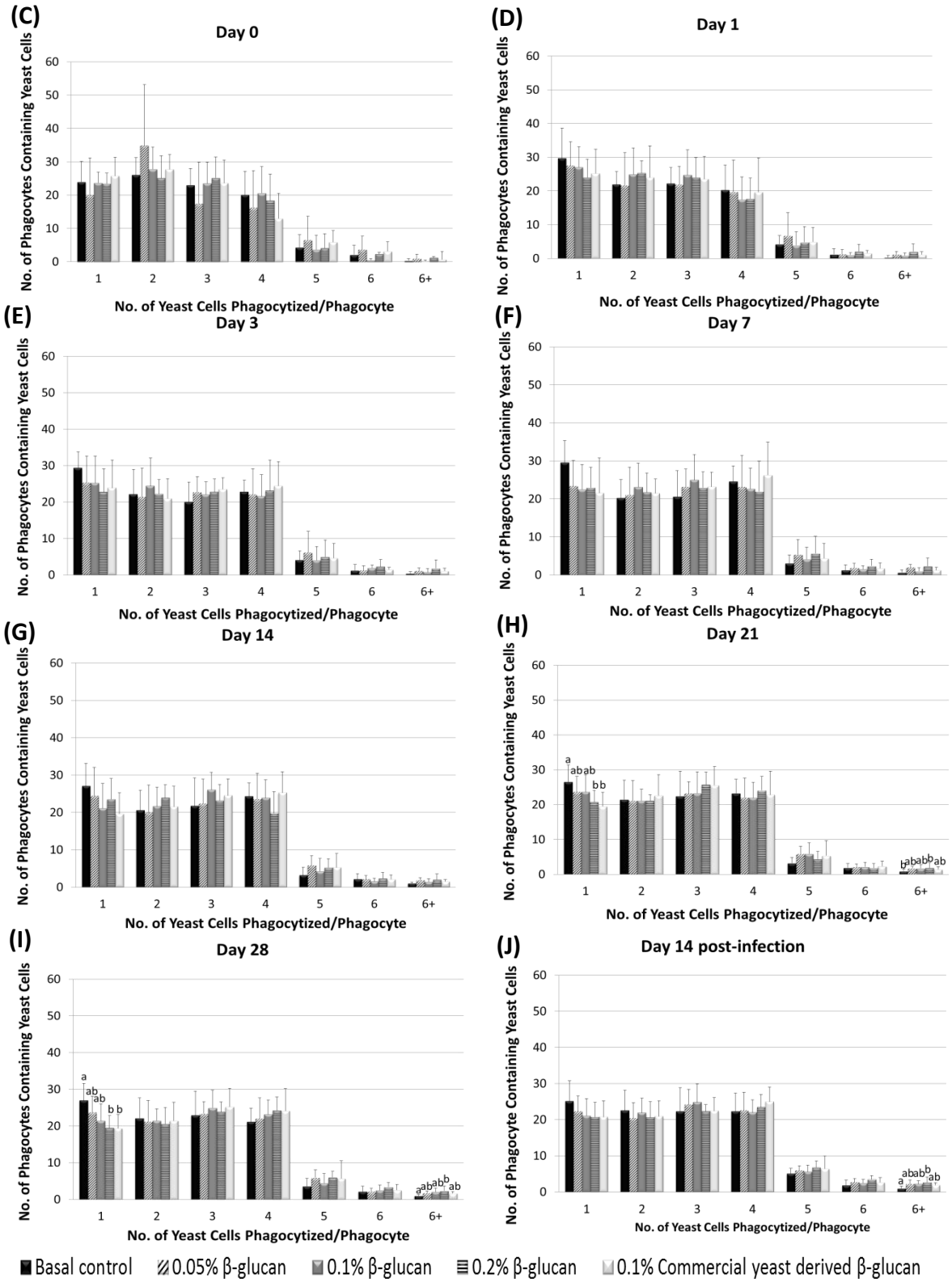


Figure 4.5(C-J; cont.). Comparison of phagocytic capacity of head kidney macrophages between groups fed different diets for upto 28 days, after which all groups were challenged with *E.ictaluri* for 14 days (Mean±SD, n=8). Different letters indicate significant differences (P<0.05) between groups for a given time point.

Table 4.4. Summary of respiratory burst and phagocytic activities of head kidney macrophages between treatments

Parameters	Day	Treatment
Respiratory burst (with NBT)	1 p.f.	A<D, B=C=E
	7 p.f.	D>B=A, C=E
Respiratory burst (with NBT+PMA)	1 p.f.	D>A=B=C=E
	3 p.f.	A<B=D, C=E
	21 p.f.	A<D, B=C=E
	28 p.f.	A<D, B=C=E
ROS	3 p.f.	D>A=B=C=E
Phagocytic activity	21 p.f.	A<B=C=D=E
	28 p.f.	A<B=C=D=E
Phagocytic index	21 p.f.	A<B=C=E=D
	28 p.f.	A<B=C=E=D
	14 p.i.	A<B=C=E=D
Phagocytic capacity	21 p.f.	Yeast (1 cell) A>E=D, C=B Yeast (6+ cells) A<D, B=C=E
	28 p.f.	Yeast (6+ cells) A<D, B=C=E
	14 p.i.	Yeast (6+ cells) A<D, B=C=E

*p.f.=post feeding, p.i.=post infection, A=Basal control diet, B=0.05% β-glucan, C=0.1% β-glucan, D=0.2% β-glucan and E=0.1% Commercial yeast derived β-glucan

4.3.3 Plasma protein activities between experimental groups

The total plasma protein levels in the 0.1% β-glucan group was significantly different to the groups fed the control diet and the diet supplemented with 0.05% β-glucan at Day 1 p.f., while fish fed the 0.05% β-glucan diet was significantly different to the group fed 0.1% commercial yeast derived β-glucan at 14 d.p.i. (Figure 4.6(A)). Plasma peroxidase activity was significantly different on Day 1 p.f. , with the 0.1% β-glucan group having higher peroxidase activity than the 0.2% β-glucan group groups (*P<0.05*), while the 0.05 % β-glucan group had statistical significantly different activities to that of the control group on 14 d.p.f. (Figure 4.6(B)). Total plasma anti-protease activity in the basal control group was significantly different to that of the groups fed 0.1% β-glucan and 0.05% β-glucan (*P<0.05*) at Day 21 (Figure 4.6(C)). The only sampling point where

there was a statistical difference in the natural antibody titre ($-Log_2+1$) was at Day 3 p.f. with highest levels recorded in the the groups fed 0.1% β-glucan (*P<0.05*) compared to those fed the 0.1% commercial yeast derived β-glucan or the basal diet (Figure 4.6(D)). The levels of natural antibodies clearly increased after infection with *E. ictaluri.* Plasma lysozyme activity of experimental fish were significantly different (P<0.05) on Day 7 p.f. in the 0.2% β-glucan group compared with fish fed the basal control diet (Figure 4.6(E)). Significant differences in lysozyme activity were evident between groups over time (P<0.05). The plasma complement activity, measured as the mean number of ACH50 units/ml plasma, was significantly higher at Day 7 p.f. (P<0.05) in the 0.1% and 0.2% β-glucan fed-groups compared to fish fed the control diet, while the 0.1% β-glucan fed group was also significantly different (P<0.05) to basal control fed fish at 14 d.p.i. Generally, the group fed the basal diet had lower $ACH_{50\%}$ levels than the immunostimulated groups (Figure 4.6(F)).The only time-points where there was a statistical difference in the total IgM levels between groups was at Day 28 p.f., when the total plasma IgM levels were significantly higher (*P<0.05*) in 0.1% β-glucan fed fish compared to the other three groups (basal control, 0.05% β-glucan and 0.1% commercial yeast derived β-glucan) (Figure 4.6(G)). A summary of the differences found between the various plasma parameters is shown in Table 4.5.

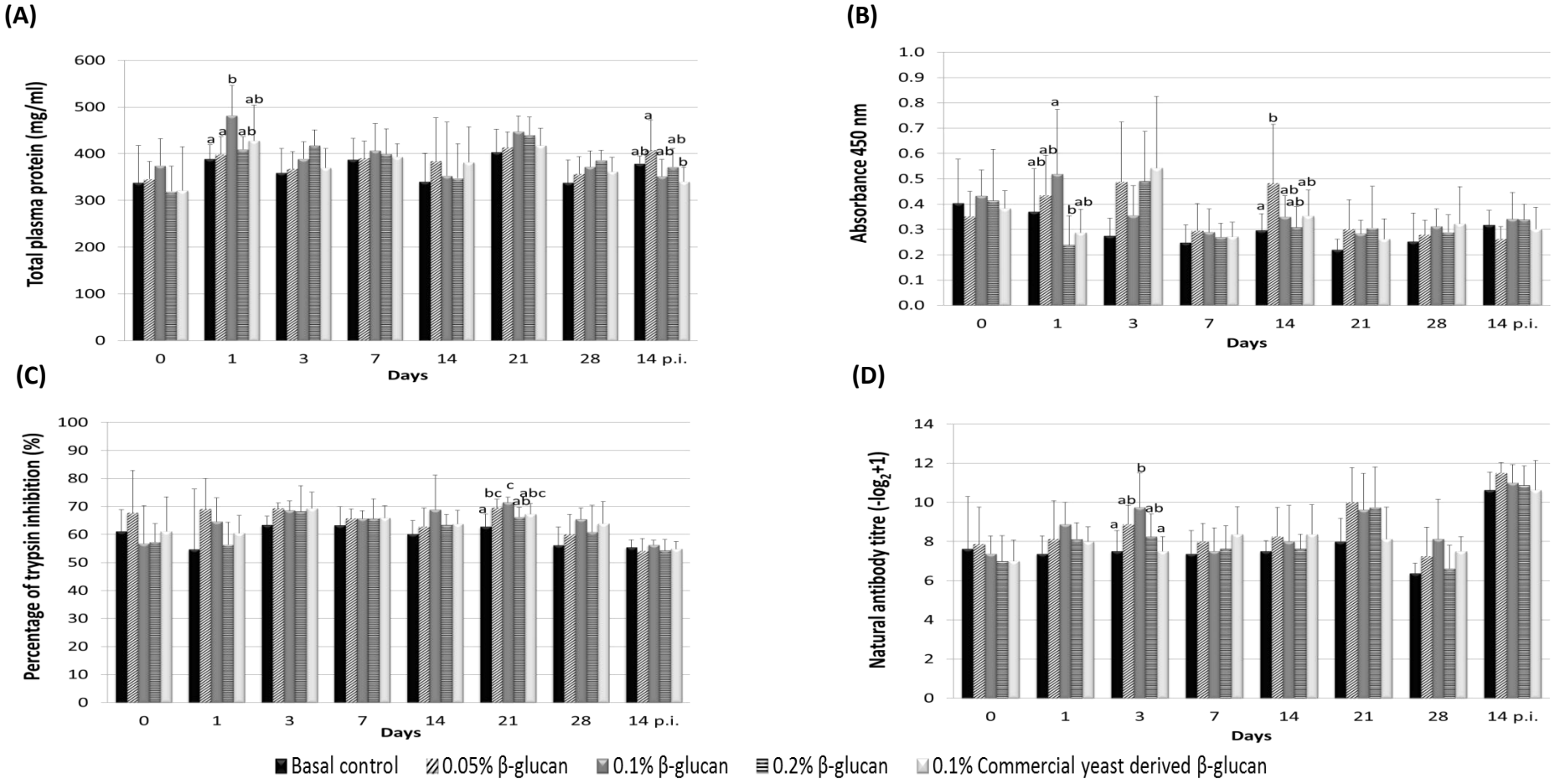


Figure 4.6(A-D). Comparison of (A) total plasma protein (mg/ml), (B) plasma peroxidase activity, (C) total plasma anti-protease activity (%) and (D) natural antibody levels ($-Log_2+1$) between experimental groups fed different diets for upto 28 days, after which all groups were challenged with *E.ictaluri* for 14 days (Mean±SD, n=8). Different letters indicate significant differences ($P<0.05$) between groups for a given time point.

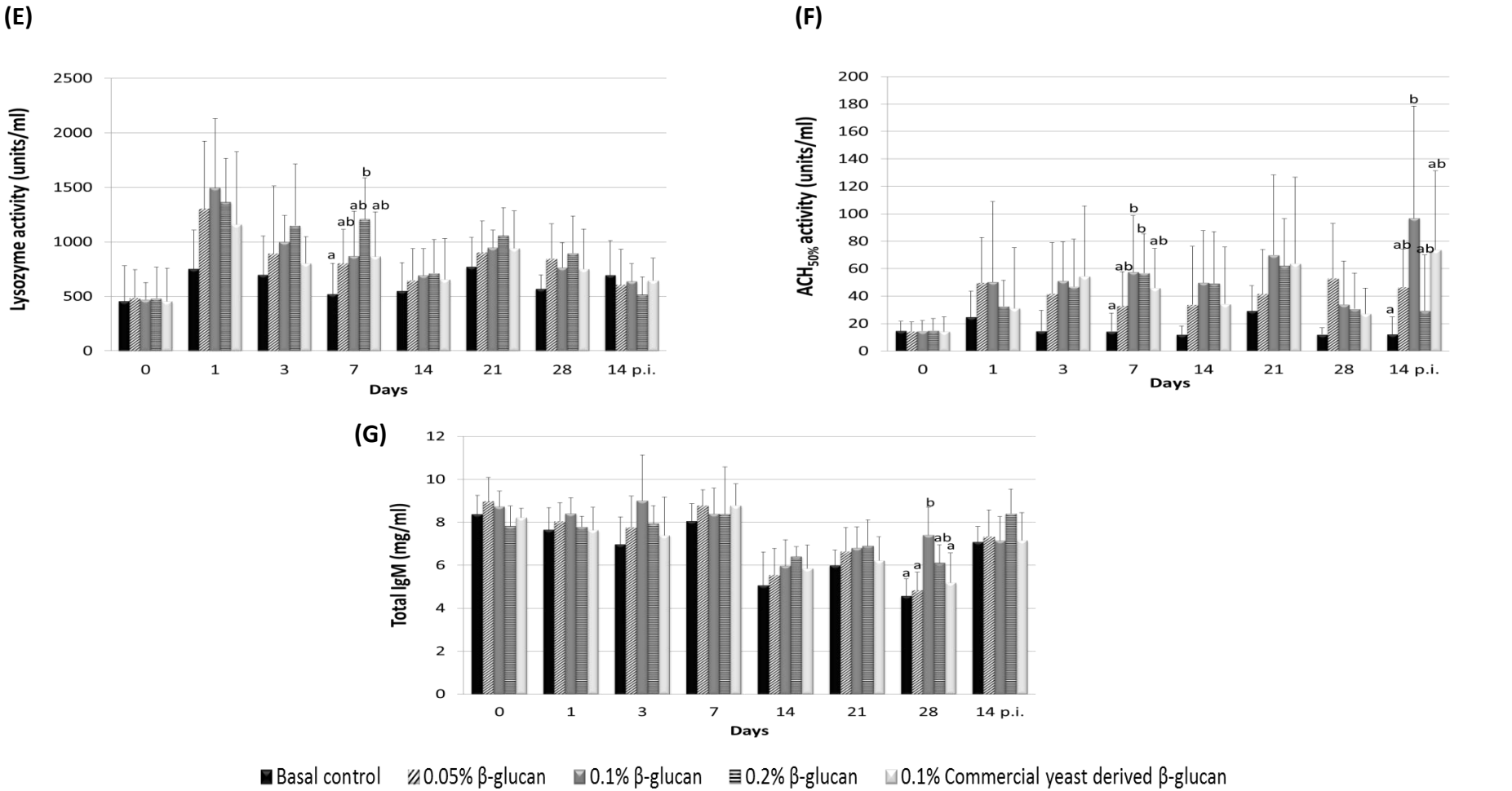


Figure 4.6(E-G; cont.). Comparison of (E) plasma lysozyme activity, (F) plasma complement activity ($ACH_{50\%}$ units ml^{-1}) and (G) total Immunoglobulin M levels (µg ml^{-1}) between experimental groups fed different diets for upto 28 days, after which all groups were challenged with *E.ictaluri* for 14 days (Mean±SD, n=8). Different letters indicate significant differences (P<0.05) between groups for a given time point.

Table 4.5. Summary of the significant differences in plasma peroxidase activity, total protein, total anti-protease activity, lysozyme activity, complement activity and total immunoglobulin M in plasma among days and treatments

Parameters	Day	Treatment
Plasma peroxidise	1 p.f.	C>D, A=B=E
	14 p.f.	B>A, C=D=E
Total protein	1 p.f.	C>A=B, D=E
	14 p.i.	B>E, A=C=D
Antiprotease	21 p.f.	C>A, B=D=E
Natural antibody titre	3 p.f.	C>A=E, B=D
Lysozyme	7 p.f.	A<D, B=C=E
Complement	7 p.f.	A<C=D, B=E
	14 p.i.	A<C, B=D=E
Total IgM	28 p.f.	C>A=B=E, D

*p.f.=post feeding, p.i.=post infection, A=Basal control diet, B=0.05% β-glucan, C=0.1% β-glucan, D=0.2% β-glucan and E=0.1% Commercial yeast derived β-glucan

4.3.4 Disease resistance to experimental *E. ictaluri* infection

4.3.4.1 Percentage mortality between experimental groups

The percentage mortality was not significantly different between the five groups (Table 4.6) following challenge with *E. ictaluri*, although the group fed 0.1% commercial yeast derived β-glucan had the lowest level of mortalities between the dietary groups. The percentage cumulative mortalities are presented in Figure 4.7(A). Unfortunately there was a large variation in the percentage mortalities between tanks within groups as shown in Figure 4.7(B), and this affected the overall level of mortality seen between groups fed the different immunostimulant diets.

4.3.4.2 Specific antibody titre against *E. ictaluri*

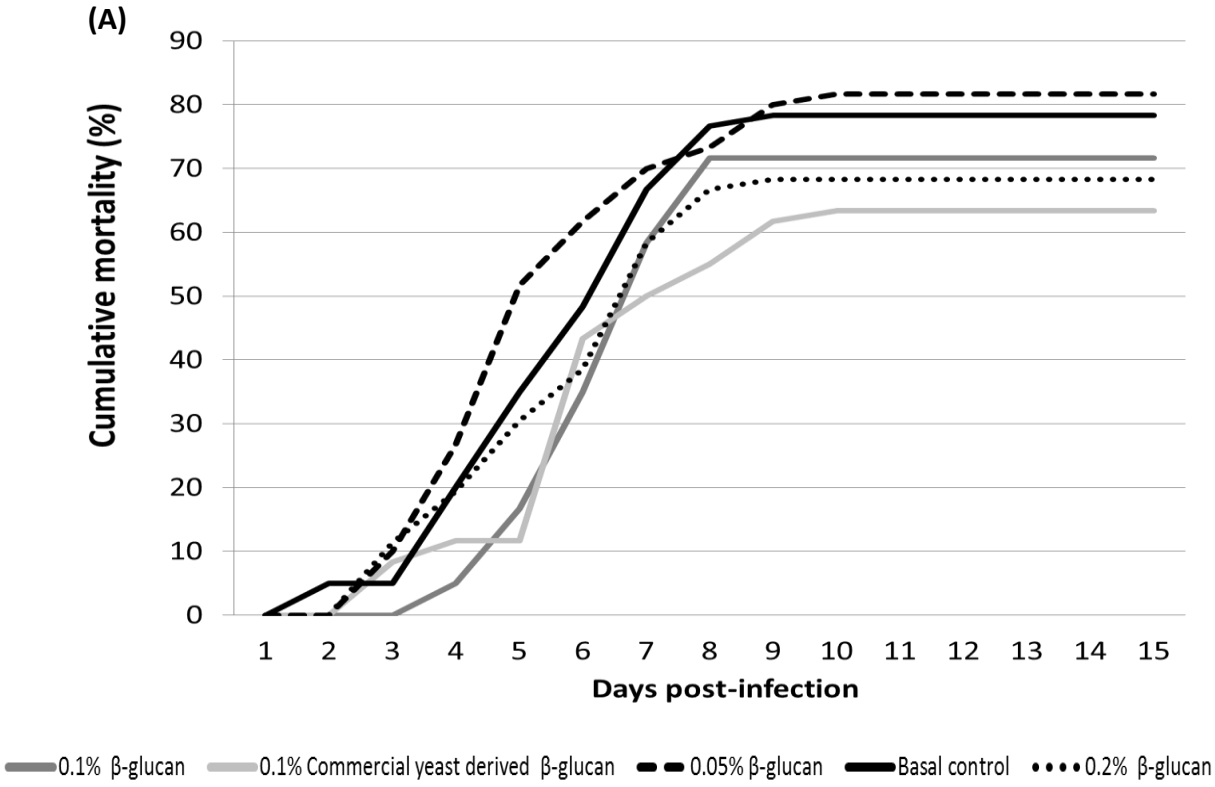
The antibody titre ($-Log_2+1$) between fish before and after experimentally infecting with *E. ictaluri* were not significantly different ($P<0.05$) between the five experimental groups, as shown in Table 4.7. However, the level of specific antibody titre against *E. ictaluri* in the post-challenge groups was up regulated to levels prior to-challenge.

Table 4.6. Percentage mortality (Mean±SD, n=15) between experimental groups following challenge with *Edwarsiella ictaluri*

	(A) Basal control	Fungal derived β-glucan			(E) 0.1% Commercial yeast derived β-glucan
		(B) 0.05%	(C) 0.1%	(D) 0.2%	
Cumulative mortality (%)	78.3 ± 14.8	81.7 ± 25.2	71.7 ± 28.0	70.0 ± 35.1	63.3 ± 30.1

Table 4.7. Comparison of average antibody titre (Mean ±SD, n=8) between experimental groups before and after infection with *Edwardsiella ictalutri*

Antibody titre against *E.ictaluri* ($-Log_2+1$)	(A) Basal control	Fungal derived β-glucan			(E) 0.1% Commercial yeast derived β-glucan
		(B) 0.05%	(C) 0.1%	(D) 0.2%	
Pre-challenge (28 d.p.f.)	6.5 ± 0.8	6.6 ± 1.4	7.0 ± 1.1	6.4 ± 0.5	6.5 ± 0.8
Post-challenge (14 d.p.i.)	10.5 ± 1.4	11.1 ± 2.5	10.3 ± 1.6	10.0 ± 2.6	10.5 ± 1.9



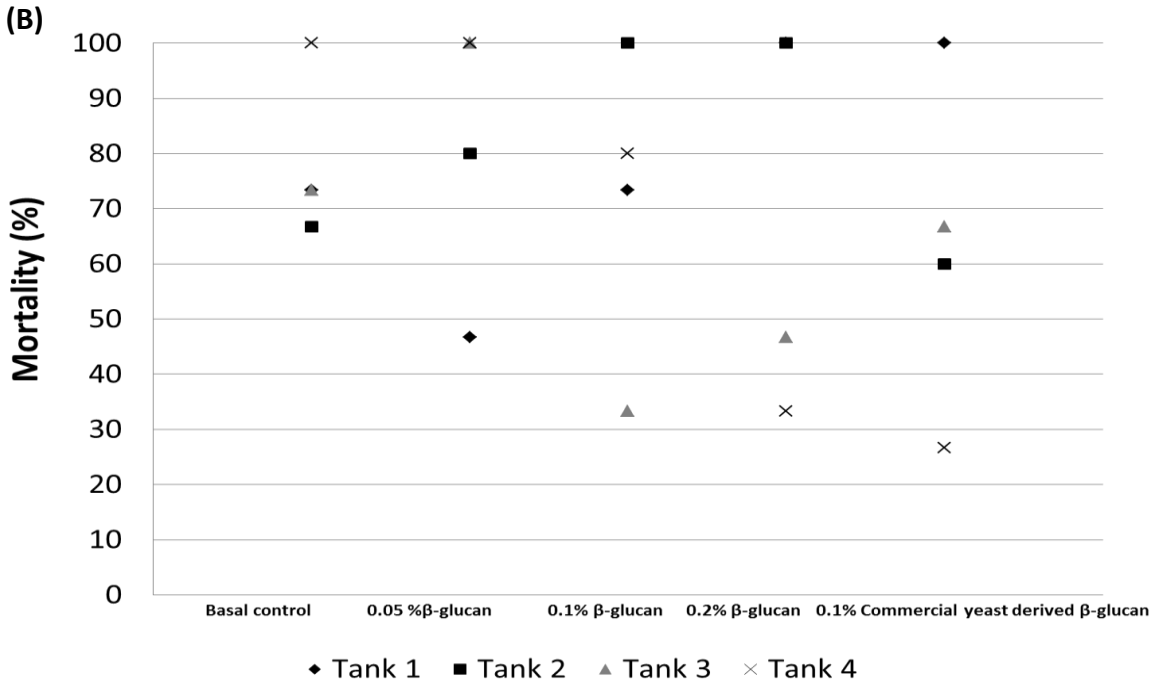


Figure 4.7(A-B). Comparison of (A) total percentage and (B) percentage cumulative mortality of *Pangasianodon hypophthalmus* between groups fed different diets for up to 28 days, after which all groups were challenged with *Edwardsiella ictaluri* for 14 days.

4.3.5 Statistical analysis

4.3.5.1 Interaction between days

The overall statistically significant (*P<0.05*) variation in immunological parameters is presented in Table 4.8 and 4.9. The variation between sampling days prior to the challenge is shown in Table 4.8 with significant differences (*P<0.05*) seen in haematocrit values (HCT), red blood cell counts (RBC), white blood cell counts (WBC), mean corpuscular values (MCV), lysozyme activity, complement activity, natural antibody titre, plasma anti-protease activity, respiratory burst, plasma peroxidise activity, total protein levels, phagocytic activity, total IgM, and differential white blood cell counts. The significant difference (*P<0.05*) in variation of immunological parameters between pre-infected, sampled at Day 28 p.f. and infected fish 14 d.p.i with *E. ictaluri* is shown in Table 4.9, with differences seen in HCT values, RBC counts, WBC counts), lysozyme activity, complement activity, natural antibody titres, plasma anti-protease activity, respiratory burst activity, phagocytic activity, total IgM and antibody titres against *E. ictaluri*.

4.3.5.2 Interaction between treatments

The statically significant (*P<0.05*) variation in immunological parameters between treatments A (basal control), B (0.05% Fungal derived β-glucan), C (0.1% Fungal derived β-glucan), D (0.2% Fungal derived β-glucan) and E (0.1% Commercial yeast derived β-glucan) in fish sampled at the various time points prior to the challenge (*i.e*. Days 0, 1, 3, 7, 14, 21 and 28 p.f.) is shown in Table 4.8. Significantly differences were seen in lysozyme activity, complement activity, natural antibody titre, plasma anti-protease activity, respiratory burst, total IgM, differential white blood cell counts:

percentage of monocytes. The significant differences (*P<0.05*) in immunological parameters between pre-infection and 14 d.p.i with *E. ictaluri* is shown in Table 4.9. Differences were seen in complement activity, respiratory burst activity, total plasma IgM, phagocytic activity and differential white blood cell counts.

4.3.5.3 Interaction between days and treatments

The significant differences (*P<0.05*) in variation of immunological parameters between Days (0, 1, 3, 7, 14, 21 and 28 p.f.) and Treatment (A, B, C, D, and E) are shown in Table 4.8 and includes white blood cell counts , plasma peroxidase, total IgM and differential white blood cell count with percentage of monocytes. Statistically different variations in immunological parameters between pre-infection and 14 d.p.i. with *E. ictaluri* were seen with MCV, total IgM and total plasma protein.

4.3.5.4 Interaction between tanks

Significant differences (*P<0.05*) in immunological parameters between tanks under treatment A, B, C, D or E at Days 0, 1, 3, 7, 14, 21 and 28 p.f. was seen in mean corpuscular value, haematocrit, phagocytic activity, complement activity, total IgM and differential white blood cell count (proportions of monocytes, lymphocytes, neutrophils and thrombocytes). Differences in immunological parameters between tanks pre and post-infection with *E. ictaluri* at Day 14 p.f. can be seen with regard to differential white blood cell counts (proportions of lymphocytes and thrombocytes).

4.3.5.5 Survival analysis by Cox Regression model

There were no statistically significant (*P<0.05*) differences in survival between treatments using a Cox Regression model. The null model -2 Log Likelihood (-2xLL) of 2318. Including treatment in the null model was not significant (-2xLL = 2309; X_4^2 =

9.44; P=0.051) in a likelihood ratio test with a pseudo r^2 value of 0.0041. There was statistically significant variation in survival between tanks (*P<0.01*). Tanks in the null model was significant (-2xLL = 2171; X_{15}^2 = 138.32; P=0.00) in a likelihood ratio test with a pseudo r^2 value of 0.0597.

Table 4.8. Statistical analysis for interaction between Day (0, 1, 3, 7, 14, 21 and 28) post feeding), Treatment (A, B, C, D and E), Day-Treatment and Tank (1-4) by using Minitab ver16 (ANOVA, general linear model, pairwise comparisons Tukey)

Parameters	Day	Treatment	Day* Treatment	Tank
HCT	**P=0.001**	P=0.086	P=0.253	**P=0.026**
RBC	**P=0.001**	P=0.885	P=0.950	P=0.094
WBC	**P=0.001**	**P=0.026**	**P=0.001**	P=0.112
MCV	**P=0.001**	P=0.262	P=0.977	**P=0.045**
Lysozyme	**P=0.001**	**P=0.006**	P=0.716	P=0.099
Complement	**P=0.001**	**P=0.031**	P=0.792	**P=0.019**
Natural antibody titre	**P=0.001**	**P=0.006**	P=0.232	P=0.497
Plasma anti-protease	**P=0.001**	**P=0.019**	P=0.395	P=0.165
Respiratory burst :				
NBT	**P=0.001**	**P=0.001**	P=0.889	P=0.632
NBT+PMA	**P=0.001**	**P=0.001**	P=0.262	P=0.960
Plasma peroxidase	**P=0.001**	P=0.133	**P=0.005**	P=0.116
Total plasma protein	**P=0.001**	**P=0.039**	P=0.503	P=0.336
Phagocytic activity	**P=0.001**	P=0.344	P=0.997	**P=0.001**
Total IgM	**P=0.001**	**P=0.023**	**P=0.040**	**P=0.012**
Differential white blood				
cell count : Monocyte	P=0.062	P=0.094	**P=0.006**	**P=0.001**
Lymphocyte	**P=0.001**	P=0.074	P=0.961	**P=0.001**
Neutrophil	P=0.250	P=0.806	P=1.000	**P=0.001**
Thrombocyte	**P=0.035**	P=0.302	P=0.626	**P=0.001**

Table 4.9. Statistical analysis for interaction between Day 28 post-feeding and 14 post-infection, Treatment (A, B, C, D and E), Day-Treatment and Tank (1-4) by using Minitab ver16 (ANOVA, general linear model, pairwise comparisons: Tukey)

Parameters	Day	Treatment	Day* Treatment	Tank
HCT	**P=0.001**	P=0.167	P=0.408	P=0.303
RBC	**P=0.002**	P=0.940	P=0.351	P=0.403
WBC	**P=0.001**	P=0.284	P=0.517	P=0.440
MCV	P=0.537	P=0.646	**P=0.035**	P=0.299
Lysozyme	**P=0.019**	P=0.887	P=0.116	P=0.420
Complement	**P=0.031**	**P=0.011**	P=0.057	P=0.627
Natural antibody titre	**P=0.001**	P=0.178	P=0.226	P=0.242
Plasma anti-protease	**P=0.001**	P=0.186	P=0.197	P=0.305
Respiratory burst :				
NBT	**P=0.001**	P=0.230	P=0.700	P=0.829
NBT+PMA	**P=0.001**	**P=0.001**	P=0.085	P=0.973
Plasma peroxidase	P=0.280	P=0.375	P=0.517	P=0.463
Total plasma protein	P=0.400	P=0.174	**P=0.011**	P=0.362
Phagocytic activity	**P=0.046**	**P=0.007**	P=0.601	P=0.103
Antibody titre against *E. ictaluri*	**P=0.001**	P=0.824	P=0.845	P=0.421
Total IgM	**P=0.001**	**P=0.016**	**P=0.001**	P=0.093
Different white blood cell count :				
Monocyte	P=0.613	**P=0.049**	P=0.932	P=0.121
Lymphocyte	P=0.929	P=0.221	P=1.000	**P=0.001**
Neutrophil	P=0.973	P=0.813	P=0.988	P=0.237
Thrombocyte	P=0.967	P=0.279	P=0.991	**P=0.003**

4.4 Discussion

The success of immunostimulants depends on many factors including the duration of feeding the immunostimulant, water temperature, fish species, dose fed, and duration of the effectiveness of the immunostimulant, circadian rhythms and seasonality. The cost of the immunostimulants is also important (Anderson 1992; Maqsood*, et al.* 2011). The present study clearly showed that β-glucan can stimulate many different immune parameters in pangasius catfish, in line with similar research carried out on other fish species (Lin*, et al.* 2011; Sharifuzzaman and Austin 2009). A range of blood parameters were measured in this study to examine the functional activity of various blood cells and to determine immune response (Pavlidis*, et al.* 2007). Haematocrit value is an indicator of the health status of the fish; the haematocrit values obtained indicated that there were were no significant differences between the treatment groups for the first 3 d.p.f., however, the values for the 0.1% β-glucan-fed groups increased at 7 and 21 d.p.f. Leukocytes and thrombocyte are also used to indicate the health status of the fish and can be used to evaluate their immune response (Tavares-Dias and Moraes 2007). The numbers of WBCs increased in the groups fed the β-glucan-supplemented diet compared to the basal control group within the first day of feeding the experimental diets. However, the differential white blood cell counts of these fish indicated it was only the percentage of monocytes that changed as a result of being fed the diets. Harikrishnan *et. al.* (2011) showed a significant increase in haematocrit values and WBC counts (with changes in the proportions of monocytes, lymphocytes and neutrophils) in *Oplegnathus fasciatus* 1-6 weeks after having fed

them diets containing an immunostimulant from the herb Baical skullcap (*Scutellaria baicalensis*).

Macrophages and neutrophils produce bactericidal ROS during respiratory burst when stimulated by foreign substances as a result of phagocytosis (Ellis 2001). Respiratory burst activity of phagocytes is increased in the presence of PMA. After feeding the pangasius with β-glucan it was found that their respiratory burst activity increased within one week of being fed the diets with the highest respiratory burst activity seen at Day 7 after feeding, similar to what was seen in Koi carp fed with either β-1,3-glucan, chitosan or raffinose in the research by Lin, *et. al* (2011). Phagocytic activity by head kidney macrophages was also higher in the fish fed β-glucan compared to the basal control group, although no difference was seen between the four immunostimulated groups. They did find a significant difference in the phagocytic activities between four immunostimulated groups and basal control group after Day 21 and 28 of feeding.

The total plasma protein levels analysed in the present study show that the major plasma proteins are involved in the immune response of the fish (Harikrishnan*, et al.* 2011); it was found that these levels were higher in fish fed with the 0.1% β-glucan supplemented diets compared to the basal control group and 0.05% β-glucan on 1 d.p.f. There was, however, no difference in these levels between 0.1% β-glucan, 0.2% β-glucan and 0.1% commercial yeast-derived β-glucan fed fish after one day of feeding the diets. The total plasma protein levels were shown to be higher 6 weeks after feeding the immunostimulant supplement in the study of Harikrishnan *et. al.* (2011).

Lysozyme is an important protein found in the plasma of fish, and is an enzyme which degrades the peptidoglycan layer of bacterial cell walls, causing lysis of the bacterial cell, and in turn, activation of the complement system and phagocytosis by head kidney macrophages (Ellis 2001). In this study, lysozyme activity was significantly higher in the 0.2% β-glucan fed fish compared to the basal control group at Day 7 of the trial, after which the trend in enzyme activity decreased. However, there was no significant difference between any of the treatment groups. In contrast, lysozyme activity measured in rainbow trout fed with a probiotic (*Kocuria*) was higher at Week 2 of feeding in the study by Sharifuzzaman and Austin (2009).

Complement activity in fish can be activated by lipopolysaccharide (LPS), which is a major constituent of the cell wall of Gram-negative bacteria (Ellis 2001). In the present study β-glucan was found to increase complement activity via the alternative pathway after 7 day of feeding and the highest activity was detected in fish after they were infected with *E. ictaluri.* This differs from the results of Cook *et. al.* (2003b), who found nodifferences in the alternate complement activity in fish fed with commercial β-glucan-based immunostimulant preparation (EcoActiva™).

Fish plasma also contains a number of protease inhibitors, principally α1-anti-protease, α2-anti-plasmin and α2-macroglobulin (α2M), which restricts the ability of bacteria to invade and grow within fish by acting against the proteases produced by the invading pathogens (Ellis 2001). In the present study administration of 0.05% and 0.1% β-glucan diets significantly enhanced plasma anti-protease activity on Day 21 of feeding. This is agreement with the observation made by Harikrishnan *et*. *al*. (2011) who found significantly enhanced serum anti-protease activity on Weeks 3 or 6 of

feeding *O. fasciatus* with diets supplemented with a probiotic or the herb (Baical skullcap; *S. baicalensis*).

Peroxidase in the plasma of fish reflects the levels of peroxidase produced by macrophages (Sitjà-Bobadilla*, et al.* 2005). In the present study, the 0.05% β-glucan fed groups had higher plasma peroxidase levels than the basal control group on 14 d.p.i., but there were no differences between the β-glucan groups. Sitja-Bobadilla *et. al*., (2005) studied the effect of meal replacement with different concentrations of plant proteins on innate defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Their data showed there was no significant effect of the diets on the myeloperoxidase activity produced by their head kidney macrophages. However, significant differences in myeloperoxidase activity were observed in the plasma of fish fed the diet with 100% plant protein replacement (PP100) compared to fish meal diet and 50% plant protein replacement (PP50). Sharifuzzaman and Austin (2009) found macrophage peroxidase content to be more pronounced in rainbow trout fed *Kocuria*-supplemented diets after two weeks of feeding the probiotic.

Immunoglobulin M (IgM) is the major immunoglobulin class in teleost fish (Morrison and Nowak 2002). In this study, the total IgM and natural antibody titres were found to be at higher levels in fish fed with the 0.1% β-glucan supplemented diets compared to the basal control group, the natural antibody titres were significantly different at 3 d.p.f. and total IgM at 28 d.p.f. Higher levels of plasma IgM were seen 14 days in all fish after infecting them with *E. ictaluri*. However, there was no significant difference between the treatment groups.

There was considerable variation in the results between fish and between time points for all of the assays performed; and this may reflect whether statistical differences could be detected between groups. More fish may need to be used per group to establish significant differences for some of the assays. A clear effect of immunostimulation was seen with many assays; however, this appeared to be dose dependent with regard to β-glucan. Generally, the greatest immunostimuation was seen with 0.2% β glucan with higher respiratory burst activity on all days examined and higher plasma lysozyme activity on Day 7 of feeding, although the 0.1% β-glucan groups had the greatest immuostimulation with regard to plasma anti-protease activity on Day 21, natural antibody titre on Day 3 and complement activity Day 7 and 14 p.i. A dose of 0.05% β-glucan appeared insufficient to effectively stimulate the fish's immune response.

Not all assays appeared useful for studying the effects of immunostimulation. Some assays were useful as an early indication of immunostimulation *e.g*. white blood cell count, respiratory burst, lysozyme activity and complement activity, showing stimulation from the first day of feeding the experimental diets. Immunology parameters which appeared useful for comparing between the treatment groups included respiratory burst activity, lysozyme activity, complement activity, plasma anti-protease, natural antibody titre and total protein.

No statistical differences were seen in the fish's resistance to the experimental infection between the different dietary groups. There was, however, a great deal of variation in the level of mortalities within the four replicate tanks for each dietary group, which affected the mean mortality obtained per group. The mortality levels

recorded were high reflecting a substantial challenge. For future challenges, the addition of a lower dose (aimed at reaching 40% mortality, for example) may allow more subtle effects of the immunostimulants to be seen. In addition, fish should be pre-screened for the presence of anti-*E. ictaluri* antibodies to confirm that fish are not infected with this pathogen before starting the experiment.The results here indicated that *E. ictaluri*-specific antibody response of fish was relatively low prior to challenge *i.e*. 1/32, suggesting that the fish were not infected prior to starting the challenge.

In conclusion, these studies clearly indicate that both the non-specific humoral and cellular immune responses of pangsius, measured in this study, were differentially stimulated by different concentrations of the immunostimulant β-glucan with doses of 0.1 or 0.2% fungal derived β-glucan obtaining optimal immunostimulation.

Chapter 5 Effects of β-glucan on the response of *Pangasianodon hypophthalmus* immune genes and their resistance to *Edwardsiella ictaluri*

5.1 Introduction

Previously in Chapter 4 the effects of feeding β-glucan on the immune function of *P. hypophthalmus* was examined using functional assays (haematocrit (Hct), mean corpuscular volume (MCV), WBC and RBC counts, differential WBC counts, phagocytic activity, respiratory burst activity, plasma lysozyme activity, complement activity, plasma peroxidase activity, total immunoglobulin M, natural antibody titre, total plasma protein and plasma anti-protease) to assess this response, and it was clearly shown that both the humoral and cellular responses of the innate immune response of fish were differentially stimulated by different concentrations of fungal-derived β-glucan. The percentage of cumulative mortalities after experimentally infecting the stimulated fish with *E. ictaluri,* by immersion, were not significantly different between the dietary groups (*i.e* between fish fed 0.05%, 0.1%, 0.2% fungal derived β-glucan, 0.1% commercial yeast derived β-glucan or a basal control diet with no glucans), possibly due to the large variation in mortalities between tanks within groups.

The immunostimulant activity of β-glucans is mediated through the modulation of gene expression of pro-inflammatory cytokines and chemokines (Biswas*, et al.* 2012; Falco*, et al.* 2012). For example, it has been shown that injection of β-glucan induces the expression of interleukin (IL) 1β genes in head kidney macrophages of carp (Selvaraj*, et al.* 2005), or expression of complement factors (*i.e.* C3 and factor B), and acute phase proteins (*i.e*. hepcidin, precerebellin and transferrin) 24 h after stimulation of rainbow trout (*Oncorhynchus mykiss*) fry with β-glucans (Chettri*, et al.* 2012). Falco, *et al*., (2012) found that diets supplemented with β-glucans reduced the inflammatory response of common carp during *Aeromonas salmonicida* infection using

a quantitative PCR (qPCR) to assess the gene expression patterns of tumour necrosis factor-α1 (TNF-α1), tumour necrosis factor-α2 (*TNF-α2*), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-10 (IL-10). A significant reduction in the level of mortalities was observed in zebrafish (*Danio rerio*) experimentally infected with *A. hydrophila* after they had been injected intraperitoneally with β-glucan, derived from *Saccharomyces cerevisiae*, at 5 mg ml^{-1} (Rodríguez*, et al.* 2009). However, the β-glucan used in the study did not appear to affect the expression of tnfα or IL-1β, but did modulate interferon-γ (IFN-γ) and chemokine expression in the kidney. Kim, Ke and Zhang (2009) showed that when grass carp (*Ctenopharyngodon idella*) were injected intraperitoneally with β-glucan derived from the mycelia of *Poria cocos* 85 at 10 mg kg^{-1}, the fish exhibited enhanced anti-viral activity against grass carp haemorrhage virus through an increased Mx expression. Diets containing 0.5 or 1 g kg^{-1} of β-1, 3-glucan derived from *Laminaria digitata* fed to white shrimp (*Litopenaeus vannamei*) resulted in significantly higher level of hemocyanin, lipopolysaccharide (LPS), β-glucan binding protein (LGBP), serine proteinase, prophenoloxidase (proPO) and superoxide dismutase (SOD) expression in stimulated shrimp exposed to 120 h of nitrite stress compared to unstimulated shrimp (Zhao*, et al.* 2012).

There are only a few studies examining the expression of immune genes in *P. hypophthalmus.* Huong Giang, *et al.* (2010) examined serum amyloid P component (SAP) and C-reactive protein (CRP) genes in *P. hypophthamus*, while Sinha, *et al*. (2010) monitored heat shock protein 70 (HSP70), growth hormone, acetylcholinesterase (AChE), trypsinogen, cytochrome P4501B (CYP1B) and cytochrome oxidase submit-1 (COI) expression as potential biomarkers after exposure to trichlorfon.

The aim of the present Chapter was to identify sequences (through the use of an EST library) that matched *P. hypophthalmus* immune genes so that these could be utilised to assess gene expression following β-glucan stimulation and infection with *E. ictaluri*. Thus, after, designing suitable primers for the specific genes identified, qPCR assays were used to assess their level of expression in *P. hypophthalmus*-fed diets supplemented with β-glucans from two different sources (0.1% fungal derived β-glucan and 0.1% commercial yeast derived β-glucan). Ultimately the effect of feeding the β-glucan supplemented diets on the fish's resistance to *E. ictaluri* was examined by experimentally infecting the fish with the bacterium using an immersion challenge.

5.2 Materials and methods

The work presented in this chapter is divided into two sections. It was first section immune genes were identified from *P. hypophthalmus* based on the identity of EST sequences. Having identified appropriate sequences for the genes, primers were designed for analysis of their expression in qPCR. In the second part of this chapter the effects of β-glucan on the expression of these immune genes prior to and after exposure to *E. ictaluri* was examined.

5.2.1 Identification of immune genes, primer design and optimization of qPCR assays

5.2.1.1 Expressed sequence tags (ESTs)

Expressed Sequence Tags (ESTs) were kindly provided by Ei Lin Ooi from Novus Aqua Research Center (NARC), Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam (unpublished data). Briefly, an EST library was prepared from 500g striped catfish challenged with *E. ictaluri* for 24 h; tissues (intestine, head kidney, liver and muscle)

were collected, RNA extracted and a cDNA library sequenced by pyrosequencing. The resulting sequences were processed to produce four EST data-sets (intestine 2,532 ESTs; head kidney 3,179 ESTs; liver 1,314 ESTs and muscle 1,070 ESTs).

5.2.1.2 Identification of immune gene sequences from EST sets

An overview of the process involved in the identification of immune sequences and qPCR development is shown in Figure 5.1. A list of immune genes of interest to examine the effect of β-glucan stimulation was first compiled from the various sources shown in Table 7.1 (Appendix 2). Using this list, all the homologous genes present in the NCBI RefSeq databases (genome, RNA and EST) were identified using BLASTN (Altschul*, et al.* 1990); their gene IDs were retrieved to make the reference list of genes. In parallel, all 8,095 EST sequences (despite their high redundancy) were also compared with the same NCBI RefSeq databases (genome, RNA and EST) using BLASTN; the gene ID of each best match was retrieved.

The list of best matches and reference list of genes were compared; if the best match of an EST was found in the reference list of genes, the EST was considered to be an acceptable homolog to this gene. This procedure maximised the likelihood that the EST was a fragment of an immune gene of interest. A more direct approach, *i.e.* comparing the ESTs to the reference list of immune genes only, would identify EST exhibited sequences similar to one of the immune gene selected, but would not ensure that this similarity was not exceeded by the non-immune gene sharing only a sequence motif.

All BLASTN searches were used with default parameters; the similarity thresholds used to filter the results were: expectation-value (e-value) lowers than 5

x10^{-5}, total coverage over 33% of the query sequence and for over 100 nucleotides in length (*i.e*. aliment longer than 100).

5.2.1.3 Design of RT-qPCR primers

The sequences of immune related genes of interest obtained in Section 5.2.1.2 were used to design the RT-qPCR primers using The NCBI Primer-Blast software [http://www.ncbi.nlm.nih.gov/tools/primer-blast/] (Ye*, et al.* 2012). The criteria for good primer design was 3' self-complementarity less than 1, a melting temperature of 60^{o}C, an amplified region of less than 200 base pairs, which was located in the middle part of the sequence region, 50-60 % GC content and length of 18-24 nucleotides.

5.2.1.4 Optimisation of RT-qPCR assays

A master mix containing both the forward and the reverse primer pairs for a2 MHC class II integral membrane protein alpha chain 2, complement (C3 and factor B/C2A), transferrin, C-reactive protein 2, precerebellin-like protein (cerebellin 14), interleukin-1β and interferon γ 2a and 2b, translation elongation factor-1α mRNA, 18S rRNA gene and β-actin mRNA were prepared (*i.e.* a stock primer solution at a final concentration of 10 µmol). The master mix was added to the wells of a 96 well qPCR plate (4 µL well^{-1}) together with 1 µl cDNA, prepared from normal *P. hypophthalmus* liver and kidney tissue as described in Section 5.2.5 and 5.2.6, and 5 µL SYBR green (Bioline, UK). The plates were sealed with a plate sealer and centrifuged with 258 xg for 30 s. The plates were placed into the qPCR machine (Eppendrof® realplex AG, USA), and the following programme used to run the assay (preheating to 95^{o}C then pause, activation 95^{o}C for 10 min, denaturing 95^{o}C for 15 sec, gradient annealing temperature 55-65^{o}C for 15 sec

and extension at 72^{o}C for 20 sec). The quality of RT-qPCR products were checked by gel electrophoresis using a 1.5% agarose gel.

5.2.1.5 RT-qPCR standard curve

Samples of cDNA *i.e*. normal liver and kidney tissue of *P. hypophthalmus*) (see Section 5.2.2.5 and 5.2.2.6) were diluted with cDNA dilution solution (lamda DNA 5 ng/µl, 2 mmol/L Tris pH 8.0) at concentrations of 1x, 5x, 50x, 500x and 5,000x. The synthesised cDNA was added into the wells of a 96 well qPCR plate (2 µl well^{-1}). A master mix of the primers was prepared (forward primer 0.25 µl, reward primer 0.25 µl, SYBR (Bioline, UK) 5 µl and Nano-pure water 3.5 µl per reaction) and 9 µL of this mix was added into the plate, which were then sealed with a plate sealer and centrifuged at 258 xg for 30 sec. The qPCR reaction used consisted of preheating for 95^{o}C, activation for 95^{o}C for 10 min, denaturing 95^{o}C for 15 sec, annealing at 57^{o}C for 15 sec and extension at 72^{o}C for 20 sec.

5.2.2 Analysis of *P. hypophthalmus* immune genes stimulated with fungal derived β-glucan

5.2.2.1 Experimental animals

Pangasianodon hypophthalmus were purchased from a local fish farm and transported to the Novus Aqua Research Center (NARC), Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam. The fish were quarantined and acclimated to laboratory conditions for at least 2 weeks prior to starting the experiment and were fed with control feed at 3 % body weight per day. The weight of the animals was 36 ±0.34 g at the start of the experiment.

5.2.2.2 Formulation of experimental diets and feeding regimes

Three dietary groups were prepared using the same β-glucan supplements as used in the dietary trial performed in Chapter 4 *i.e.* a basal control group; 0.1 % w/w fungal derived β-glucan and 0.1 % w/w commercial yeast drived β-glucan. The standard basal diet was prepared using the formulation showed in Table 5.1, and was supplemented with the fungal-derived β-glucan or the commercial yeast-derived β-glucan to prepare the experimental diets. The details about the procedure used to make these feeds are detailed in Chapter 4 section 4.2.2. Fish received the basal control diet at 3% body weight per day for 14 days during the acclimation period prior to starting the experiment.

5.2.2.3 Experimental plan

5.2.2.3.1 Experimental design

The experimental design consisted of the three dietary groups outlined above. All treatment groups were maintained in four replicate 340 L fibre tanks (75 fish per tank). After acclimation for 14 days, fish were fed with the experimental feeds at 3% body weight per day for 14 days. Two fish per tank per time point were collected, from which spleen, head kidney and liver were collected at 14 days post-feeding (d.p.f.) and 24 h post-infection (h.p.i.). Samples were stored in RNA later at -80^{o}C for analysis of immune genes.

5.2.2.3.2 Challenge with *E. ictaluri*

Pangasianodon hypophthalmus were challenged by immersion exposure for 30 min with a freshly prepared culture of *E. ictaluri* (NLF33). The procedure for preparing the bacteria is described in Chapter 4 section 4.2.5. For the challenge, 1 x10^{6} cfu ml^{-1} *E.*

ictaluri was added to the tank (10 L), while bacterial culture medium was added to the uninfected group. Fish were separated into two sets, the first set was transferred to 30 L fibre tanks to monitor immune gene expression in response to the infection, and the second set was transferred to 80 L fibre tanks to determine the level of specific cumulative mortalities and the temperature was maintained at 26 ±1^{o}C during the infection trial. Tissue samples were collected from the fish in the 30 L fibre tanks at 24 h. The mortality in the infected and uninfected groups in the 80 L fibre tanks was recorded up to 14 d.p.i. and specific cumulative mortalities determined.

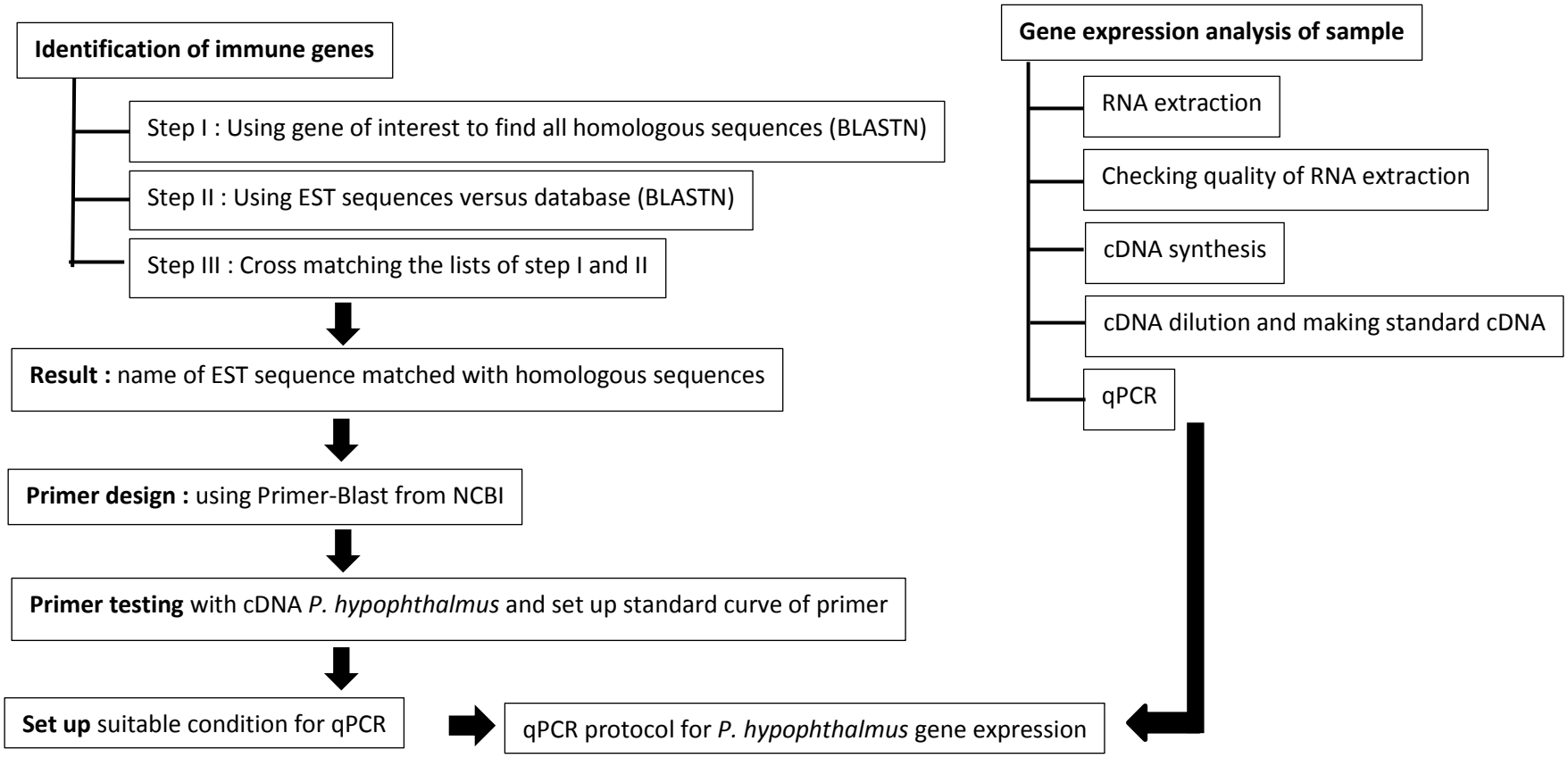


Figure 5.1. Identification of immune genes and gene expression analysis of *Pangasianodon hypophthalmus*

Table 5.1. Feed formulation

Ingredients	(%) of total weight	Weight of ingredients (g)		
		Basal control	0.1% Fungal derived β-glucan	0.1% Commercial yeast derived β-glucan
Fungal derived β-glucan	-	0	2.5	5.0
Commercial yeast drived β-glucan	-	0	0	0
Cassava	13.7	479.5	476.0	476.0
Soybean meal (48%)	46.7	1,634.5		
Rice bran solvent extract	12.2	427.0		
Canola meal	8.0	280.0		
Wheat flour	5.9	206.5		
Fish meal	3.0	105.0		
Soy protein concentrate	2.73	95.55		
Fish oil	2.54	88.9		
Soybean oil	2.53	88.55		
Dicalcium phosphate (DCP)	1.2	42.0		
Mineral premix	0.5	17.5		
Vitamin premix	0.5	17.5		
Methionine Hydoxy Analogue (84%)	0.3	10.5		
Ascorbic phosphate (25%) (Stay C)	0.1	3.5		
Choline Chloride (50%)	0.1	3.5		
Total	**100**	**3,500**		

5.2.2.3.3 Sample collection

Tissues were collected from head kidney, liver and spleen into RNAonshore (0.5 M Na_2EDTA, 1 M sodium citrate, ammonium sulphate and nuclease free H_2O) (Dr John Taggart, Institute of Aquaculture, University of Stirling, personal communication), and stored at -20^{o}C until analysed. The samples were collected at 14 d.p.f. and 24 h.p.i for immune gene analysed.

5.2.2.4 RNA isolation

Tissue samples were weighed (< 100 mg) and 1 ml of TRI reagent® added (Sigma, UK) and incubated on ice for 45 min. The samples were then homogenised with a bead beater (Mini-Beadbeater 24, USA) for 45 sec or until tissue was disrupted.

The homogenized samples were incubated at 20^{o}C for 5 min, then 50 µl of 1-bromo-3-chloropropane (Sigma, UK) was added and the tube was shaken vigorously for 15 sec. The samples were incubated at 20^{o}C for 15 min and centrifuged (Centrifuge, Sigma, UK) at 20,000 xg for 15 min at 4^{o}C. The aqueous (upper) phase *i.e*. 200 µl was remove from the transparent phase using a wide-bore pipette tip and transferred into a new tube and 100 µl of RNA precipitation solution (1.2M NaCl, 0.8M Sodium citrate sequinhydrate ($C_6H_6Na_2O_7.1.5H_2O$), nuclease-free water 100 ml) added, together with 100 µl of isopropanol (Fluka, UK). The tubes were gently inverted 4-6 times. The samples were incubated for 10 min at 20^{o}C and centrifuged at 20,000 xg for 10 min at 4^{o}C.

The supernatant was removed and the pelleted washed with 1 ml of 75 % ethanol, for 15 min at 20^{o}C. They were then flicked to detach the pellet from the bottom of the tube, inverted a few times and centrifuged at 20,000 xg for 5 min at

20^{o}C. Finally the RNA pellet was air dried at 20^{o}C for 3-5 min until all visible traces of ethanol were removed.

The samples were suspended in 100 µl of RNAase free-water and incubated at 20^{o}C for 30-60 min with gentle flicking of the tubes every 10 min to aid re-suspension. The concentration of samples were measured using a nanodrop spectrophotometer (ND-100; USA) and adjusted to a final concentration 500 ng µl^{-1} for liver and kidney tissue samples, and 300 ng µl^{-1} for spleen using RNAase-free water. The samples were finally stored at -70^{o}C.

The quality of RNA extracted was checked on a 1.5% agarose gel (Sigma, UK). Samples were prepared by using 2 µl of 2x loading dye (Biolabs; UK) and mixed with 2 µl of sample. The aliquots of RNA with the loading buffer were heated for 5 min at 75^{o}C, chilled on ice and run on a 1.5% agarose gel using a gel electrophoresis system (BIO-RAD® HU13; USA) at 75V for 30 min.

5.2.2.5 cDNA synthesis

Pooled RNA samples (two fish per pool) were used for cDNA synthesis using a high capacity cDNA reverse transcription kit (Applied biosystem, UK). A master mix was prepared containing 10 xRT buffer 2 µl, 25 xdNTPs 0.8 µl, 10x Random primer 2.0 µl, multiscribe™ reverse transcriptase 1 µl and RNAase free water 10.2 µl. The pooled RNA samples (4 µl well^{-1}) together with the master mixed (16 µl well^{-1}) were added to the wells of a 96 well qPCR plate, which was sealed with a plate seal and centrifuged at 258 xg for 30 sec. The plate was placed into the PCR machine (Biometra® T-gradient, USA) with a thermoregulation programme set as preheat at 25^{o}C, activation for 10 min

at 25^{o}C, denaturing for 120 min at 37^{o}C, annealing for 15 sec at 85^{o}C and extension at 4^{o}C for ∞). The synthesised cDNA samples were stored at -70^{o}C until used.

The cDNA prepared from the liver and kidney of each group was pooled and a series dilution of this was prepared (1x, 5x, 500x and 5,000x) by diluting the pooled cDNA in cDNA dilution solution (lamda DNA (Biolabs, UK) 5 ng µl^{-1}, 2 mM Tris pH 8.0), and 50 µl aliquots of each dilution was placed in tubes and stored at -70^{o}C.

5.2.2.6 RT-qPCR

The cDNA standards prepared in Section 5.5.2.5 and cDNA prepared from samples being analysed were added to 96 well qPCR plates at 2 µl well^{-1}. A master mix containing primers (forward primer 0.04 µl, reverse primer 0.04 µl, SYBR 5 µl and Nano pure water 292 µl reaction^{-1}) was prepared and 8 µl of this was added into the wells of the plate, which was subsequently sealed with a plate sealer and centrifuged at 258 xg for 30 sec. The running conditions of the qPCR (preheating at 95^{o}C with a pause step, activation at 95^{o}C for 10 min, denaturing at 95^{o}C for 15 sec, annealing at 57^{o}C for 15 sec and extension at 72^{o}C for 20 sec) and was carried out in a real time qPCR machine (Eppendrof® realplex AG, USA). Standard curves were generated for relative quantitation of gene expression on each assay plate using the serial dilutions of a reference cDNA described in Section 5.2.1.5. Each dilution of the standard curve was run in duplicate. Two time points for each treatment *i.e.* 14 d.p.f. and 24 h.p.i., were evaluated per tissue. The relative concentration of each total mRNA sample for each gene was calculated from the respective standard curve by iCycler iQ Real-Time PCR Detection System software version 3.0a (Bio-Rad). Data normalization was carried out by dividing the value obtained for each gene sample by the respective value obtained

for the αtranslation elongation factor-1α mRNA, 18S rRNA gene and Beta-actin mRNA. The resulting ratios were used to compare expression of the genes of interest in different tissues over a period of time.

5.2.3 Statistical analysis

The gene expression data were $\log_{10}$ transformed, to provide normal distribution for analysis and were normalised against a reference set of these immune genes by using three reference genes. Each sample represents the mean ratio of three replicates where each replicate is derived from a sample pool of two fish from each relevant treatment group. The level of expression observed within the control condition is designated a value of "1" and thereby the expression observed in the treatments is expressed as a ratio in relation to the control. Significant differences in expression between the basal control and the immunostimulant diet-fed groups on 14 d.p.f. and in the groups infected or not infected with *E. ictaluri,* at 24 h.p.i. were analysed using an analysis of variance (ANOVA) general linear model in Minitab (Version 16 © University of Stirling, 2013). Survival analysis with cox regression within SPSS (Version 19 © University of Stirling, 2013) was used to assess differences in survival. Differences were considered significant when the p-value < 0.05. Statistica (version 8.0 ©Statsoft, Inc., 1984-2008, Tulsa, Oklahoma, USA) was used to conduct the multivariate test principal component analysis (PCA) on square root transformed data. PCA was used to look for data clusters within the dataset and to obtain the factor scores for the first three principal components, which as a data reduction method summarises the variation between specimens. A Permanova (version 1.6; M.J. Anderson, Department of Statistical, University of Auckland, 2005) *i.e.* a non-parametric with linear ANOVA

model test was then applied to the factor scores from the first three principal components to test for significance between the groups of fish. Differences were considered significant when the Monte Carlo (p_mc) < 0.05.

5.3 Results

5.3.1 Identification of immune genes, primer design and optimization of qPCR assays

5.3.1.1 Identification of immune gene sequences from EST sets

The initial list of 33 immune genes of interest is provided in Table 7.1 (Appendix 2). The procedure identified 24 ESTs covering 9 unique genes (Appendix 2, Table 7.2); Intestine: 5 ESTs (2 genes); Head Kidney: 4 ESTs, 2 genes); Liver: 15 ESTs, 7 genes), Muscle: nothing. The sequences obtained from the ESTs are shown in Table 5.2; they were used for designing the RT-qPCR primers.

5.3.1.2 Primer design for immune gene expression

Primers were designed for the immune related genes obtained from EST sequences Primer-Blast (Ye*, et al.* 2012). Suitable primers were identified for various *P. hypophthalmus* immune genes including genes of the adaptive response (a2 MHC class II integral membrane protein alpha chain 2), the innate response, including complement (C3 and factor B/C2A), the acute phase response proteins (transferrin, C-reactive protein 2, precerebellin-like protein [cerebellin 14]), and cytokines (IL-1β and INF-γ 2a and 2b). Primers were also designed for housekeeping genes (translation elongation factor-1α mRNA, 18S rRNA gene and β-actin mRNA) for normalizing the data (Table 5.3).

The only genes which did not show expression in RT-qPCR were the IFN-γ 2a and 2b genes, in both normal and infected *E. ictaluri* liver and kidney tissues.

5.3.1.3 Effect of β-glucan supplemented diets on the expression of selected immune genes

There were no statistical differences between the 0.1% commercial yeast derived β-glucan, 0.1% fungal derived β-glucan and basal control diet ($P<0.05$) in the relative gene expression for MHC II, transferrin, c-reactive protein, precerebellin, IL-β, or complement (C3 and factor B) in liver tissue or IL-1β and MHC II in spleen and kidney from after feeding for 14 days, as shown in Figure 5.2. The group fed with the 0.1% fungal derived β-glucan showed a higher trend in the expression of IL-1β and MHC II genes in their spleen and liver (Figure 5.2(A and B)) when compared with the other dietary group, but this was not statistically different, while the IL-1β and MHC II genes expressed in kidney showed a higher trend in the 0.1% commercial yeast derived β-glucan fed fish than in the other two dietary groups, but again these levels were not statistically different (Figure 5.2(C)).

Table 5.2. Suggested immune-related genes from final cross matching between *P. hypophthalmus* EST sequences and list of homologous immune gene sequences from the NCBI database

Activity	Gene name	Target tissue	EST ID	Ref Seq_Description	Ref Seq_gene ID	Ref Seq_Species
Adaptive response	a2 MHC class II integral membrane protein alpha chain 2	Spleen	HEADKIDNEY00000404 [3 - 737] HEADKIDNEY00000405 [3 - 737]	major histocompatibility complex class II integral membrane alpha chain gene	30783	*Danio rerio*
Complement	C3	Liver	INTESTINE00000802 [1 - 771] LIVER00000045 [25 - 4365] LIVER00000046 [25 - 4365] LIVER00000047 [25 - 4665] LIVER00000048 [25 - 4665]	complement C3-H2-like complement component c3a	100331492 321046	*Danio rerio*
	Factor B/C2A	Liver	LIVER00000028 [11 - 1858] LIVER00000029 [11 - 1495]	complement factor B/C2A	100862742	*Ictalurus punctatus*
Acute phase response	Transferrin	Liver	LIVER00000118 [911 - 2050] LIVER00000119 [1 - 1041]	Transferring	100335020	*Ictalurus punctatus*
	C-reactive protein	Liver	LIVER00000908 [6 - 542]	C-reactive protein 2	327615	*Danio rerio*
	precerebellin-like protein	Liver	LIVER00000631 [3 - 581]	cerebellin 14	100007164	*Danio rerio*
Cytokines	Interleukin-1β	Spleen	LIVER00000992 [3 - 503]	interleukin 1, beta	100304696	*Ictalurus punctatus*
	Inferferon γ 2a	Spleen	Partial sequence from GenBank: JN185453			
	Interferon γ 2b	Spleen	Partial sequence from GenBank: JN185454			

Table 5.3. Primers used for *P. hypophthalmus* immune gene expression

Activity	Gene name	Primer Sequence (5'->3')	Length	EST ID	Ref Seq_gene ID	Ref Seq_Species
Adaptive response	a2 MHC class II integral membrane protein alpha chain 2	Pair 2 FWD: GAGCTCAACACTCAGCCAGT REV: CACACCAGGAAGCTCCACAT	172	HEADKIDNEY00000405 [3 - 737]	30783	*Danio rerio*
Complement	C3	Pair 1 FWD: TCCACCAGAGCCATCCCATA REV: CACAACTTGAACGCCACCAG	198	LIVER00000048 [25 - 4665]	100331492 321046	*Danio rerio*
	Factor B/C2A	Pair 1 FWD: CAAAGTGCGTGTGTGTCAGG REV: AACTGCTAAAAGCCTCCGCT	110	LIVER00000028 [11 - 1858]	100862742	*Ictalurus punctatus*
Acute phase response	Transferrin	Pair 1 FWD: CACCCCATAACCTTCACCCC REV: CGCAGTTTTCCCCAAACCAG	149	LIVER00000118 [911 - 2050]	100335020	*Ictalurus punctatus*
	C-reactive protein 2	Pair 1 FWD: AGGAGTCCGACACTGCCTAT REV: CCCGCTGCTTCTCAGGTAAA	200	LIVER00000908 [6 - 542]	327615	*Danio rerio*
	Precerebellin-like protein (Cerebellin 14)	Pair 1 FWD: GGCATTCTGGGCCATACCTT REV: CCAGTTGCAAGGGAGTTTGC	195	LIVER00000631 [3 - 581]	100007164	*Danio rerio*

Table 5.3(cont.). Primers used for immune *P. hypophthalmus* genes expression

Activity	Gene name	Primer Sequence (5'->3')	Length	EST ID	Ref Seq_gene ID	Ref Seq_Species
Cytokine	Interleukin-1β	Pair 1 FWD: CAGAGGCTGAAGCACACTCA REV: CCTTGTCCTGCCTGCTGTAA	148	LIVER00000992 [3 - 503]	100304696	*Ictalurus punctatus*
	Interferon γ 2a and 2b	Pair 1 FWD: TATGTCACTGAGCTGCTGGC REV: TTAGCTTGACGTCGTCTCCG Pair 2 FWD: TCCCAACCCTGCCAAATTGT REV: GCCTCATTCTCCATCCAGGT	143 150	-	346990968 346990970	*Pangasianodon hyphthalmus*
Housekeeping gene (Reference gene)	Translation elongation factor-1α mRNA	Pair 3 FWD:TGAAATTGCCGCACTGGTTG REV: CTGGGCCTCATCACCAACAT	169	HEADKIDNEY00000687 [53-1444]	30516	*Danio rerio*
	18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene	Pair 2 FWD: GGTCGGCGTCCAACTTCTTA REV: GCAATCCCCAGTCCCAATCA	192	-	836268556	*Pangasianodon hyphthalmus*
	β-actin mRNA	Pair 3 FWD: ATTGATGCCCCTGGACACAG REV:GGGTCTGTCCGTTCTTGGAG	133	HEADKIDNEY00000644 [227-1459]	57934 100534412	*Danio rerio* *Oreochromis niloticus*

Principal component analysis (PCA) was used to investigate immune gene expression in the three different tissues (liver, kidney and spleen). A total of 11 variables, detailing the relative expression of each target gene in the liver (C-reactive protein, transferrin, complement factor B and C3, precerebellin, IL-1β and MHC class II), the kidney (IL-1β and MHC class II) and in the spleen (IL-1β and MHC class II) were used as input data and were derived from a total population of 24 fish. The experimental samples were derived from three pools of fish sampled at 14 d.p.f., one pool of eight fish receiving a basal control diet, a second pool of eight fish receiving a 0.1% commercial yeast derived β-glucan diet and a third pool receiving a 0.1% fungal derived β-glucan diet. A summary of the derived data are shown in Table 5.4. The coefficient of variation (CV) for each variable was determined to ascertain the amount of variation about the mean and whether the variable should be included within the PCA analysis. The CVs determined using the raw data was large and so a series of data transformations were explored, with a square root transformation decreasing the size of all CVs except for the two kidney variables which remained over 30 %. The component loadings for each variable and the percentage of the variance explained by each principal component ("factor") were determined for each successive round of principal components analysis and are presented. The first round of PCA considered all 11 measured variables using the square root transformed data whilst, the second round of PCA considered only those variables with a coefficient of variation (CV) below on arbitrarily set level of ≤ 33% (*i.e.* the two kidney variables with CV values exceeding this were excluded prior to analysis); the output from these analyses are shown in Table 5.5. The plot of the first two principal components (*i.e.* Factor 1 vs. Factor 2) is

shown in Figure 5.3, and these two factors account for 43.9% of the variation between specimens. The plot shows that there is a high degree of overlap between each of the diets at 14 d.p.f., suggesting that the diets do not effect a significant shift in the expression of all immune genes. The two kidney variables were then removed from the dataset and a second PCA analysis based on nine square root transformed variables at 14 d.p.f. was run. The plots for the second PCA analysis show two projections of the first three principal components with a high degree of overlap between diets as shown in Figure 5.4(A and B).

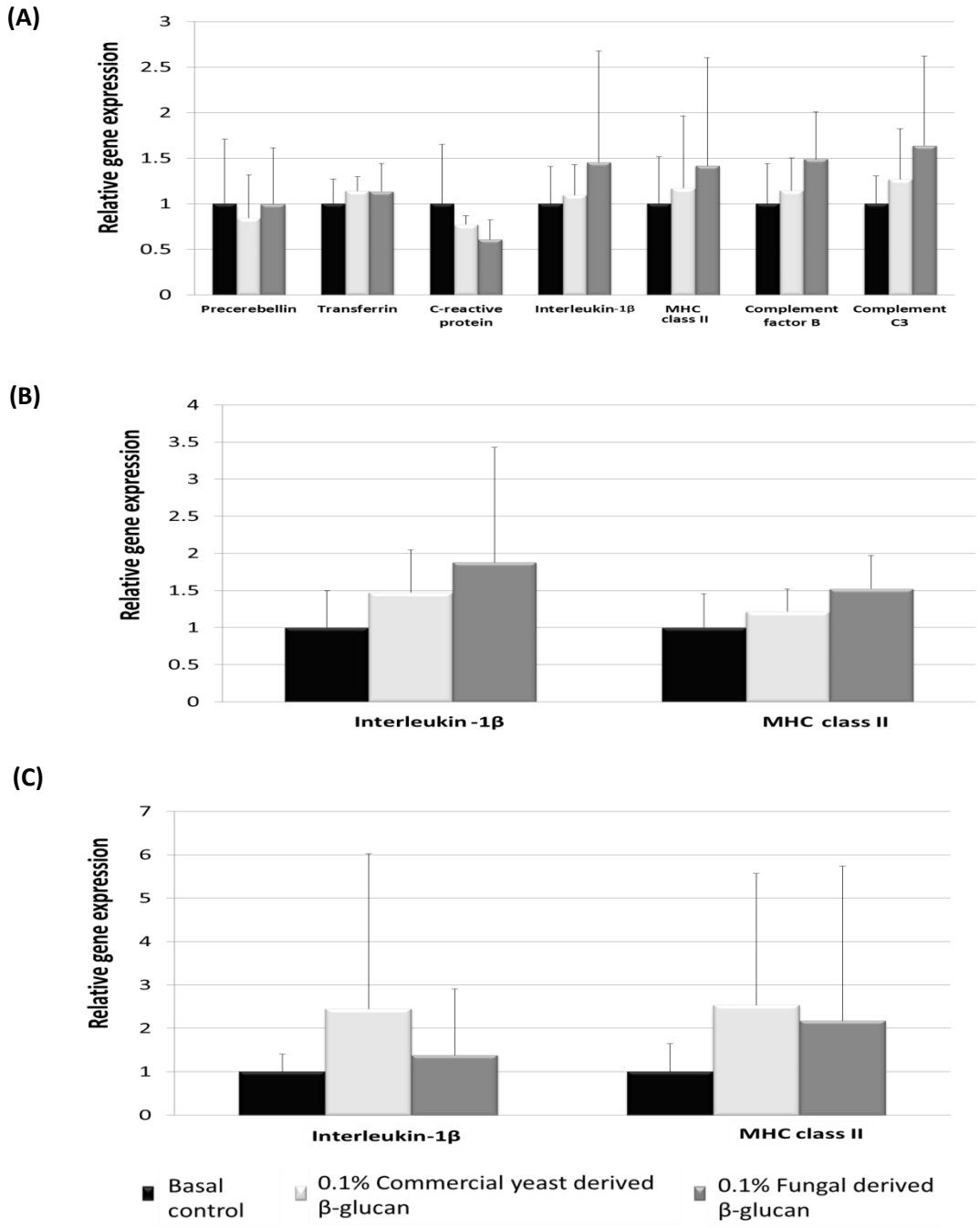


Figure 5.2(A-C). Relative gene expression between the immunostimulant diets (0.1% commercial yeast derived β-glucan and 0.1% fungal derived β-glucan) and basal control diet at 14 d.p.f (Mean±SD, n=8). (A) in liver tissue, (B) in spleen tissue and (C) in kidney

Table 5.4. Univariate statistics for 11 variables representing the immune gene expression in three different tissues, namely the liver (C-reactive protein, transferrin, complement factor B and C3, precerebellin, IL-1β and MHC class II), the kidney (IL-1β and MHC class II) and the spleen (IL-1β and MHC class II). The experimental samples were derived from three pools of fish sampled at 14 d.p.f., one pool of fish receiving a basal control diet (n=8), a second pool receiving a 0.1% commercial yeast derived β-glucan diet (n=8), and, a third pool receiving a 0.1% fungal derived β-glucan diet (n=8).

Tissue	Variable	Mean ± SD	Range		% CV (Raw)	% CV (Sqrt)
			Minimum	Maximum		
Liver	Precerebellin	3.03 ± 1.88	1.11	8.25	62.01	28.78
	Transferrin	2.06 ± 0.47	1.13	2.80	22.92	11.98
	C-reactive protein	6.39 ± 3.34	1.66	15.34	52.34	25.98
	IL-1β	2.10 ± 1.35	0.63	7.85	64.43	26.43
	MHC class II	3.85 ± 2.75	1.04	13.28	71.45	32.74
	Complement factor B	1.02 ± 0.39	2.13	0.40	39.08	20.02
	Complement C3	2.15 ± 1.15	0.95	6.40	53.67	23.54
Spleen	IL-1β	2.45 ± 1.72	0.65	8.96	70.34	31.12
	MHC class II	8.95 ± 3.22	1.99	14.34	35.95	20.42
Kidney	IL-1β	34.94 ± 48.71	9.45	230.60	139.24	53.55
	MHC class II	0.24 ± 0.34	0.07	1.39	141.58	60.40

The coefficient of variation (CV) given here is expressed as a percentage and is given by the standard deviation divided by the mean. Coefficient of variations for both the raw data and square root transformed data are given.

Table 5.5. The component loadings and the percentage of the variance explained by each variable for each successive round of principal components analysis. The first round of PCA (PCA 1; n=24) considers all measured variables (square root transformed data) whilst, the second round of PCA (PCA 2; n=24) considers only those variables with a coefficient of variation (CV) below on arbitrary set level of ≤ 33% (*i.e*. the two kidney variables with CV values exceeding this were excluded prior to analysis). Values above ±0.70 are shown in a bold font.

Tissue	Variable	PCA 1			PCA 2		
		Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3
Liver	Precerebellin	-0.38	-0.61	-0.07	-0.69	-0.06	0.07
	Transferrin	0.25	0.44	0.64	-0.07	**-0.79**	-0.23
	C-reactive protein	0.17	-0.29	**0.71**	-0.03	**-0.80**	0.34
	IL-1	-0.45	0.30	0.59	-0.12	-0.32	-0.35
	MHC class II	-0.48	-0.55	-0.30	**-0.74**	0.20	0.44
	Complement factor B	-0.53	-0.64	0.33	**-0.80**	-0.40	0.10
	Complement C3	-0.69	-0.32	-0.08	**-0.73**	0.15	-0.27
Spleen	IL-1	-0.03	-0.07	0.22	-0.07	-0.19	**-0.84**
	MHC class II	-0.50	0.06	0.46	-0.44	0.53	-0.35
Kidney	IL-1	-0.64	0.55	0.21	-	-	-
	MHC class II	-0.66	0.66	0.25	-	-	-
Total variance (%)		22.91	20.98	16.71	26.83	21.02	15.44
Cumulative (%)		22.91	43.89	60.60	26.83	47.85	63.29

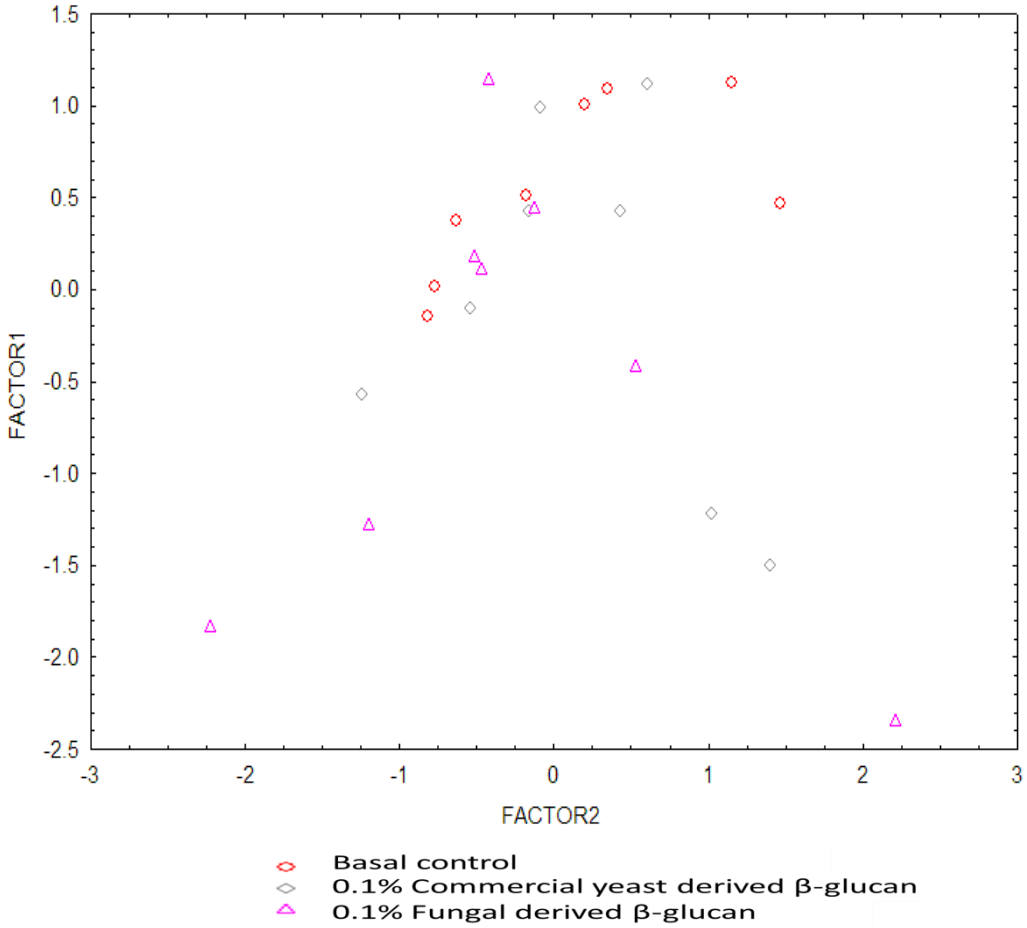


Figure 5.3. Principal component analysis (PCA) of all 11 selected variables based on square root transformed variables. Only the first two principal components are shown (*i.e.* Factor 1 *vs*. Factor 2) which accounts for 43.89% of the variation between specimens. The graph shows that there is a high degree of overlap between each of the diets at 14 d.p.f.

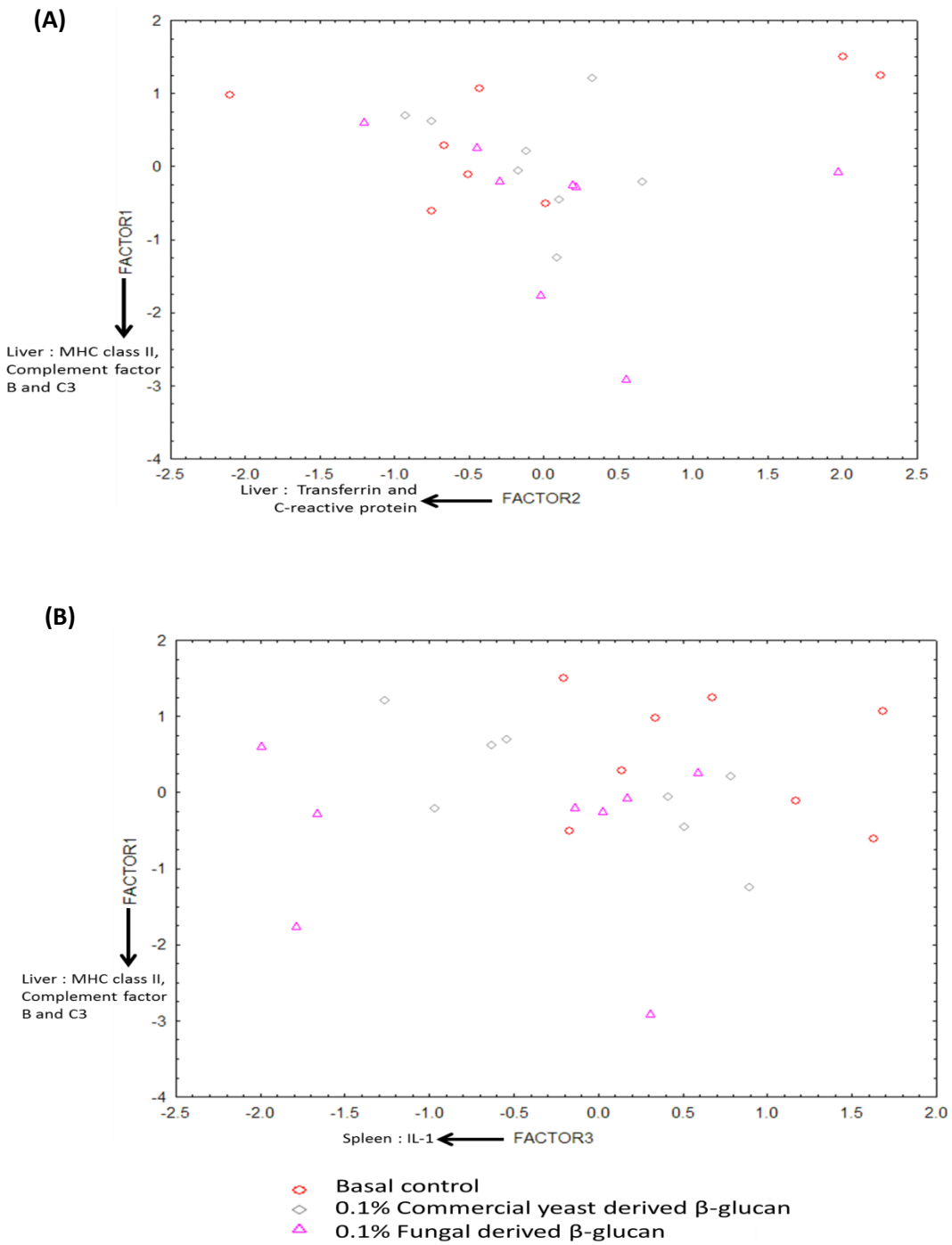


Figure 5.4(A-B). Principal component analysis (PCA) based on nine square root transformed variables at 14 d.p.f. No variables from the kidney were included because of their high coefficient of variation values (*i.e.* ≥ 33%). The graph shows two projections of the first three principal components *i.e.* Factor 1 *vs.* Factor 2 (A) and Factor 1 vs. Factor 3 (B). The plot shows a high degree of overlap between diets.

5.3.2 Inflammatory-related immune gene expression 24 hours after challenging with *E. ictaluri*

The effect of *E. ictaluri* challenge on immune gene expression was determined at only 24 h.p.i. The relative gene expression (acute phase response genes, MHC II and complement factor B) in liver tissue, in the spleen (MHC II) and in the kidney (IL-1β) were shown to be statistically different between the infected and uninfected groups ($p<0.05$). The expression of precerebellin, C-reactive protein, MHC class II, complement factor B in the liver and IL-1β in the kidney were up-regulated in the *E. ictaluri*-infected group as shown in Figures 5.5(A, C, E and F) and 5.7(A). There was no significant difference, however, in the expression of IL-1β in the liver and spleen, complement C3 in the liver or the MHC class II gene in the kidney between the uninfected and the *E. ictaluri*-infected groups as shown in Figures 5.5(D and G), 5.6(A) and 5.7(B), respectively. Using ANOVA, there were no significant differences between the group fed immunostimulant and the control group. Only the level of expression of the transferrin gene in basal control diet in the uninfected group was significantly higher than the 0.1 % fungal-derived β-glucan group ($p<0.05$) as shown in Figure 5.5(B).

Principal component analysis (PCA) was then used to investigate the immune gene expression in the three different tissues (*i.e*. liver, kidney and spleen), sampled from fish fed three different experimental diets that were then subsequently challenged with *E. ictaluri* or not. The same 11 variables relating to gene expression in the liver (C-reactive protein, transferrin, complement factor B and C3, precerebellin, IL-1β and MHC class II), the kidney (IL-1β and MHC class II), and the spleen (IL-1β and MHC class II) were used. A total of 48 experimental fish were divided into six pools of

fish sampled at 24 h.p.i., with one pool receiving a basal control diet without challenging with *E. ictaluri*, a second pool receiving a 0.1% commercial yeast derived β-glucan diet without challenging with *E. ictaluri*, a third pool receiving a 0.1% fungal derived β-glucan diet without challenging with *E. ictaluri*, a fourth receiving basal control diet followed by a challenge with *E. ictaluri*, a fifth pool receiving a 0.1% commercial yeast derived β-glucan diet challenged with *E. ictaluri*, and, a sixth pool of fish receiving a 0.1% fungal derived β-glucan diet before being challenged with *E. ictaluri.* A summary of the data is shown in Table 5.6. The component loadings of each variable and the percentage of the variance explained by each principal component (*i.e.* "factor") on the square root transformed data) determined by the first principal components analysis which considered all six pools of fish (*i.e.* both uninfected and *E. ictaluri*-infected fish) is shown in Table 5.7. The plot of the first two principal components, which accounts for 51.01% of the variation between fish, shows a clear separation between the uninfected and infected *E. ictaluri* groups (Figure 5.8.). A permanova test on the factor scores of the first three principal components analysis confirmed that there was a statistical significant difference in the levels of gene expression (based all on 11 variables) between the group of uninfected fish and those groups of fish that were infected with *E. ictaluri* ($p = 0.0002$). The genes having the strongest influence within the PCA were the liver precerebellin, C-reactive protein, complement factor B and transferrin through Factor 1 and IL-1 from both the liver and the spleen acting through Factor 2. The three projections of the first three principal components (*i.e.* Factors 1-3) representing all six different groups of fish (uninfected; basal control, 0.1% commercial yeast derived β-glucan, 0.1% fungal derived β-glucan

and infected; basal control, 0.1% commercial yeast derived β-glucan and 0.1% fungal derived β-glucan) are shown in Figure 5.9 (A-C). The plot shows there is some overlap between the groups, however, the PCA plot of Factor 1 *vs.* Factor 3 (see Figure 5.9 (B)) shows that the group of eight uninfected fish receiving the basal control diet could be separated from the uninfected fish receiving either a 0.1% commercial yeast derived β-glucan and / or 0.1% fungal derived β-glucan diet. This separation and its significance was assessed through further rounds of PCA and permanova tests. The genes having a major influence through Factor 1 were precerebellin, C-reactive protein and complement factor B in the liver, and MHC class II through Factor 2 in the kidney.

Following the first PCA of all fish groups, two further PCAs were conducted, which considered first the uninfected (*i.e.* non-*E. ictaluri* challenged fish) three groups of fish alone and then in a second PCA, the infected (*i.e. E. ictaluri* challenged fish) groups of fish to look for further structuring within the data. The PCA analysis and plot of the uninfected fish, which is based on all 11 variables, is shown in Table 5.8 and in Figure 5.10(A-C). The PCA plots show three projections of the first three principal components. Figure 5.10(A) of Factor 1 *vs.* Factor 2 shows that the uninfected fish receiving the 0.1% fungal derived β-glucan diet is separated and is significantly different (permanova p_mc = 0.013) from the other two pools of uninfected fish receiving different diets. The main genes having a major effect on this separation are the liver C-reactive protein through Factor 1 and the liver precerebellin acting through Factor 2. Figures 5.10(B and C) look at these groups in two different projections and show that there is some overlap between all three groups. Table 5.9 and Figure 5.11 (A-C) explore the structuring between the three groups of *E. ictaluri* challenged fish.

The component loadings for each variable and the percentage of the variance explained by each factor within the principal components analysis based on the square root transformed are shown in Table 5.9. The PCA plots presented in Figures 5.11(A-C) show that there is some overlap between all three groups of challenged fish and that there are statistically significant differences in expression between the control group and the group receiving the 0.1% commercial yeast derived β-glucan diet (permanova p_mc = 0.0008) and also between the control group and the group receiving the 0.1% fungal derived β-glucan diet (permanova p_mc = 0.005). There was no statistically significant difference, however, between the groups of fish receiving the 0.1% commercial yeast derived β-glucan diet and the 0.1% fungal derived β-glucan diet (permanova p_mc = 0.183). The results suggest that the immunostimulant diets have a significant effect on the total immune gene expression in the liver, kidney and spleen of fish challenged with *E. ictaluri* when compared to fish receiving a standard (control) diet.

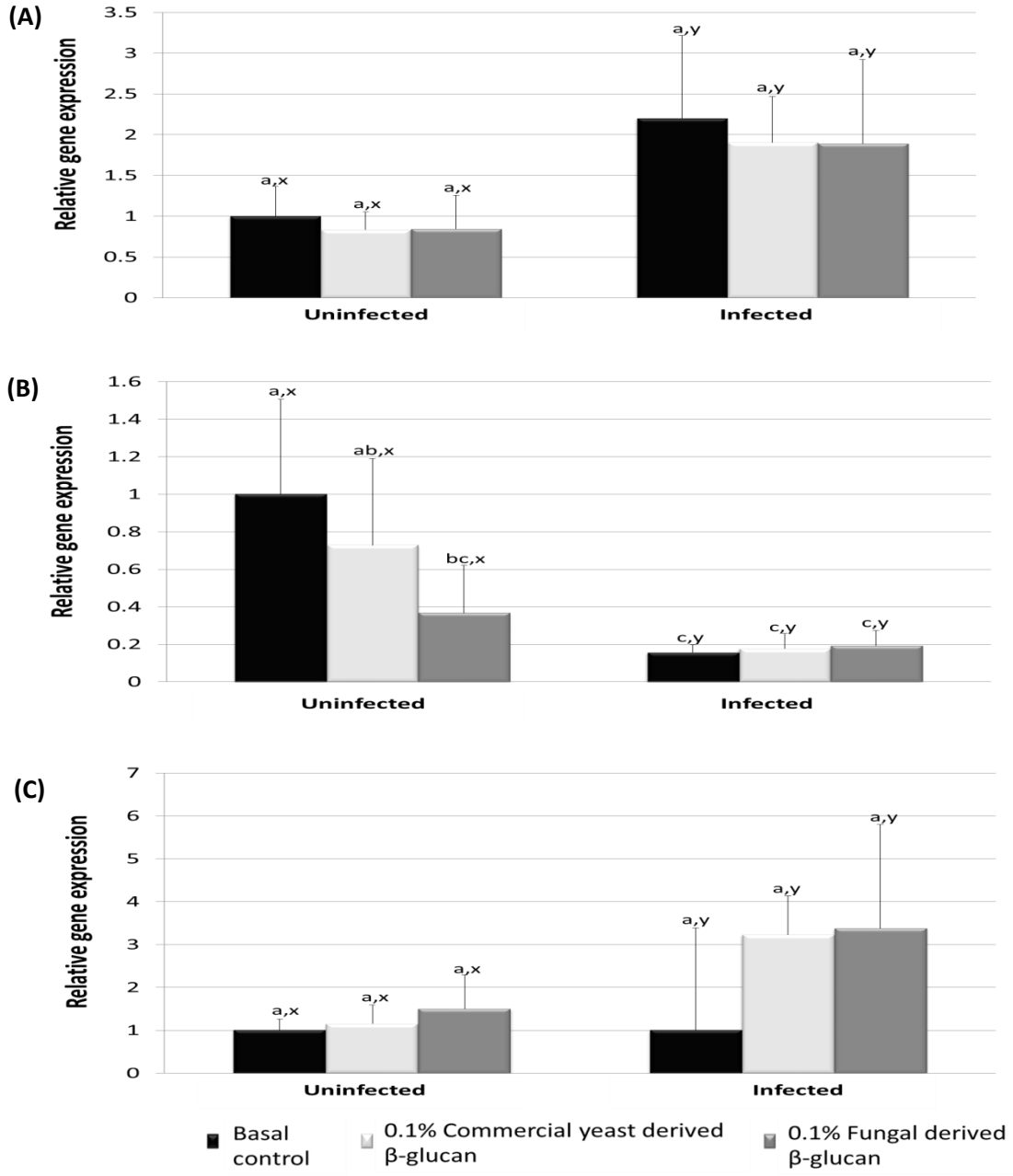


Figure 5.5(A-C). The relative gene expression in liver tissue between the experimental diets (0.1% commercial yeast derived β-glucan, 0.1% fungal derived β-glucan and basal control) with uninfected and infected with *E. ictaluri* at 24 h.p.i (Mean±SD, n=8). Acute phase responses (A) Precerebellin gene, (B) Transferrin gene and (C) C-reactive protein. Different letters indicate significant differences (P<0.05) between groups of experimental diets (a,b,c) or infected and uninfected with *E. ictaluri* (x,y). A significant interaction between experimental diets and infection was found only in Transferrin

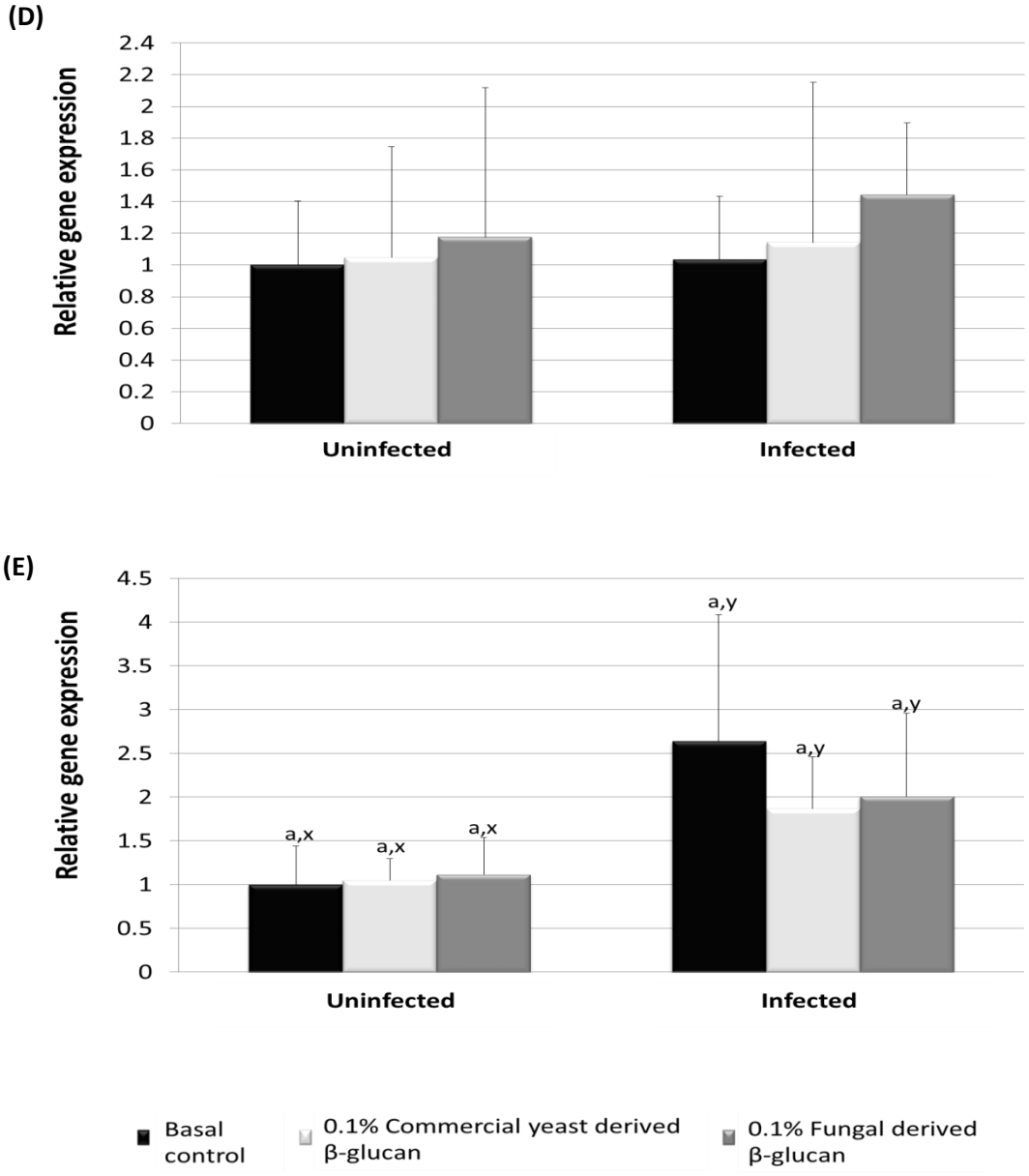
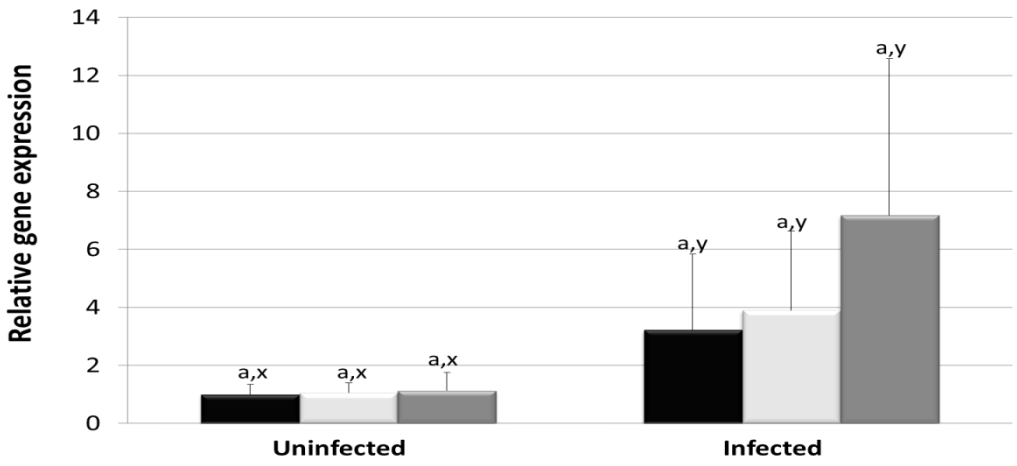


Figure 5.5(D-E; cont.). The relative gene expression in liver tissue between the experimental diets (0.1% commercial yeast derived β-glucan, 0.1% fungal derived β-glucan and basal control) with uninfected and infected with *E. ictaluri* at 24 h.p.i (Mean±SD, n=8). Cytokine and adaptive immune response (D) cytokine was IL-1β and (E) adaptive immune response was a2 MHC class II integral membrane protein alpha chain 2. Different letters indicate significant differences (P<0.05) between groups of experimental diets (a,b,c) or infected and uninfected with *E. ictaluri* (x,y).

(F)



(G)

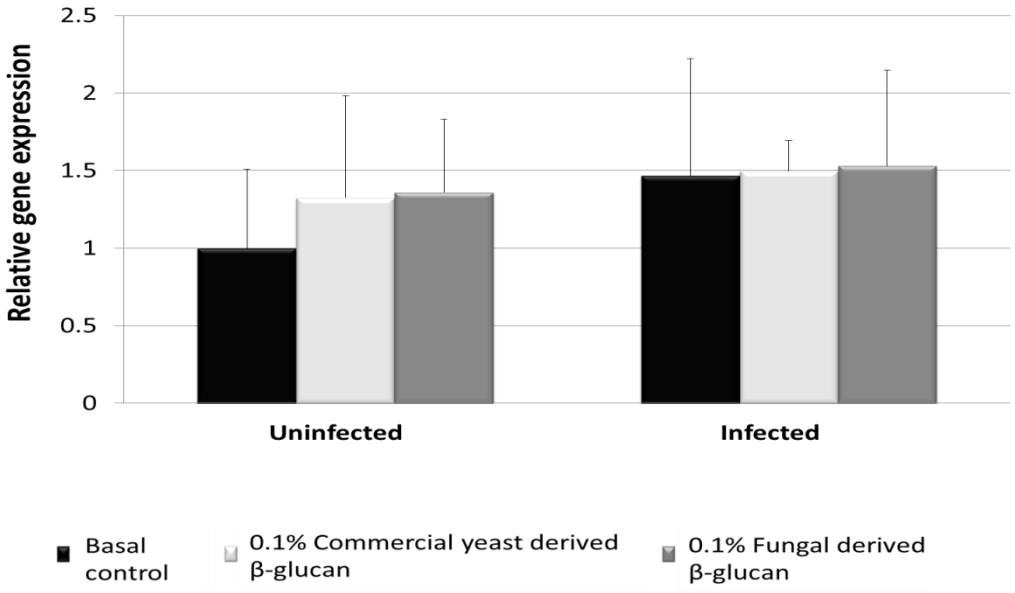


Figure 5.5(F-G; cont.). The relative gene expression in liver tissue compared between the experimental diets (0.1% commercial yeast derived β-glucan, 0.1% fungal derived β-glucan and basal control) with uninfected and infected with *E. ictaluri* at 24 h.p.i (Mean±SD, n=8). Complement (F) factor B and (G) C3. Different letters indicate significant differences (P<0.05) between groups of experimental diets (a,b,c) or infected and uninfected with *E. ictaluri* (x,y).

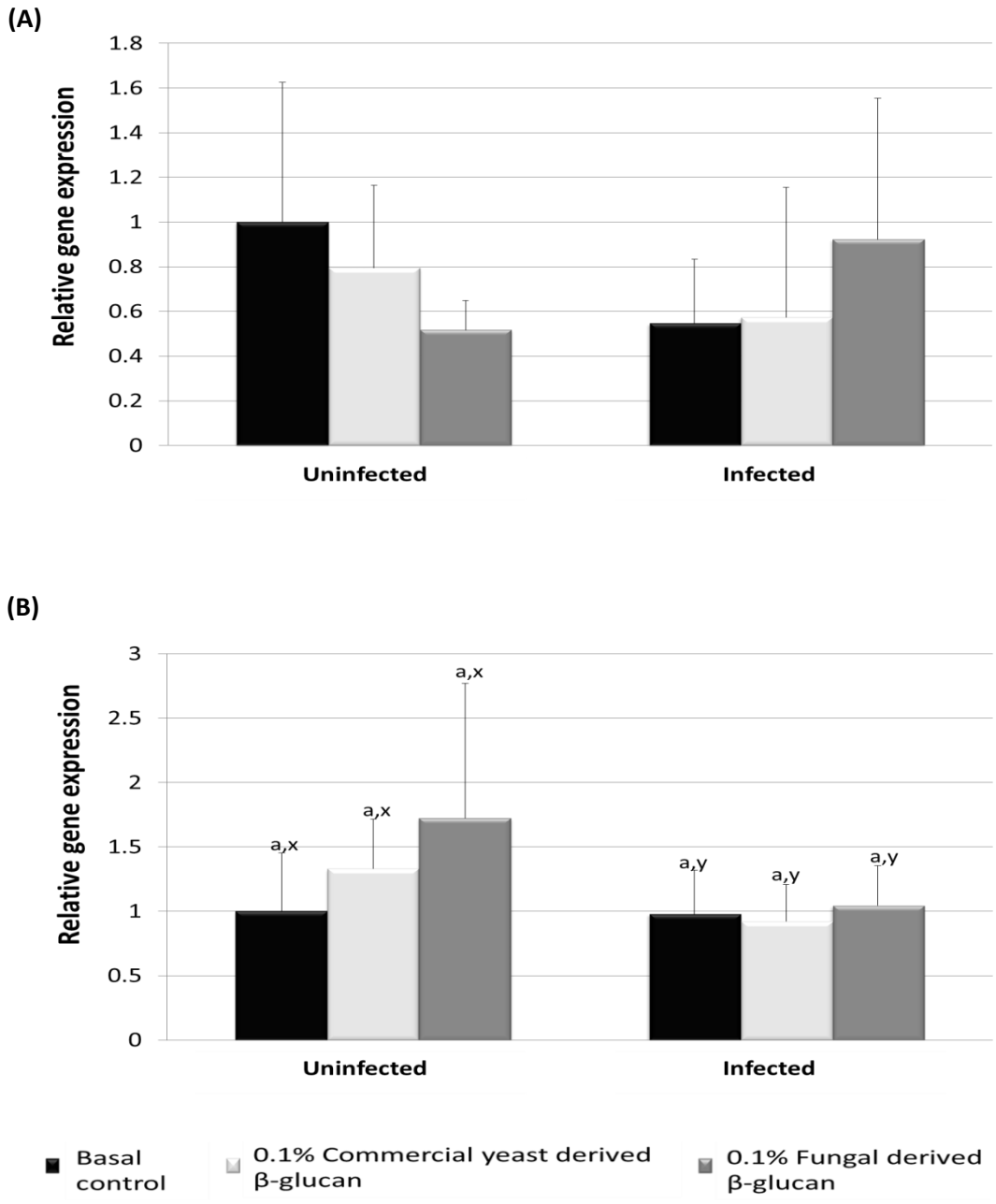


Figure 5.6(A-B). The relative gene expression in spleen compared between the experimental diets (0.1% commercial yeast derived β-glucan, 0.1% fungal derived β-glucan and basal control) with uninfected and infected with *E. ictaluri* at 24 h.p.i. (A) IL-1β and (B) a2 MHC class II integral membrane protein alpha chain 2 (Mean±SD, n=8). Different letters indicate significant differences (P<0.05) between groups of experimental diets (a,b,c) or infected and uninfected with *E. ictaluri* (x,y).

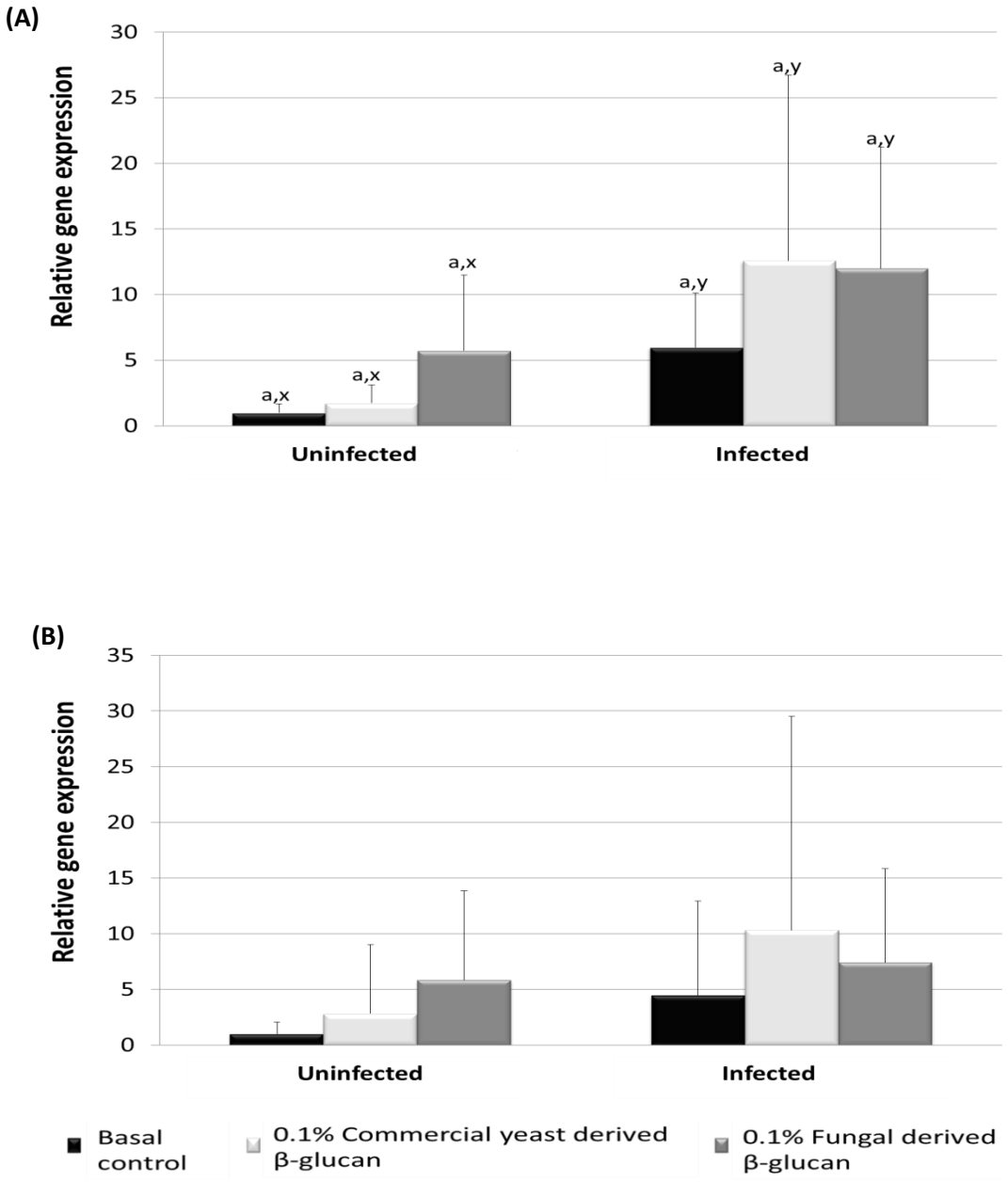


Figure 5.7(A-B). The relative gene expression in kidney compared between the experimental diets (0.1% commercial yeast derived β-glucan, 0.1% fungal derived β-glucan and basal control) with uninfected and infected with *E. ictaluri* at 24 h.p.i. (A) IL-1β and (B) a2 MHC class II integral membrane protein alpha chain 2 (Mean±SD, n=8). Different letters indicate significant differences (P<0.05) between groups of experimental diets (a,b,c) or infected and uninfected with *E. ictaluri* (x,y).

Table 5.6. Univariate statistics for 11 variables representing the immune gene expression in three different tissues, namely the liver (C-reactive protein, transferrin, complement factor B and C3, precerebellin, IL-1β and MHC class II), the kidney (IL-1β and MHC class II), and, the spleen (IL-1β and MHC class II). The experimental samples were divided from six pools of fish sampled at 24 h.p.i. with one pool receiving a basal control diet without challenging with *E. ictaluri* (n=8), a second pool receiving a 0.1% commercial yeast derived β-glucan diet without challenging with *E. ictaluri* (n=8), a third pool receiving a 0.1% fungal derived β-glucan diet without challenging with *E. ictaluri* (n=8), a fourth receiving basal control diet followed by a challenge with *E. ictaluri* (n=8), a fifth pool receiving a 0.1% commercial yeast derived β-glucan diet challenged with *E. ictaluri* (n=8), and, a sixth pool receiving a 0.1% fungal derived β-glucan diet before being challenged with *E. ictaluri* (n=8).

Tissue	Variable	Mean ± SD	Range		% CV (Raw)	% CV (Sqrt)
			Min.	Max.		
Liver	Precerebellin	1.39 ± 0.82	0.37	4.12	59.45	28.62
	Transferrin	2.83 ± 2.79	0.40	13.23	98.71	47.29
	C-reactive protein	1.60 ± 1.24	0.43	6.13	77.79	36.63
	IL-1β	1.33 ± 0.79	0.46	4.06	59.28	27.85
	MHC class II	3.73 ±2 .24	0.80	12.12	60.05	27.89
	Complement factor B	4.36 ± 5.10	0.73	24.62	116.84	52.50
	Complement C3	2.20 ± 0.90	0.60	4.40	41.18	20.35
Spleen	IL-1β	10.7 ± 7.0	3.45	34.85	67.62	30.41
	MHC class II	7.81 ± 3.91	2.58	26.46	50.00	22.84
Kidney	IL-1β	58.37 ± 75.52	2.06	297.57	129.38	60.58
	MHC class II	0.42 ± 0.82	0.02	4.28	189.24	90.98

The coefficient of variation (CV) given here is expressed as a percentage and is given by the standard deviation divided by the mean. Coefficient of variations for both the raw data and square root transformed data are given.

Table 5.7. The component loadings and the percentage of the variance explained by each variable within the principal components analysis applied to all six groups of fish. The first round of PCA (uninfected with *E. ictaluri*; n=24, and infected with *E. ictaluri*; n=24) considers all variables, the data of which have been square root transformed. Values above ±0.70 are shown in a bold font.

Tissue	Variable	PCA		
		Factor 1	Factor 2	Factor 3
Liver	Precerebellin	**-0.82**	0.10	-0.20
	Transferrin	**0.70**	-0.05	-0.11
	C-reactive protein	**-0.88**	0.17	-0.17
	IL-1β	-0.04	**-0.80**	-0.38
	MHC class II	-0.62	0.20	-0.42
	Complement factor B	**-0.81**	-0.15	-0.03
	Complement C3	-0.58	-0.22	-0.18
Spleen	IL-1β	0.30	**-0.77**	-0.15
	MHC class II	0.25	0.32	0.16
Kidney	IL-1β	-0.60	-0.26	0.57
	MHC class II	-0.37	-0.28	**0.82**
Total variance (%)		36.24	14.77	13.37
Cumulative (%)		36.24	51.01	64.38

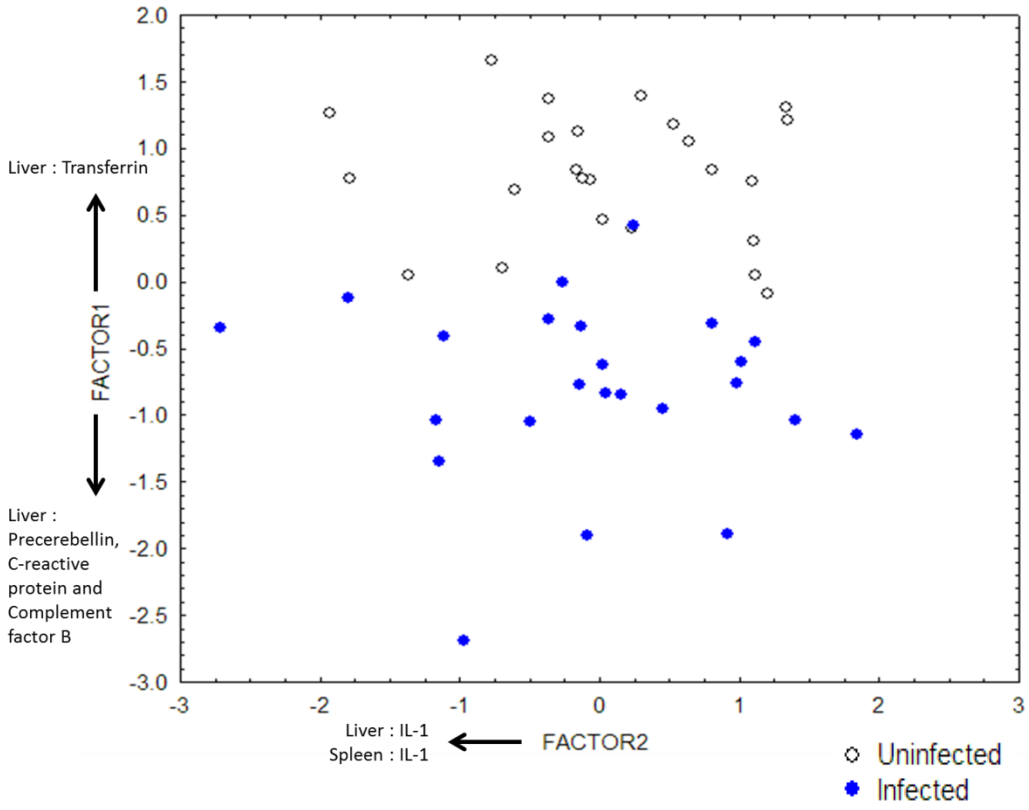
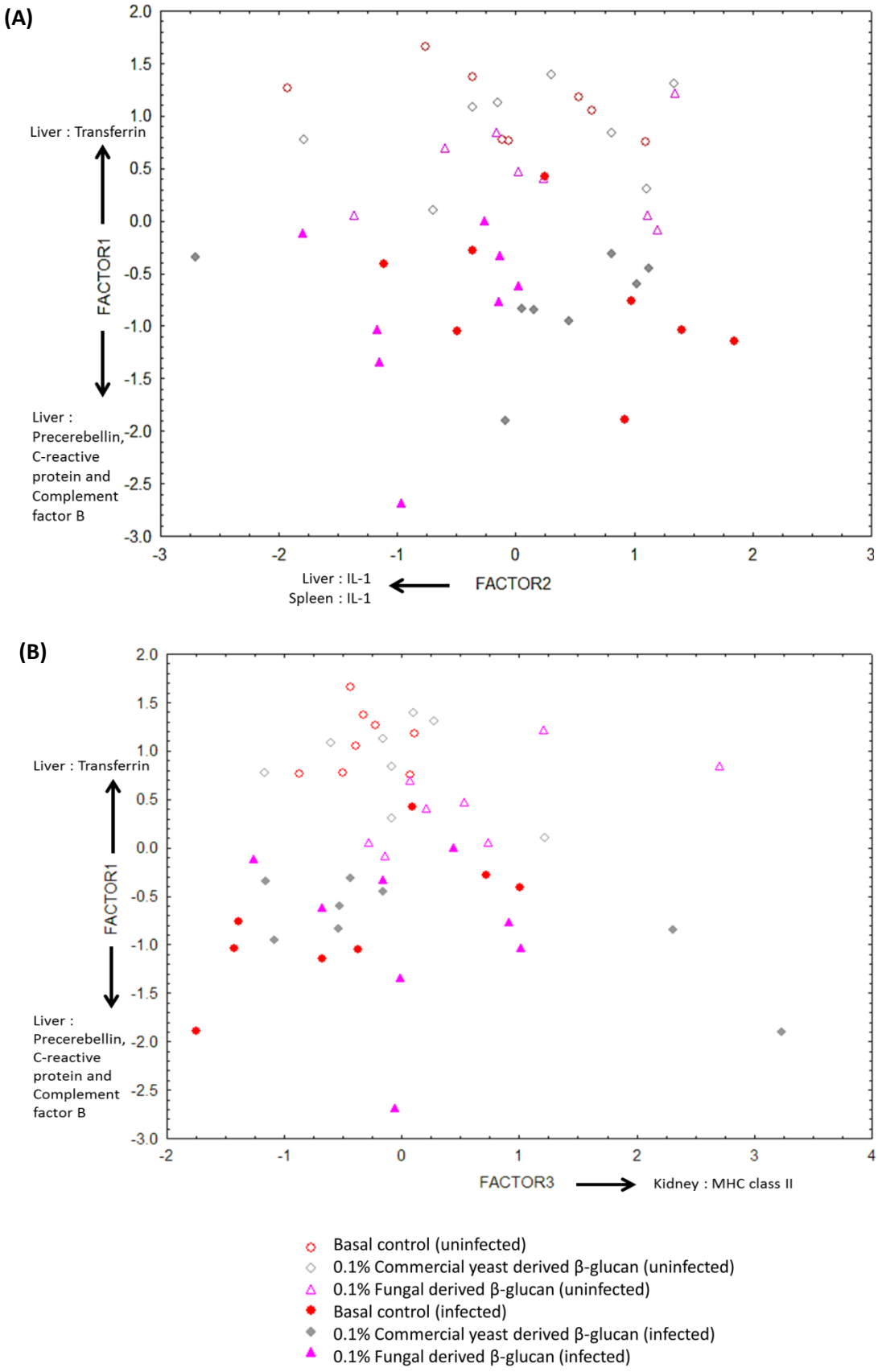


Figure 5.8. Principal component analysis (PCA) of all 11 selected variables based on square root transformed data at 24 h.p.i. Only the first two principal components are shown (*i.e.* Factor 1 *vs.* Factor 2) which accounts for 51.01% of the variation between specimens. The graph shows that there is a degree of separation between the uninfected and infected *E. ictaluri* groups indicating that infection elicits a change in gene expression.


(A)
Liver : Transferrin
FACTOR1
Liver : Precerebellin, C-reactive protein and Complement factor B
Liver : IL-1
Spleen : IL-1
FACTOR2
(B)
Liver : Transferrin
FACTOR1
Liver : Precerebellin, C-reactive protein and Complement factor B
FACTOR3
Kidney : MHC class II
Basal control (uninfected)
0.1% Commercial yeast derived β-glucan (uninfected)
0.1% Fungal derived β-glucan (uninfected)
Basal control (infected)
0.1% Commercial yeast derived β-glucan (infected)
0.1% Fungal derived β-glucan (infected)

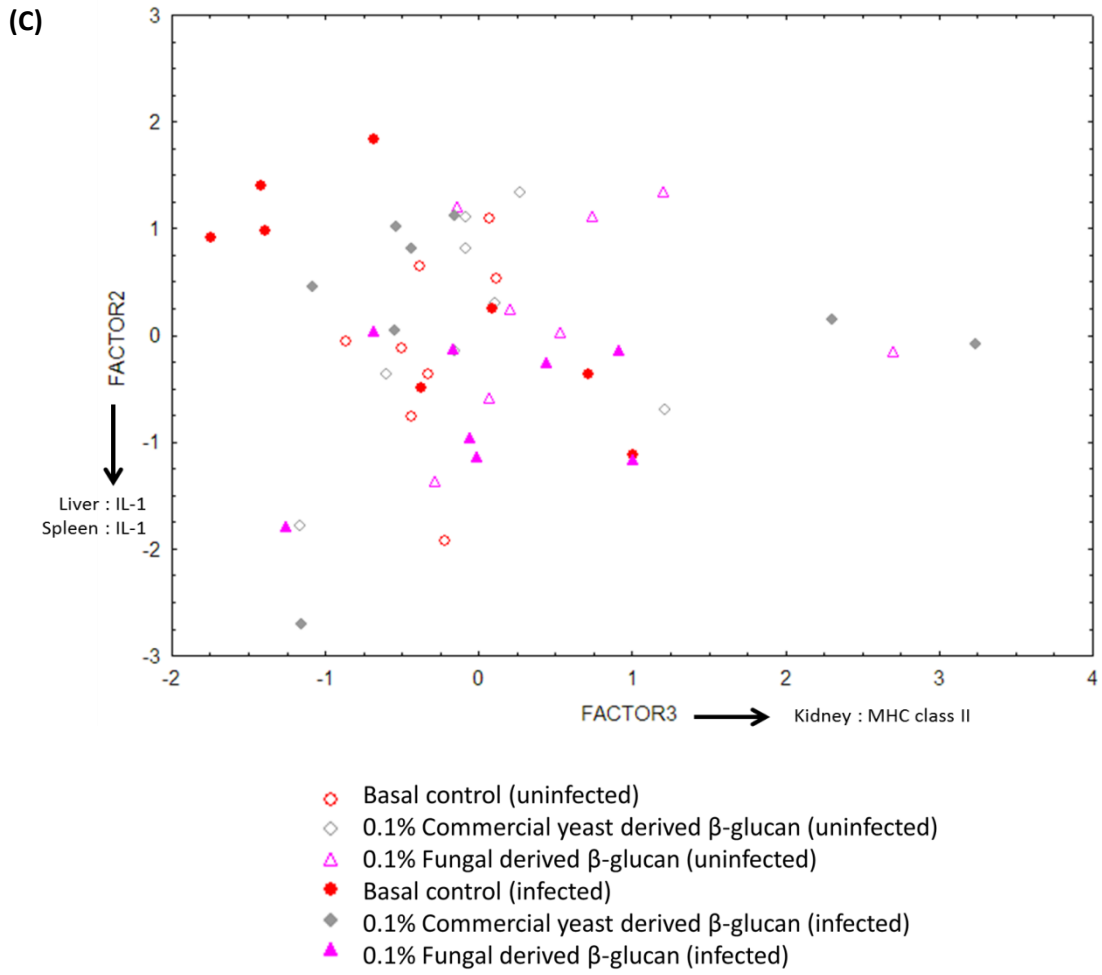
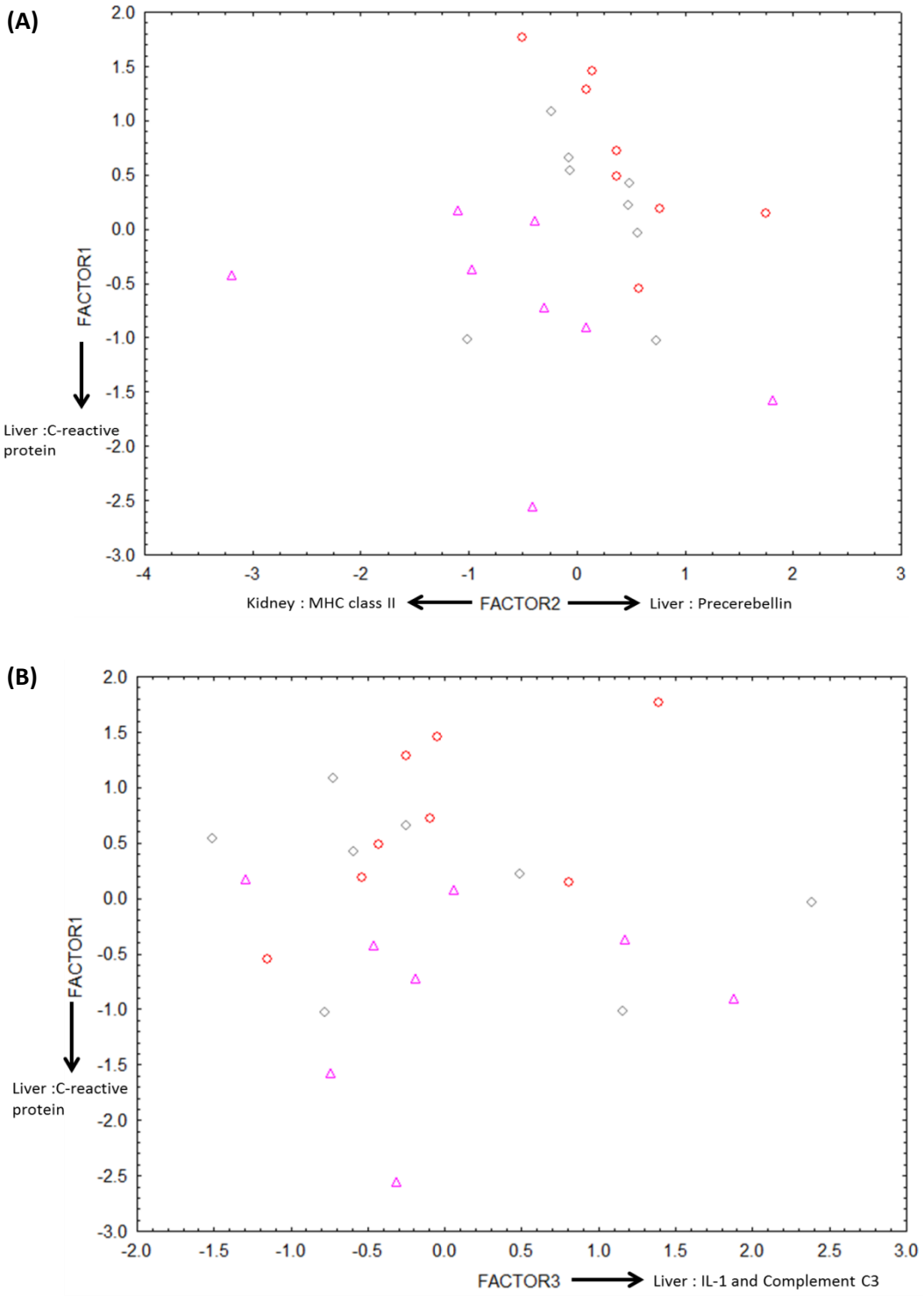


Figure 5.9(A-C). Principal component analysis (PCA) of all 11 selected variables based on square root transformed data at 24 h.p.i. derived from all six pools of fish (*i.e.* uninfected and those challenged with *E. ictaluri*). The graph shows three projections of the first three principal components *i.e.* Factor 1 *vs.* Factor 2 (A), Factor 1 *vs.* Factor 3 (B) and Factor 2 *vs.* Factor 3 (C).

Table 5.8. The component loadings and the percentage of the variance explained by each factor within a principal components analysis which considers only the three uninfected groups of fish (*i.e.* those not challenged with *E. ictaluri*; n=24). The analysis considers all 11 variables, the data of which have been square root transformed. Values above ±0.70 are shown in a bold font.

Tissue	Variable	PCA		
		Factor 1	Factor 2	Factor 3
Liver	Precerebellin	-0.37	**0.72**	0.07
	Transferrin	0.56	0.17	0.10
	C-reactive protein	**-0.80**	0.16	0.16
	IL-1	0.065	-0.015	**0.85**
	MHC class II	-0.60	0.27	-0.35
	Complement factor B	-0.46	0.56	0.21
	Complement C3	-0.46	0.13	**0.73**
Spleen	IL-1	0.53	-0.11	0.49
	MHC class II	-0.57	-0.33	-0.18
Kidney	IL-1	-0.56	-0.66	0.07
	MHC class II	-0.34	**-0.82**	0.20
Total variance (%)		26.55	19.97	16.04
Cumulative (%)		26.55	46.52	62.56


(A)
FACTOR1
Liver :C-reactive protein
Kidney : MHC class II
FACTOR2
Liver : Precerebellin
(B)
FACTOR1
Liver :C-reactive protein
FACTOR3
Liver : IL-1 and Complement C3
Basal control (uninfected)
0.1% Commercial yeast derived β-glucan (uninfected)
0.1% Fungal derived β-glucan (uninfected)

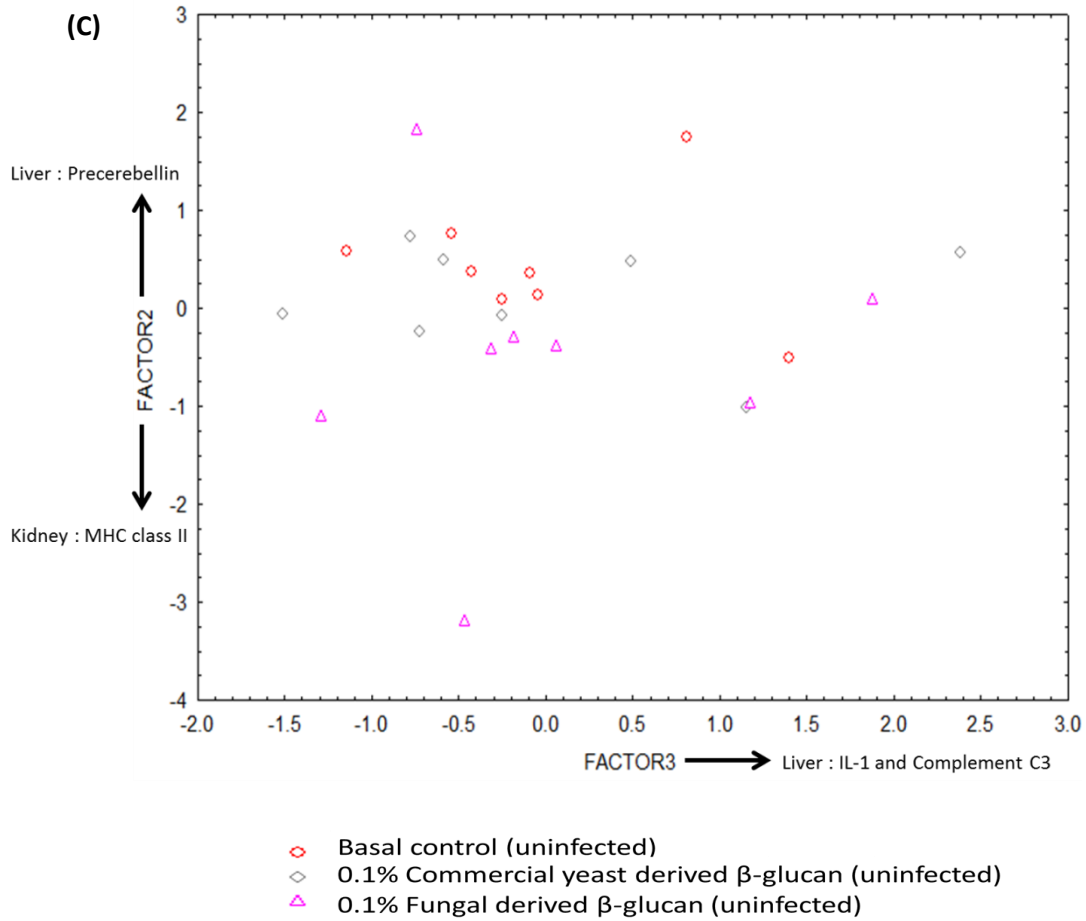
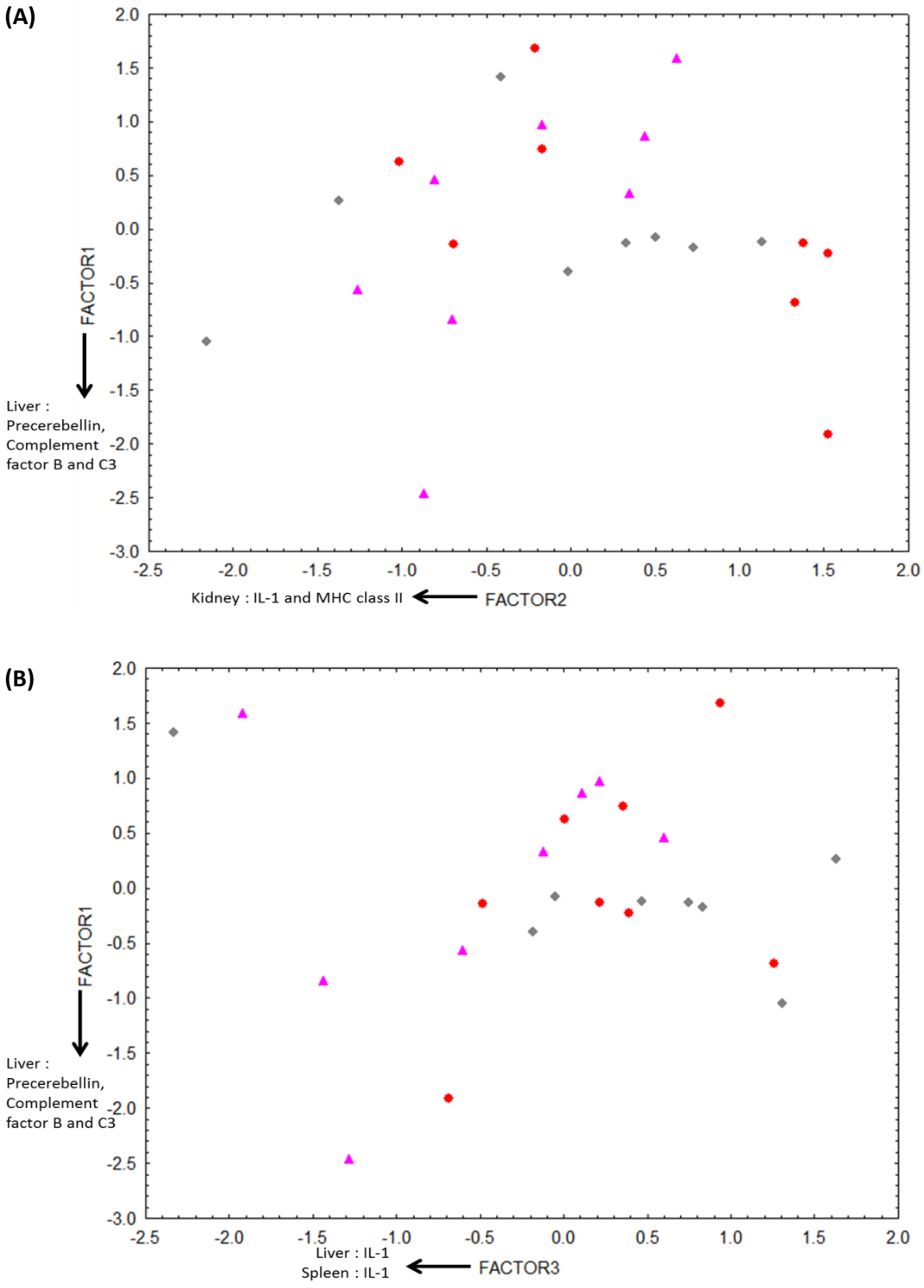


Figure 5.10(A-C). Principal component analysis (PCA) of all 11 selected variables based on square root transformed uninfected data at 24 h.p.i. The graph shows three projections of the first three principal components *i.e.* Factor 1 *vs.* Factor 2 (A), Factor 1 *vs.* Factor 3 (B) and Factor 2 *vs.* Factor 3 (C).

Table 5.9. The component loadings and the percentage of the variance explained by each factor within the principal components analysis which explores sub-structuring in the three pools of *E. ictaluri* challenged fish. The PCA (infected with *E. ictaluri*; n=24) considers all variables, the data of which have been square root transformed. Values above ±0.70 are shown in a bold font.

Tissue	Variable	PCA		
		Factor 1	**Factor 2**	**Factor 3**
Liver	Precerebellin	**-0.71**	0.18	-0.075
	Transferrin	-0.28	0.20	-0.24
	C-reactive protein	**-0.84**	0.25	-0.02
	IL-1	0.38	-0.06	**-0.84**
	MHC class II	-0.10	0.62	-0.22
	Complement factor B	**-0.70**	-0.37	-0.35
	Complement C3	**-0.73**	-0.004	-0.35
Spleen	IL-1	0.47	-0.17	**-0.71**
	MHC class II	0.16	0.52	0.16
Kidney	IL-1	0.01	**-0.77**	0.07
	MHC class II	-0.28	**-0.81**	0.15
Total variance (%)		25.41	20.01	14.78
Cumulative (%)		25.41	45.42	60.20


(A)
FACTOR1
Liver : Precerebellin, Complement factor B and C3
Kidney : IL-1 and MHC class II
FACTOR2
(B)
FACTOR1
Liver : Precerebellin, Complement factor B and C3
Liver : IL-1
Spleen : IL-1
FACTOR3
Basal control (infected)
0.1% Commercial yeast derived β-glucan (infected)
0.1% Fungal derived β-glucan (infected)

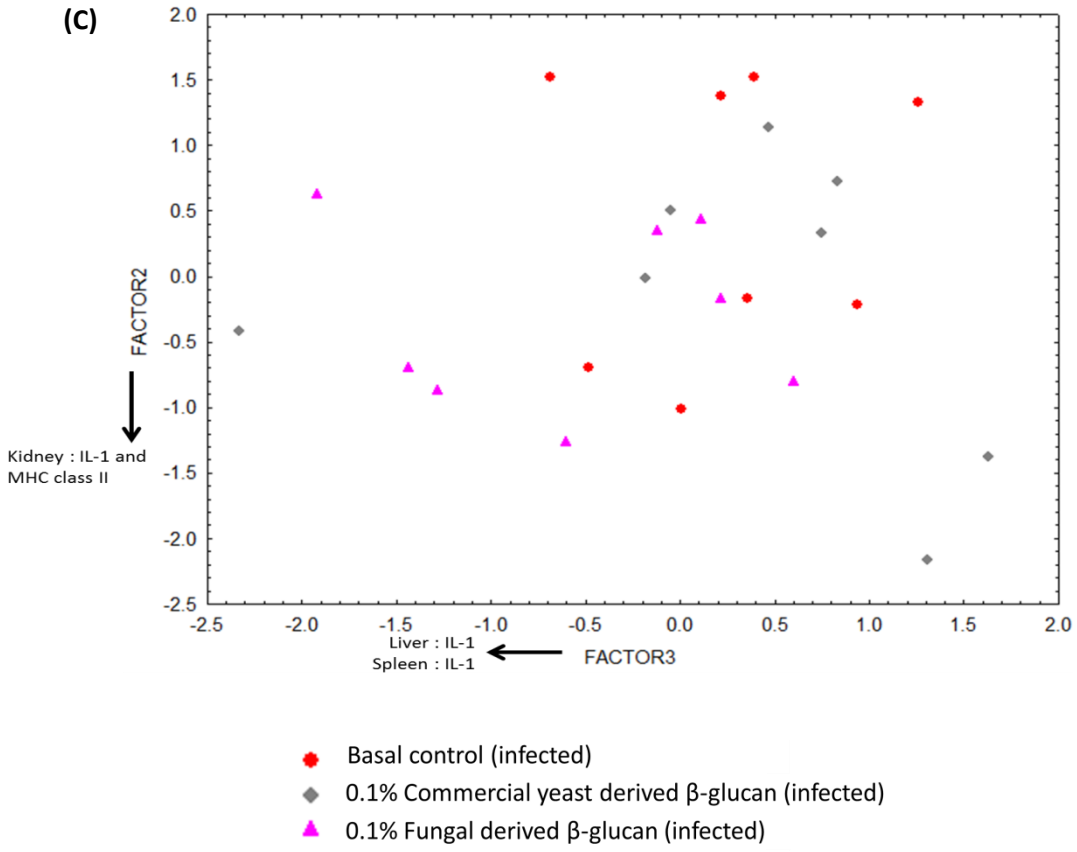
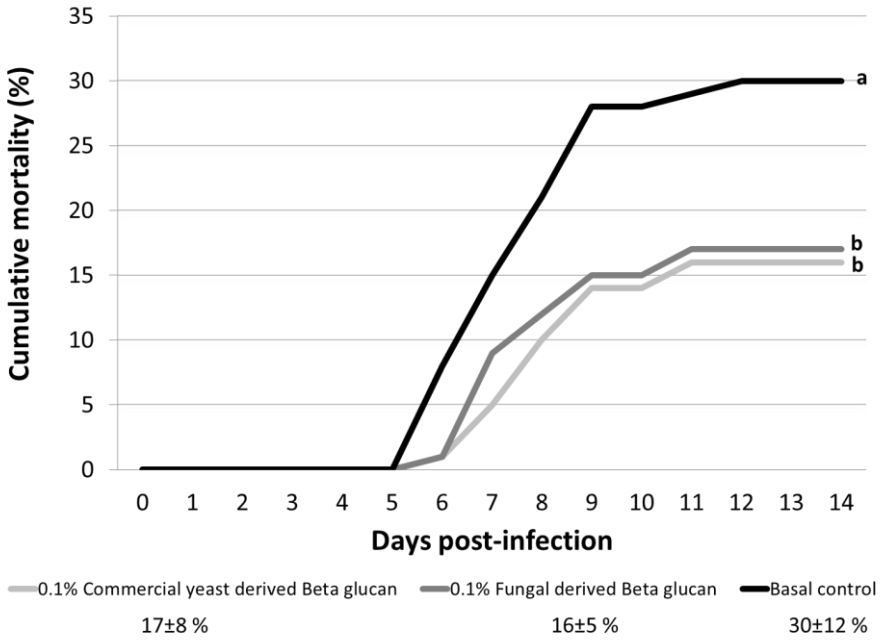


Figure 5.11(A-C). Principal component analysis (PCA) of all 11 selected variables based on square root transformed infected data at 24 h.p.i. The graph shows three projections of the first three principal components *i.e.* Factor 1 *vs.* Factor 2 (A), Factor 1 *vs.* Factor 3 (B) and Factor 2 *vs.* Factor 3 (C).

5.3.3 Survival post-challenge with *E. ictaluri*

Mortalities of *P. hypophthalmus* exposed to *E. ictaluri* were shown in Figure 5.12(A). Average cumulative mortality was 30 ±12% in the group fed the basal control diet, 17 ±8% in group fed fungal-derived β-glucan 0.1 % and 16 ±5% in the group fed commercial yeast-derived β-glucan 0.%. The data showed that the fish in 0.1% fungal-derived β-glucan and 0.1% commercial yeast-derived β-glucan group had a significantly lower levels of cumulative mortality ($P<0.05$) than the fish fed the basal control diet. The variation between the tanks 14 d.p.i is shown in Figure 5.12(B). The survival between treatments was statistically significant ($P<0.05$) using Cox Regression model *i.e* the null model -2 Log Likelihood (-2xLL) was 685.674. Including treatment in the null model was significant (-2xLL = 678.807; X_2^2 = 6.867; P=0.32) in a likelihood ratio test with a pseudo r^2 value of 0.010. There was statistically significant variation in survival between tanks (*P<0.01*). Additionally, including tank variation between the group of treatment was not significant (-2xLL = 669.334; X_9^2 = 9.473; P=0.395) in a likelihood ratio test with a pseudo r^2 value of 0.024.

(A)



(B)

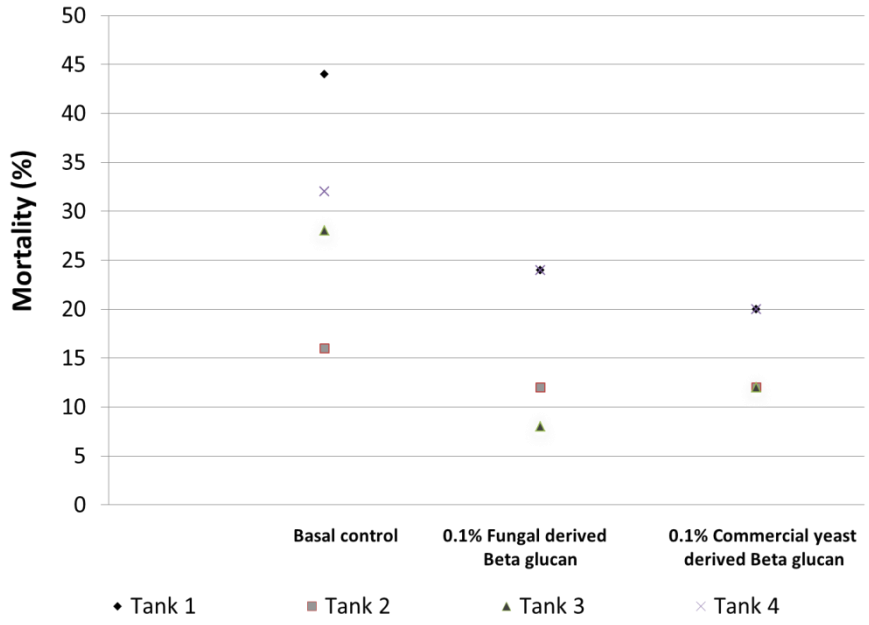


Figure 5.12(A-B). (A) The percentage cumulative mortality between experimental groups (0.1% commercial yeast derived β-glucan, 0.1% fungal derived β-glucan and basal control), (B) the variation between the tanks of experiment groups. Different letters indicate significant differences (P<0.05) between groups of fish fed experimental diets (a,b,c).

5.4 Discussion

In the previous chapter, the effect of β-glucans on the immune function of *P. hypophthalmus* and their resistance to *E. ictaluri* infection was examined. The results clearly indicated that both the humoral and cellular immune responses of *P. hypophthalmus* were differentially stimulated by different concentrations of the β-glucan, with increased immune responses observed within two weeks of feeding *P. hypophthalmus* the β-glucan-supplemented diets. Some immune parameters such as respiratory burst activity, WBC counts, total plasma protein and plasma peroxidase activity were stimulated after only one day of feeding fish the immunostimulant diets. Doses of 0.1 or 0.2% fungal-derived β-glucan appeared to give optimal immunostimulation, both the 0.1 and 0.2% fungal-derived β-glucan increased the fish's resistance to *E. ictaluri* infection. Although doses of both 0.1 and 0.2% fungal-derived β-glucan were found to stimulate the immune response of *P. hypophthalmus*, a dose of 0.1 % is economically more suitable for a commercial application in aquaculture. The effect of feeding the same β glucan-supplemented diets (at the optimal doses of 0.1%), on the immune genes in *P. hypophthalmus,* together with their resistance to *E. ictaluri* was investigated in the present study.

Samples for analysis were collected after 14 days of feeding fish the immunostimulant diets, and then again 24 h. after infecting the fish with *E. ictaluri*. Although the sequences for a few immune genes had already been identified for *P. hypophthalmus* from the NCBI database (unpublished data) *i.e*. interferon γ 2a (JN185453) and 2b (JN185454) and immunoglobulin heavy chain (JN106388), attempts were made in this study to find sequences for additional immune genes using EST

sequences provided by Novus International. This process first involved compiling a list of immune genes of interest, taken from the literature, to examine the effects of β-glucan stimulation on the *P. hypophthalmus* immune gene response. Then, all the homologous genes in NCBI RefSeq databases relating of the genes of interest were identified so as to prepare list of reference genes. In parallel, all 8,095 EST sequences were compared with genes contained in the NCBI RefSeq databases. Finally, the list of best matches between these and the list of reference genes were compared. As a result of this selection seven immune genes (C-reactive protein, precerebellin, transferrin, IL-1, MHC class II, complement factor B and C3) and three reference genes (translation elongation factor-1α mRNA, 18S rRNA and Beta-actin mRNA genes) were identified and used to design primers for studying the expression of these genes by qPCR analysis. Partial cds sequences were also found in GeneBank of NCBI RefSeq databases for *P. hypophthalmus* IFN-γ 2a (JN185453) and 2b (JN185454) mRNA, which were used to design primers for studying the expression of these immune genes. However, testing of the chosen primer sequences for IFN-γ 2a and 2b did not result in any expression of these genes in RT-qPCR using normal liver or kidney tissue or liver sampled for *E. ictaluri* infected fish. Interferon tends to expressed during virus infections rather than bacterial infections (Robertsen*, et al.* 1990), although IFN-γ has been shown to be activated during the expression of bactericidal activity by macrophages (Schroder*, et al.* 2004). Furnes, *et al.* (2009) found IFN-γ gene expression to be up-regulated in kidney and spleen of cod injected with the dsRNA polyinosinic: polycytidylic acid (poly I:C), which is a strong inducer of type I IFNs. Also, IFN-γ expression was up regulated in kidney of cod injected with formalin-killed *Vibrio*

anguillarum, but this expression was lower than seen with poly I:C stimulation, suggesting that IFN-γ is involved in the innate immune response against both viral and bacterial infections in Atlantic cod.

Suitable qRT-PCR assays were developed to analysis the expression of the seven immune genes (C-reactive protein, precerebellin, transferrin, IL-1, MHC class II, complement factor B and C3) selected from the ESTs prepared from striped catfish. These assays were used to characterize the differential expression of those genes in liver, spleen or head kidney after 14 d.p.f. and also 24 h.p.i. with *E. ictaluri*. Translation elongation factor-1-alpha mRNA, 18S rRNA and Beta-actin mRNA genes were used as constitutively expressed gene for normalization of the RT-qPCR results. After 14 days of feeding the β-glucan supplemented diets, there appeared to be no statistically significant differences in the levels of expression in genes encoding IL-1β, MHC class II, complement factor B and C3, transferrin, precerebellin and C -reactive protein between dietary groups and between tissues. Falco, *et.al*. (2012) examined a number inflammatory genes in common carp fed with a 0.1% β-glucan supplemented diet (MacroGard®) for 14 days, after which the fish were infected with *A. salmonicida* by intraperitoneal injection. They found that after feeding the fish with glucan-supplemented diets for 14 days only tumor necrosis factor-1α (tnf1α) and IL-10 in the gut and tumor necrosis factor-2α (tnf2α) in head kidney were significantly down-regulated compared to fish fed the basal control diet. However, there are no significant differences in IL-1β, IL-6 and IL-10 expression in either the gut or the kidney between the groups fed the supplemented diets and those fed the control diets. β-glucan from algae (*Euglena gracilis*) was also found to stimulate the expression of

complement factors (C3 and factor B) and acute phase proteins (hepcidin, precerebellin and transferrin) in rainbow trout bathed with the β-glucan at 100 μg ml^{-1} for 24 h (Chettri*, et al.* 2012), while Kadowaki, *et al*. (2013) showed that orally administered of lipopolysaccharide from *Pantoea agglomerans* for 60 days stimulated an up-regulation in the expression of IL-1β and TNF -α), but down-regulated the expression of IL-6 in the head kidney of common carp.

An effect of feeding β-glucans on the immune genes expressed during *E. ictaluri* infection was clearly shown in this study. In addition, the expression of genes in fish experimentally infected with *E. ictaluri* after feeding them for 14 days with the immunostimulant diets were statistically significant different to the genes expressed in the uninfected groups.

The acute phase response is described as a group of proteins that react in the host in response to infection or injury, and together they are one of the components of the innate immune response (Bayne and Gerwick 2001). In this present study, the expression of acute phase genes precerebellin and C-reactive protein, present in the liver, showed a statistically significant up-regulation between the infected and the uninfected groups of fish, while the expression of transferrin was significantly down-regulated. There was also a down-regulation in transferrin expression in the uninfected group fed with 0.1% fungal derived β-glucan compared to the basal group prior to infection.

Transferrin is associated with a negative acute phase response in reaction to stress or inflammation, but can also act as a positive acute phase protein (Neves*, et al.* 2009). Transferrin is important in iron metabolism and is involved in the defense

response of the host against bacterial infections. The regulation of iron homeostasis is an important aspect of the acute phase response in catfish (Peatman*, et al.* 2007). Liu, *et al* (2010) found the transferrin gene to be highly expressed in the liver of channel catfish, while it's expression was low in most other tissues tested examined (*i.e*. blood, brain, gill, heart, head kidney, trunk kidney, intestine, muscle, skin, spleen, stomach and ovary). They also showed that it was significantly up-regulated on 3 and 7 days after infection with *E. ictaluri*. Transferrin appears to act as a positive acute phase protein in channel catfish in order to increase iron storage to make it unavailable for bacterial growth (Liu*, et al.* 2010). On the other hand, in this study it appeared as a negative acute phase protein in striped catfish during the early stages of infection with *E. ictaluri*. Only one time point was used in this present for the analysis *i.e*. 24 h.p.i., although, it would have been useful to analyze further time points later in the infection.

There was no significant difference between uninfected and infected groups with regard to the levels of IL-1β and complement C3 gene expression in the liver, but there was a trend for increased levels in the infected groups fed with the immunostimulant diets (*i.e.* 0.1% fungal derived β-glucan and 0.1% commercial yeast derived β-glucan). Peatman, *et al*. (2007) found an up-regulation in the expression of the acute phase response, *i.e*. transferrin, intelectin, toll-like receptor 5, complement C3, ceruloplasmin and fibrinogen in the liver of channel catfish infected with a Gram negative bacterium at 3 d.p.i.

A significant down–regulation in MHC class II expression was found in the spleen of fish infected with *E. ictauri* at 24 h.p.i. compared with the uninfected fish,

and a significant up-regulation of IL-1β was shown in the kidney. However, the expression of these genes was not significantly different between fish fed with the immunostimulant diets and the control diet. The higher level of expression in the anterior kidney of infected fish was probably the result of higher concentrations of macrophages and neutrophils in this tissue than found in the liver or spleen. Elibol-Flemming, *et al.* (2009) reported that the level of IL-1β and end-binding protein-1 expression were significant higher in head kidney and spleen than that measured in the gut or liver of channel catfish during infection with *E. ictaluri,* while the level of heat shock protein 70 expression was significant higher in head kidney and liver than that of gut and spleen.

MHC class I molecules bind to and present a large number of different peptides to cytotoxic T cells, that specialize in displaying proteins that are manufactured within the cell. On the other hand, MHC class II molecules are designed to present peptides to T-helper cells (Sompayrac 2008). In this present study, only MHC class II gene expression was examined after challenging with *E. ictaluri* because it was not possible to identify the MHC class I gene sequence from the EST sequence data base. MHC class II expression was significantly up-regulated in the liver and down-regulated in the spleen of infected groups, with a clear cut difference in expression between tissues. Bao, *et al* (2005) reported that the channel catfish hepcidin gene (an antimicrobial peptide gene) was up-regulated in the spleen and head kidney of fish 1-3 d.p.i. with *E. ictaluri*, but not in their liver. Jaafar, *et al.* (2011) found liver to be a good tissue for studying the expression of immune genes in response to β-glucan absorbed through the intestine, after feeding the immunostimulant to rainbow trout. Using a murine

model, it was shown that oral administration of particulate beta-1,3-glucan was absorbed by intestinal macrophages and transported to the spleen, lymph nodes and bone marrow (Hong*, et al.* 2004). They found that effect of the β-glucan, the infection or both, was dependent on the organ, the time and targeted gene being examined.

The PCA was used to investigate the immune gene in the three different tissues (*i.e.* liver, kidney and spleen) using a total of 11 variables (liver 7 variables (C-reactive protein, transferrin, complement factor B and C3, precerebellin, IL-1β and MHC class II), kidney 2 variables (IL-1β and MHC class II) and spleen 2 variables (IL-1β and MHC class II), sampled from fish fed the three different immunostimulant diets on 14 d.p.f. and 24 h.p.i. with *E. ictaluri*. At 14 d.p.f., the PCA analysis showed a high degree of overlap between each of the diets, suggesting that the experimental diet did not cause a significant shift in the expression of all immune genes between the dietary groups. However, at 24 h.p.i., the PCA analysis shows a statistically significant differences between 0.1 % fungal derived β-glucan diet from basal control diet and 0.1 % commercial yeast derived β-glucan in the uninfected group. Moreover, the infected group, which had been fed the immunostimulant diet containing 0.1% fungal, derived β-glucan diet and 0.1% commercial yeast derived β-glucan diet showed a statistically significant effect on the overall expression of immune genes in the liver, kidney and spleen of fish challenged with *E. ictaluri* when compared to fish receiving a basal control diet.

There are many published studies relating to the effect of β-glucan on the immune response of fish and the effect that these have on improving the fishes resistance against different pathogens and their survival during infection (Jørgensen

and Robertsen 1995; Selvaraj*, et al.* 2005; Whittington*, et al.* 2005; Misra*, et al.* 2006b; Welker*, et al.* 2007; El-Boshy*, et al.* 2010; Gopalakannan and Arul 2010; Chettri*, et al.* 2012; Falco*, et al.* 2012; Pionnier*, et al.* 2013).

The present investigation showed that dietary β-glucan significantly improved the survival rate of *P. hypophthalmus* to *E. ictaluri* infection. The percentage mortality in the fish fed either 0.1% fungal derived β-glucan and 0.1% commercial yeast derived β-glucan was 16 ±5% and 17 ±8% respectively, compared to basal the control diet (30 ±12%). Furthermore, the data from the challenge experiment and the overall gene expression at 24 h.p.i. in *P. hypophthalmus* confirm that 0.1% fungal derived β-glucan diet and 0.1% commercial yeast derived β-glucan diet had an effect on the immune response of the fish in response to the *E. ictaluri* infectiion. Chen and Ainsworth (1992) reported that yeast glucan administered to channel catfish by intraperitoneal injection increased the fish's resistance against *E. ictaluri*. The relative percentage survival of common carp fed dietary β-(1,3) glucan or whole cell yeast (*Sacharomyces uvarum*) then infected with *A. hydrophila* was 75-80 % and 54-60 % respectively (Gopalakannan and Arul 2010). Nile tilapia fed on a diet supplemented with *Saccharomyces* cerevisiae, β-glucans and laminaran, then experimentally infected with *A. hydrophila* by interperitoneal injection resulted in a significant difference in the relative percentage survival between the fish fed the supplemented diet and the control group (Boshy*, et al.* 2010). The present study revealed that the β-glucan from fungal and yeast can improve the relative percentage survival *P. hypophthalmus* to *E. ictaluri* infection, although some other authors have found them to have a limited effect on the disease

resistance of channel catfish against an *E. ictaluri* infection (Welker*, et al.* 2007; Peterson*, et al.* 2010) and carp against *A. hydrophila* (Selvaraj*, et al.* 2005).

The ability of an immunostimulant to increase immune gene expression and improve disease resistance to various pathogens depends on a variety of factors such as the composition and concentration of the active ingredients present in the immunostimulant, the fish species being examined, the route of administration (oral, immersion or injection), the target pathogen (viral, bacterial and parasite), concentration of pathogen used for the experimental infection, and duration of stimulation. For example, Chettri *et al.* (2012) reported significant up-regulation of immune genes of rainbow trout fry (*i.e*. complement C3 and factor B and acute phase proteins hepcidin, precerebellin and transferrin) after 24 h bath stimulation with β-glucan at 100 mg ml^{-1}. Also, common carp, fed 0.1% β-glucan diet (MacroGard®) for 14 days and then injected intraperitoneally with *A. salmonicida* (1 $x10^{8}$ cfu ml^{-1}) had significantly higher levels of both C-reactive protein and alternative complement profiles in their serum after infection compared with the control fish not fed the immunostimulant-supplemented diet. A distinct organ and time-dependent expression profile pattern was detected in carp for all the genes selected; a peak in gene expression first occurred in the head kidney (6 h.p.i. or 12 h.p.i.), then an up-regulation was observed in the liver several hours later (24 h.p.i.) and finally up- or down-regulation of genes was observed in the mid-gut 24 h.p.i. and 72 h.p.i. (Pionnier*, et al.* 2013).

5.5 Conclusions

It may be concluded from the results obtained from this work that dietary administration of β-glucan to *P. hypophthalmus* enhanced their resistance to infection by *E. ictaluri*, and there were significant differences in immune gene expression (IL-1β, MHC class II, complement factor B and C3, transferrin, precerebellin and C-reactive protein) between uninfected and infected groups. In addition, 0.1% fungal derived β-glucan diet and 0.1% commercial yeast derived β-glucan diets stimulated the overall expression of immune genes in the liver, kidney and spleen of fish infected with *E. ictaluri* compared with groups fed the basal control diet, at 24 h.p.i. Although the immunostimulant diets did not induce any change in the gene expression at 14 day post-feeding, the fish fed with 0.1% fungal derived β-glucan diet in the uninfected group at 24 h.p.i. displayed a significantly different pattern of expression for the immune genes examined compared to the other dietary groups.

Chapter 6 General discussion and final conclusions

6.1 General discussion

Aquaculture of striped catfish, *P. hypophthalmus*, began in Vietnam in early 1960 in small scale extensive farms (Phuong and Oanh 2010), and they have been bred in Thailand since 1967 (Boonbrahm*, et al.* 1967). The main growth in the culture of this species, principally for export, has occurred in Vietnam (Bui*, et al.* 2010; De Silva and Phuong 2011), although it is also considered to be an economically important freshwater species in Thailand (Lin and Kaewpaitoon 2000; Menasveta 2000), Bangladesh (Ali*, et al.* 2012) and India (Singh and Lakra 2012). This species is ideal for aquaculture as it is fast-growing and can tolerate poor water quality (Paripatananont 2002).

Knowledge of the immune system of striped catfish is limited. Understanding the immune response is very important in order to evaluate the health status of the fish and assist in control of disease (including prevention), so that production levels can be sustained in the aquaculture industry. The aims of this thesis were to develop and standardise methods to elucidate and measure the immune responses in *P. hypophthalmus* and then to use these with relevant disease models (*A. hydrophila* and *E. ictaluri*) and immunodoulators (β-glucans from different sources and at different doses) to determine if bacterial diseases can be controlled, and which functional immune responses and immune genes could be correlated with disease resistance.

As a variety of different species from family Pangasiidae are economically important for aquaculture initial work focused on the characterisation of IgM in these species and anti-P. *hypophthalmus* IgM mAbs were tested to determine if these cross reacted between species (Chapter 2). Although affinity purification of the IgM from the different fish species resulted in a purer preparation, ammonium sulphate

precipitation (14 % w/w) was shown to be faster and easier to perform. Recently Sudhagar*, et al.* (2013) used ion exchange chromatography with diethylaminoethly (DEAE) cellulose following ammonium sulphate precipitation to purify pangasius IgM. This method, however, involves many steps and is therefore more complicated to perform and more expensive than the two methods reported above, although it may provide the purest IgM.

Estimation of the molecular weights of the H and L chains of IgM is straightforward and can be accomplished using SDS-PAGE (12.5%), while determining the molecular weight of whole IgM is difficult. The H and L chains of IgM from *P. hypophthalmus* were estimated to be 70-72 kDa and 25-26 kDa, respectively, with the L chains in the other Asian fish species having a similar molecular weight to that of *P. hypophthalmus*, while the H chains varied between the species. The molecular weights of both the H and L chains of *P. hypophthamus* have been previously reported by Sudhagar, *et al.* (2013) *i.e*. H chain 70.1 kDa and L chain 26 kDa, using reducing SDS-PAGE for analysis of the components, and Huong Giang, *et. al.* (2012) *i.e*. H chain 72 kDa and L chain 24, 26 and/or 28-29 kDa using the same method but a different percentage of polyacrylamide. It can clearly be seen that use of different techniques leads to different values being obtained for the molecular weight (MW) of pangasius IgM, but overall it would appear that the MW of H chain of the molecule is around 70-72 kDa and L chain was around 24-26 kDa. In contrast, determination of the molecular weight of whole IgM molecules by different methods has led to large variations in the molecular weight. In Chapter 2 SV-AUC was used to determine the molecular weight of the whole IgM molecule (the tetramer) from *P. hypophthalmus* as an alternative to the more commonly used native gels that are run under non-denaturing conditions, and

was estimated to be 848-876 kDa. This is similar to the estimate made using H and L chains to calculate the molecular weight (832-860 kDa) and lies between the molecular weights determined using gel filtration (900 kDa) by Huong Giang, *et. al.* (2012) and non-reducing gradient polyacrylamide gel electrophoresis (798 kDa) performed by Sudhagar, *et al.* (2013). These two methods can be used to quickly estimate the MW of proteins and nucleic acids (Ralston 1993), but they are empirical techniques that require calibration, and protein standards, and therefore rely on a series of assumptions that are often invalid (Ralston 1993; Rhodes*, et al.* 2009). For example, the MW of the reference standards for electrophoresis and chromatography are determined originally by means of using standards (Ralston 1993). In addition, one of the main errors when using gel filtration chromatography for size determination comes from not using molecules of a similar shape and density for the protein standards to that of the molecule of interest (Rhodes*, et al.* 2009). Analytical ultracentrifugation, on the other hand, is a direct measurement of the molecular weights of a solute in the native state and as they exist in solution, without having to rely on calibration and without having to make assumptions concerning their shape (Ralston 1993). One limitation of SV-AUC analysis, however, is that a sufficient quantity of a highly purified protein is needed for the analysis (Rhodes*, et al.* 2009). Nevertheless, this method provides the only primary method (*i.e*. not requiring standards) available to molecular biologists for the determination of hydrodynamic parameters. As it is based on first principles, sedimentation analysis can be applied to systems that cannot be analysed by any other means (Rhodes*, et al.* 2009). The interpretation of hydrodynamic data from sedimentation velocity uses bead modelling, with programs such as SOlution MOdeller (SOMO) (Brookes*, et al.* 2010). One

limitation using SOMO is that it is necessary to know the amino acid sequence of the target protein and this is not always possible. In addition, as IgM has a high carbohydrate composition the MW needs to be adjusted to take this into account. It is clear that a single technique cannot provide all the answers and some methods are very complex, including SV-AUC. Yoo and Jane (2002) used a combination of high-performance size-exclusion chromatography (HPSEC) with multi-angle laser-light scattering to determine weight-average MW of amylopectin from selected starches.

In terms of applying this work to investigate the health status of fish, it was established (in Chapter 3 and 4) that antibodies developed against the IgM molecule of *P. hypophthalmus* will provide useful tools to elucidate the immune response to pathogens and to assist in vaccine development. There are two previous reports in which polyclonal anti-*P. hypophthalmus* IgM or monoclonal anti-*P. hypophthalmus* IgM have been prepared and used to characterize the IgM molecule from *P. hypophthalmus* (Huong Giang*, et al.* 2012; Sudhagar*, et al.* 2013). In the present study, six anti- *P. hypophthalmus* IgM mAbs were shown to react with the IgM of other Asian fish species, some reacting with the L chain and others with the H chain of the molecule, indicating common epitopes between the L chain and H chains of IgM of family Pangasiidae and other fish species by Western blot. A standardised ELISA was developed using these mAbs (Chapter 2).

A series of functional immune assays was also standardised and optimised for use in *P. hypophthalmus* in Chapter 3 to enable both innate and adaptive immune responses to be elucidated following bacterial challenge and vaccination (Chapter3), and following immunomodulation by β-glucans and bacterial challenge (Chapter 4).

The intensification of culture practices has resulted in an increase in disease outbreaks in striped catfish farms associated with fungi, parasites as well as bacteria. Among the infectious diseases reported, bacterial agents have been responsible for the major epizootics affecting striped catfish farming (Phuong and Oanh 2010). The clinical signs caused by bacterial diseases in striped catfish farming include white spots on the internal organs caused by *E. ictaluri* (Ferguson*, et al.* 2001; Crumlish*, et al.* 2002), haemorrhagic symptoms caused by *A. hydrophila* (Subagja*, et al.* 1999; Roberts 2012), columnaris infection caused by *Flavobacterium columnare* (Tien*, et al.* 2012) and reddish lesions near the pectoral fin and belly regions caused by *Enterobacter cloacae* (Kumar*, et al.* 2013). The outbreaks of disease have been primarily caused by stress-induced factors such as poor water quality, pollution from agricultural activities, poor husbandry practices, high stocking densities, and low seed quality, making the stock susceptible to infectious pathogens. Due to the disease outbreaks, the use of drugs and chemicals is commonplace in catfish farms to treat the infection (Phuong and Oanh 2010). However, only a few antibiotics are permitted to be used because drug resistance has become a major problem in fish culture (Björklund*, et al.* 1991; Aoki 1992; Phuong and Oanh 2010). Thus, other methods of control are sought.

Vaccination is one of the methods of choice for preventing infectious diseases on farms, with a variety of commercial vaccines already available for a number of diseases *e.g*. vibriosis, furunculosis, and infectious pancreatic necrosis (IPN) in salmonids (Gudding*, et al.* 1999). Commercial vaccines are available in various parts of the world, such as for Yersiniosis (Europe, Chile, Canada and USA), Piscirickettsiosis (Chile), Flavobacteriosis (Chile, Canada and USA), Columnaris (Chile and USA), Enteric septicaemia of catfish (USA), Bacterial kidney disease (Chile, Canada and USA),

Lactococciosis (Italy, France, UK and Japan), Pasteurellosis (Mediterranean), Streptococciosis (Asia), Pancreas disease (PDV; UK, Ireland and Norway), Infectious salmon anaemia (ISAV; Canada, East of USA, Norway and UK), Infectious haematopoietic necrosis (IHNV; Canada and West of USA), Iridoviral disease (RSIV; Asia) and Grass carp haemorrhage disease (GCHDV; China)(Sommerset*, et al.* 2005; Toranzo*, et al.* 2005; Brudeseth*, et al.* 2013). Several hurdles have to be overcome for the successful application of vaccination prophylaxis for infectious disease in aquaculture, especially regarding the production of cheap but effective antigens and adjuvants, while bearing in mind environmental issues such as water quality and temperature, dissolve oxygen and associated regulatory concerns (Sommerset*, et al.* 2005). The use of vaccination in fish has significantly reduced specific disease-related losses, resulting, in turn, in a reduction in the use of antibiotics and thus their impact on the environment and issues relating to food safety because of antibiotic residues in fish products (Vinitantharat*, et al.* 1999). Due to the wide variety of fish species and limited knowledge on their immune response, the development of the vaccines has been based largely on non-empirical strategies (Sommerset*, et al.* 2005). Recently the first vaccine for striped catfish was licensed in Vietnam against *E. ictaluri*, but functional immune and gene assays require development and optimisation for this fish species.

In Chapter 3 a bacterial (*A. hydrophila*) challenge model was used in conjunction with the standardised functional assays developed to elucidate and compare the immune response to infection versus vaccination *i.e.* live versus dead bacterial challenge. Live (a sub-clinical dose) and killed *A. hydrophila* were injected intraperitoneally into *P. hypophthalmus* and both the innate and adaptive immune

response were measured. It was shown that both live and killed *A. hydrophila* administrated via this route stimulated both their innate and adaptive immune responses, with the live *A. hydrophila* stimulating these responses earlier than the killed bacteria. It appeared that the best immunoassays for monitoring the effect of this stimulation are phagocytic and respiratory burst assays (*i.e.* to monitor cellular responses), and complement assay, lysozyme assay (at pH 5.4), total Ig M, plasma peroxidase assay and specific antibody titre against *A. hydrophila* (for monitoring the humoral responses). Overall, the basic responses expected were seen in *P. hypophthalmus* following vaccination with a live or killed extracellular bacterium. This study also provided basic information on *P. hypophthalmu*s that could be used for developing more targeted vaccines for this species, such as recombinant subunit or DNA vaccines, and tools for investigating the immune response after vaccination. Most licensed vaccines currently used on commercial fish farms are based on inactivated products (Biering*, et al.* 2004; Håstein*, et al.* 2005). Sun, *et al*. (2011) compared the use of a recombinant subunit vaccine and DNA vaccine for Edwardsiellosis (*E. tarda*) in Japanese flounder (*P. olivaceus*) by intramuscular injection. Their results showed that both recombinant subunit and DNA vaccines were able to induce innate and adaptive immune response in this fish. On the one hand, DNA vaccines can stimulate both B cell and T cell response, while recombinant subunit vaccines mainly show activation of the humoral immune response. DNA vaccines are thought to be particularly useful against viral and intracellular bacterium, by inducing cell-mediated antigen presentation (Brudeseth*, et al.* 2013). For example, an IHNV DNA vaccine was developed to protect fish from IHN virus and has been licensed for use in Canada (Alonso and C Leong 2013). Also, a VHSV DNA vaccine has been shown to protect fish against VHS virus

(McLauchlan*, et al.* 2003; Pereiro*, et al.* 2012). In the future, DNA vaccinated fish will have to be labelled as genetically modified organisms (GMO) in some countries (Brudeseth*, et al.* 2013). The application of live attenuated vaccines is not a new idea (Gudding and Van Muiswinkel 2013). The advantages of modified live vaccines is that they offer better immunity against intracellular pathogens compared with inactivated vaccines (Brudeseth*, et al.* 2013), as shown with a live *E. ictaluri* attenuated vaccine against enteric septicaemia of channel catfish (ESC) (Shoemaker*, et al.* 1999) and a modified live *Flavobacterium columnare* vaccine against columnaris disease in channel catfish (Shoemaker*, et al.* 2011). A live attenuated vaccine enables the bacteria to survive, replicate and activate the cellular immune response, and antibody and mucosal immunity. The disadvantages of the using a modified live vaccine are the concerns mainly for their safety in the animal and in the environment in case they revert back to being virulent (Shoemaker*, et al.* 2009). Both biological safety assessments and genetic modification are the factors that have to be paid attention to when using GMOs in live vaccines. However, their use in the terms of biological safety can be better controlled and safety assessed than the random use of unknown mutations in conventional live attenuated vaccines (Frey 2007).

The use of immunostimulants as dietary supplements as an alternative approach to that of drugs, chemicals, antibiotics or vaccines is currently being adopted by the aquaculture industry to help prevent and control diseases by enhancing the immune defence mechanisms of the fish, and thereby increasing immunocompetency and disease resistance (Bricknell and Dalmo 2005; Bairwa*, et al.* 2012). However, the development of immunostimulants is complicated due to the limited knowledge on their mode of action (Djordjevic*, et al.* 2009), and also β-glucan from different sources

have differences in their structure and activity (Meena*, et al.* 2012). Furthermore, their dose and route of administration are important for successful stimulation of fish's immunity (Meena*, et al.* 2012). For example, short term feeding a pellets diet containing 0.1% β-1,3 glucan from yeast, *Saccharomyces cerevisiae* to Asian catfish (*Clarias batrachus*) daily for a week and then challenging them with an intraperitoneal injection of *A. hydrophila* showed lysozyme levels, superoxide production, haemagglutination titre and level of protection against *A. hydrophila* challenge, though enhanced innate immune function and improved disease resistance (Kumari and Sahoo 2006). Moreover, long term administration 250 mg of (1,3)-β-D-glucan from barley fed daily to roho (*Labeo rohita*) for 56 days and then challenged by immersing the fingerling fish into *A. hydrophila* and *E. tarda* showed enhancement the immune response (superoxide anion production, phagocytosis, lysozyme activity, haemolytic complement activity and serum bactericidal activity), growth (specific growth rate and food conversion ratio) and survival rate (Misra*, et al.* 2006c). Limited studies have been conducted on the application of immunostimulants in *P. hypophthalmus* (Prasad and Priyanka 2011; Bich Hang*, et al.* 2012). In this present study (Chapter 4) different concentrations of β-glucan were used in the diets of striped catfish for 28 days. The optimal dose of fungal-derived β-glucan for enhancing the immune function of *P. hypophthalmus* was 1.0 and 2.0 g per kg diet, while the lowest dose used (0.5 g per kg diet) appeared insufficient to effectively stimulate the fish's immune response. WBC counts, respiratory burst of head kidney macrophages, and serum lysozyme and complement activity proved useful indictors of early immunostimulation, the first day after starting to feed immunostimulant diets. After 28 d.p.f., fish were experimentally infected with *E. ictaluri* (8×10^4 cfu ml^{-1}) by immersion and although no statistical

differences were seen in the fish's resistance to the experimental infection between the different dietary groups in the first trial performed, differences were observed in a subsequent trial where the challenge dose used was lower (1 x10^6 cfu ml^{-1}). In the later trial significant differences were shown between immunostimulant groups (0.1% fungal derived β-glucan and 0.1% commercial yeast derived β-glucan) and the basal control diet group. The lower challenge regime aimed for a 40 % mortality level, which enabled the differences between dietary groups to be detected. Many challenge models have been developed to investigate pathogenesis and to investigate the health status of the fish. There are a few reported studies relating to challenge with *E. ictaluri* in *P. hypophthalmus.* Crumlish, *et al.* (2010) performed experimental challenges with *E. ictaluri* and/or *A. hydrophila* in *P. hypophthalmus*. Immersion infection with *E. ictaluri* using 1 x10^8 cfu ml^{-1} for 60 min at 28 ±2^{o}C, gave cumulative level of mortalities of 80%, while intraperitoneal injection with 1 x10^6 cfu ml^{-1} resulted in 95% cumulative mortalities. However, a co-infection with *E. ictaluri* and *A. hydrophila* resulted in cumulative mortalities of 95% by immersion when *E. ictaluri* was added at 1 x10^8 cfu ml^{-1} and *A. hydrophila* at 2 x10^7 cfu ml^{-1}, and 100 % cumulative mortalities by intraperitoneal injection (*E. ictaluri* at 1 x10^6 cfu ml^{-1} and *A. hydrophila* 2.5 x10^3 cfu ml^{-1}).

Immunostimulants are widely used in aquaculture, but there are few reports on the immune genes that are expressed by their stimulation in *P. hypophthalmus*. In Chapter 5 EST data from pyrosequencing was used to find sequences of *P. hypophthalmus* immune genes. Seven immune gene sequences were identified and primers were designed for these to use in qRT-PCR. Another approach for identifying genes sequences is next-generation sequencing (NGS), such as the use of Illumina

sequencing by synthesis of single-molecule arrays with reversible terminators, massively parallel sequencing by hybridization–ligation, implemented in the Supported Oligonucleotide Ligation and Detection system (SOLiD) from Applied Biosystems (Morozova and Marra 2008), Ion Torrent Personal Genome (PGM), a system based on semiconductor detection and Pacific Biosciences (PacBio) have developed a process enabling single molecule real time (SMRT) sequencing (Quail*, et al.* 2012). The most valuable features of the NGS technology include higher sequencing capacity, clonal sequencing of single molecules, ability to multiplex samples, higher diagnostic sensitivity, workflow miniaturization, and cost benefits (Morozova and Marra 2008). However, errors in NGS data may arise from incorrect genome mapping, failures in base calling, or contamination with extrinsic DNA (Grumbt*, et al.* 2013).

The primers designed in Chapter 5 were used to develop new qRT-PCR assays for various the immune genes (interleukin-1-beta, MHC class II, complement factor B and C3, transferrin, precerebellin, C-reactive protein) and housekeeping genes (translation elongation factor-1α mRNA, 18S rRNA gene and β-actin mRNA) identified for *P. hypophthalmus.* These were used to compare the expression of the immune genes in response to the *P. hypophthalmus* fed with the different β-glucan supplemented diets and also the gene expression profiles in relation to *E. ictaluri* infection in *P. hypophthalmus* after being fed the diets. Although immunostimulant diets did not induce any change in the gene expression at this time point 14 days post feeding, differences were shown in total gene expression between the β-glucan supplemented diets and the basal control diet after challenge with *E. ictaluri* at 24 h.p.i. Gene expression and the relative survival rate ultimately depends on many factors such as β-glucan composition and concentration, species of fish, administration

routes (oral, immersion and injection), target pathogen (viral, bacterial and parasite), concentration of pathogen, and timing of glucan stimulation and timing of expose pathogen.

6.2 Final conclusions

Standardised and optimised functional immune assays and methods for measuring the expression of various immune genes were developed for *P. hypophthalmus* in the current study. These techniques were then applied to examine the immune response of *P. hypophthalmus* to live and killed *A. hydrophila*, elucidate response to β-glucan immunostimulant diets and their resistance to *E. ictaluri* using both functional immunological assays and gene expression assays. The results indicated that fungal derived β-glucan diets stimulated both of humoral and cellular immune response in *P. hypophthalmus* when levels were at or above 0.1% glucan, and that such diets protected against *E. ictaluri* infection if the experimental dose of the bacterium was carefully controlled (40% mortality). Experimental disease challenges enable differences in diets to be established, while measuring gene expression alone after feeding did not (or at least at the time point measured). Successful disease models depend on the concentration of bacteria, time and route of exposure and environmental factors such as water quality and temperature, and it is important that the challenge protocol is standardised, and for testing immustimulants it is recommended that the dose should be lower than that normally used to test vaccine efficacy. Further work is clearly required with regard to gene expression as only seven immune genes were identified in this study. Furthermore, this gene expression experiment only covered one time point post-feeding and one time point after challenging with *E. ictaluri*. Future studies are therefore needed to analyse what is

happening with these genes at different time points during immunodulation and infection to determine gene expression over time. It is also important in future work to try to relate immune gene expression with functional immune response. This was not possible in the present study due to variation between the experimental groups and the fact that immune gene expression did not cover with all immune functional assays that were performed. Ultimately, further work on the *P. hypoophthalmus* immune system should focus on full immunological transcriptomic analysis to enable a more complete understanding of the gene expression and regulatory networks involved in the immune response of *P. hypophthalmus* to disease.

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Appendix I

Appendix I

General buffer

Phosphate buffered saline, pH 7.2 (PBS)

(0.02M Phosphate, 0.15M NaCl, pH adjusted to 7.2 with conc. HCl)

$NaH_2PO_4.2H_2O$ (BDH)	0.876g/l
$Na_2HPO_4.2H_2O$ (BDH)	2.56g/l
NaCl (Sigma)	8.77g/l

Sodium sulphate precipitation

Soduim sulphate buffer

	14 % (w/v)	16 % (w/v)	20 % (w/v)
Na_2SO_4 (Sigma)	1.4 g	1.6 g	2.0 g
Distilled water	10 ml	10 ml	10 ml

Anesthetic

Benzocaine solution

Benzocaine (Sigma, UK)	10 g
95% Ethanol	100 ml

Anticoagulant

Heparin solution

Heparin (Sigma)	25 IU
L-15 medium (Leiboritz, Sigma)	10 ml

Affinity chromatography purification (IgM purification)

Binding buffer (20mM sodium phosphate; 0.8 M ammonium sulphate, pH 7.5)

$NaH_2PO_4.2H_2O$ (BDH)	0.876 g
$Na_2HPO_4.2H_2O$ (BDH)	2.56 g
$(NH_4)_2SO_4$ (Sigma)	105.712 g
Distilled water	1 liter

Elution buffer (20 mM sodium phosphate, pH 7.5)

$NaH_2PO_4.2H_2O$ (BDH)	0.876 g
$Na_2HPO_4.2H_2O$ (BDH)	2.560 g
Distilled water	1 liter

Regeneration buffer (20mM sodium phosphate, 30% isopropanol, pH 7.5)

$NaH_2PO_4.2H_2O$ (BDH)	0.3066 g
$Na_2HPO_4.2H_2O$ (BDH)	0.8960 g
Distilled water	350 ml
Isopropanol (Sigma)	150 ml

Affinity chromatography purification (anti- *P. hypophthalmus* IgM monoclonal antibodies; Protein G affinity column)

Binding buffer (20 mM sodium phosphate, pH 7.5)

$NaH_2PO_4.2H_2O$ (BDH)	0.876 g
$Na_2HPO_4.2H_2O$ (BDH)	2.560 g
Distilled water	1 liter

Elution buffer (0.1 M glycine-HCL; Sigma, pH 2.7)

$NaH_2PO_4.2H_2O$ (BDH)	0.876 g
$Na_2HPO_4.2H_2O$ (BDH)	2.56 g
Glycine (Sigma)	7.507 g
Distilled water	to 1 liter

Adjust pH to 2.7 with HCL

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

2x sample buffer

0.5 M Tris-HCl pH 6.8	2.5 ml
Glycerol (Sigma)	2.0 ml
10% Sodium dodecyl sulfate (SDS; Sigma)	4.0 ml
Dithiothreitol (DTT; Sigma)	0.31 g
Bromophenol blue (BDH)	2.0 mg
Distilled water	to 10 ml

Western blot

Transblot buffer (0.2 M glycine; 0.025 M Tris; 20% methanol, pH 8.3)

Glycine (Sigma)	14.4 g
Trisma base (Sigma)	3.03 g
Methanol	200 ml
Distilled water	to 1 litre

Tris buffered saline (TBS; 10 mM Tris, 0.5 M NaCl, pH 7.5)

Trisma base (Sigma)	2.42 g
NaCl (Sigma)	29.24 g
Distilled water	to 1 litre

Tris buffered saline with Tween 20 (TTBS; 0.1% Tween 20 in TBS, pH 7.5)

Trisma base (Sigma)	2.42 g
NaCl (Sigma)	29.24 g
Tween-20 (Sigma)	0.5 ml
Distilled water	to 1 litre

Enzyme-Linked Immunosorbent Assay (ELISA)

Coating buffer (Carbonate-bicarbonate solution)

Na_2CO_3 (Sigma)	1.59 g	Dissolved in 1 L distilled water
$NaHCO_3$ (Sigma)	2.93 g	Adjusted to pH 9.6 and freshly make

Or buffer tablets (Sigma)
1 tablet in 100ml distilled H_2O Prepare fresh. Do not store.

Post-coat buffer
3 % (w/v) Casein (dried milk)

Casein	3 g
Distilled water	100 ml

Or 1% (w/v) Bovine serum albumin (BSA)

BSA	1 g
Distilled water	100 ml

Low salt wash buffer x10
(LSW; 0.02M Tris base, 0.38 M NaCl, 0.05% v/v Tween-20, pH 7.3)

Trisma base (Sigma)	24.2 g Dissolved in 1 L distilled water
NaCl (Sigma)	222.2 g pH adjusted to 7.3 with conc. HCl
Tween 20 (Sigma)	5 ml

Dilute 1:10 before use

High salt wash buffer x10
(HSW; 0.02M Tris base, 0.5 M Nacl, 0.1% v/v Tween-20, pH 7.7)

Trisma base (Sigma)	24.2 g Dissolved in 1 L distilled water
NaCl (Sigma)	292.2 g pH adjusted to 7.7 with conc. HCl
Tween 20 (Sigma)	10 ml

Dilute 1:10 before use

Antibody buffer

Bovine serum albumin (Sigma)	1 g
Phosphate buffer saline (Sigma)	100 ml

Substrate buffer (Sodium acetate/ citric acid buffer)

Citric acid (Sigma)	21.0 g Dissolve in approx 900mls distilled water
Sodium acetate (Sigma)	8.2 g adjust pH to 5.4 using 1M NaOH

Make up to final volume of 1 litre Store at 4°C

Conjugate buffer

Bovine serum albumin (Sigma)	1 g
Low salt wash buffer (1X)	100 ml

Substrate
3'3'5'5'-Tetramethylbenzidine dihydrochloride (TMB) (42 mM) was added to 1:2 acetic acid: distilled water.
Prepare Acetic acid solution by adding 2mls of glacial acetic acid to 4mls water
42mM TMB dihydrochloride – 0.07896g/6mls (0.01316/ml).
Cover in foil and store at 4°C
150 µl of this solution was added to 15 ml substrate buffer and 5µl H_2O_2

Stop reagent (2M H_2SO_4)

H_2SO_4 (conc.) (Sigma)	10 ml
Distilled water	70 ml Once cooled make up to 90 ml.

White blood cell and red blood cell counts

Natt-Herricks's stain

Sodium chloride (NaCl; Sigma)	3.88 g
Sodium sulphate (Na_2SO_4; Sigma)	2.50 g
Sodium phosphate (Na_2HPO_4; Sigma)	1.74 g
Potassium phosphate (KH_2PO_4; Sigma)	0.25 g
Distilled water	1,000 ml
Methyl violet (Sigma)	0.1 g
Formaldehyde (VWR)	7.5 ml

Filter with 0.45 µm paper 2-3 times. Keep away from light and store at 20^{o}C. *N.B. This solution should be stirred overnight and filtered before use.

Differential white blood cell count

Giemsa's solution (IVD)

Giemsa's azure eosin methylene blue solution	10 ml
Buffer solution	190 ml

Mix well, leave to stand for 10 min and filter if necessary

Respiratory burst of head kidney macrophage assessed using Nitroblue tetrazolium

Lysis buffer

0.1 M citric acid (Sigma)	2.1014 g
1.0 % Tween 20 (Sigma)	1 ml
0.05 % (w/v) crystal violet (Sigma)	0.05 g
Distilled water	100 ml

70% (v/v) Methanol

Methanol (Sigma)	350 ml
Distilled water	150 ml

2M Potassium hydroxide (KOH)

2M of KOH (Sigma)	11.222 g
Distilled water	100 ml

NBT solution

NBT (Sigma)	10 mg/ tablet
Distilled water	0.5 ml
L-15 medium (Sigma)	9.5 ml

Make up fresh and store in 4^{o}C

PMA stock solution (1µg/ml)

PMA (Sigma)	1 mg
Ethanol (Sigma)	1 ml

PMA + NBT solution

PMA 1µg/ml	10 µl
NBT	9ml

Lysozyme activity

Sodium phosphate buffer (SPB)

Stock A : 0.04 M solution of $NaH_2PO_4.2H_2O$

$NaH_2PO_4.2H_2O$ (Sigma)	6.24 g
Distilled water	1 litre

Stock B : 0.04 M solution of $Na_2HPO_4.2H_2O$

$Na_2HPO_4.2H_2O$ (Sigma)	7.12 g
Distilled water	1 litre

Mix the following amounts of each solution to achieve the desired pH:

Stock A (ml)	**Stock B** (ml)	pH
0.66	250	5.4

Plasma peroxidase activity

HBSS-plus solution

HBSS without Ca^{2+} and Mg^{2+} (Sigma) 100 ml

Substrate buffer (Sodium acetate/ citric acid buffer)

Citric acid (Sigma)	21.0 g	Dissolve in approx 900 ml distilled water
Sodium acetate	8.2 g	adjust pH to 5.4 using 1 M NaOH

Make up to final volume of 1 litre. Store at 4°C

Substrate

TMB (20 mM; Sigma) was added to 1:2 acetic acid: distilled water.
Prepare 50 % Acetic acid solution (40 mM; Sigma) by adding 2 ml of glacial acetic acid to 4 ml water Prepare 20mM TMB hydrochloride – 0.063 g/5 ml (0.012/ml)
Cover in foil and store at 4°C
Add to 200 µl H_2O_2 to 3 ml of substrate buffer and 3ml of (TMB + Acetic acid)

Stop reagent (2M H_2SO_4)

H_2SO_4 (conc.) (Sigma)	10 ml
Distilled water	70 ml Once cooled make up to 90 ml.

Complement activity

0.1% Gelatin-complement fixation (G-CF) buffer

Barbitone complement fixation test diluent tablet (Oxoid)	1 tablet
Gelatin (Sigma)	0.1 g
Distilled water (warmed)	100 ml

Stir until the tablet dissolves and gelatin creates froth on the top of the mixture.
Pour the buffer into 100 ml glass container and shake vigorously and store in the fridge at 4^oC

0.1% G-CF Buffer / 20 mM EDTA

Barbitone complement fixation test diluent tablet (Oxoid)	1 tablet
Gelatin (Sigma)	0.1 g
Distilled water (warmed)	80 ml

Stir until the tablet dissolves and gelatin creates froth on the top of the mixture.

Pour the buffer into 100 ml glass container and shake vigorously and store in the fridge at 4^{o}C
Prior to using the mixture add 20 ml of 0.1 M EDTA.

0.1M EDTA solution (Molar mass of EDTA: 292.24 g/mol)

0.1 M EDTA	2.92 g
Distilled water	100 ml

The solubility of EDTA increases with increasing pH. Increase pH to 8.5 by adding drops of 10 M NaOH. Pour the EDTA solution into an 100 ml glass container, shake vigorously and store in fridge at 4^{o}C.

0.1 % (v/v) anhydrous Na_2CO_3

Na_2CO_3 (Sigma)	0.1 g
Distilled water	100 ml

Plasma antiprotease activity

2% (w/v) Azocasein

Azocasein (Sigma)	0.1 g
Phosphate buffer saline, pH 7.2	5 ml

10% (v/v) Trichloracetic acid

Trichloracetic acid (Sigma)	1 g
Distilled water	10 ml

1N Sodium hydroxide (NaOH)

Sodium hydroxide (Merck)	20 g
Distilled water	500 ml

Trypsin standard (5mg/ml)

Trypsin (Sigma)	0.005 g
Distilled water	1 ml

RNA extraction using TRI-Reagent and BCP

RNA precipitation solution

1.2 M NaCl (Sigma)	7 g
0.8 M $C_6H_6Na_2O_7$-1.5H_2O (Sigma)	21.05 g
Nuclease free H_2O	100 ml

Dissolve the NaCl in 50 ml of nuclease-free H_2O into sterile glass bottle and add the sodium citrate sesquihydrate. Using magnetic stirrer and slight heat to dissolve. Make up to 100 ml with nuclease free H_2O in measuring cylinder (which has been rinsed several times with nuclease-free-H_2O). Filter-sterilised the solution using a 0.2 µ syringe filter in to another sterile glass bottle. Store at 4^{o}C

50x TAE buffer

Trisbase	24.2 g
0.5 M Glacial acetic acid (Sigma)	5.71 ml
Na_2EDTA (pH 8.0) (Sigma)	10 ml
H_2O	up to 1,000 ml

0.5 M Na_2EDTA

Disodium ethylenediaminetetra acetate.$2H_2O$ (Sigma)	186.1 g
H_2O	800 ml

Stir vigorously and adjust the pH to 8.0 with NaOH and sterilize by autoclaving

Agarose Gel electrophoresis

50xTAE buffer

Trisbase	242g
Glacial acetic acid (Sigma)	57.1 ml
0.5 M Na_2EDTA (pH 8) (Sigma)	100 ml
H_2O	up to 1,000 ml

Prepare before use : 1X TAE buffer (50xTAE buffer 5 ml + Nuclease free H_2O 250 ml) the dilution titre 1:50

0.5 M Na_2EDTA (pH8)

Disodium ethylenediaminetetraacetate (Na_2EDTA).2 H_2O (Sigma)	186.1 g
Nuclease free H_2O	800 ml

Stir vigorously and adjust the pH with NaOH and sterilize by autoclaving

Preservation of RNA in field collected samples

0.5 M Na_2EDTA (pH 8)

Na_2EDTA	18.61 g
Nuclease free H_2O	80 ml

Stir vigorously and adjust the pH with NaOH and add distilled water to 100 ml

1 M Sodium citrate tribasic dehydrate

Sodium citrate	29.41 g
Nuclease free H_2O	100 ml

RNA onshore

0.5 M Na_2EDTA (Sigma)	4 ml
1 M Sodium citrate (Sigma)	2.5 ml
Ammonium sulphate (Sigma)	65 g
Nuclease free H_2O	93.5 ml
Final volume (approximate)	120 ml

Warm the water on a heater/stirrer to more quickly dissolve the chemicals. Add chemicals slowly. When fully dissolved – allow to cool to room temperature. Adjust pH to 5.2 with sulphuric acid. Vacuum filter through glass fibre filter. Store room temperature

Appendix II

Appendix II

Table 7.1. The list of reference genes-immune genes from closer relative of *P.* hypophthalmus (four species : *Ictalurus punctatus, Danio rerio, Oncorhynchus mykiss* and *Salmo salar*)

Parameters	Genes	Genes ID	Species
Complement	C3	100305089	*Ictalurus punctatus*
	C5	565774	*Danio rerio*
	factor B/C2B	100862743	*Ictalurus punctatus*
	factor B/C2A	100862742	*Ictalurus punctatus*
Mononuclear phagocytes Enzyme	iNOS	100136358	*Salmo salar*
	Lysozyme C	100528519	*Ictalurus punctatus*
	serine proteases-like	100034638	*Danio rerio*
Acute phase response	Hepcidin	100304640	*Ictalurus punctatus*
		100335017	*Ictalurus punctatus*
	precerebellin-like protein	100135893	*Oncorhynchus mykiss*
	CRPc-reactive protein, pentraxin-related	751795	*Danio rerio*
		100136779	*Oncorhynchus mykiss*
	Serum amyloid P-component precursor (SAP)	570524	*Danio rerio*
	transferrin	100335020	*Ictalurus punctatus*
T-cell	CD8 alpha	100415908	*Ictalurus punctatus*
	CD4-like protein 1	100304978	*Ictalurus punctatus*
	CD4-like protein 2	100304979	*Ictalurus punctatus*
	a2 MHC class II integral membrane protein alpha chain 2	64691	*Danio rerio*
	novel MHC class I antigen	565768	*Danio rerio*

Table 7.1(cont.). The list of reference genes-immune genes from closer relative of *P.* hypophthalmus (4species : *Ictalurus punctatus, Danio rerio, Oncorhynchus mykiss* and *Salmo salar*)

Parameters	Genes	Genes ID	Species
Cytokine	il-6 interleukin-6	100136689	*Oncorhynchus mykiss*
	interleukin-1-beta	100136024	*Oncorhynchus mykiss*
	tnf-alpha tumour necrosis factor alpha-like	100136034	*Oncorhynchus mykiss*
	chemokine-like (IL-8)	100136039	*Oncorhynchus mykiss*
	interleukin 8	100002946	*Danio rerio*
	interleukin 10	553957	*Danio rerio*
	tgfbr1b transforming growth factor, beta receptor 1 b	792928	*Danio rerio*
	tgfbr2 transforming growth factor, beta receptor II	30739	*Danio rerio*
	tgfb3 transforming growth factor, beta 3	369195	*Danio rerio*
Antimicrobial peptide	cathelicidin antimicrobial peptide	100136187	*Oncorhynchus mykiss*
	cathelicidin antimicrobial peptide defbl1 defensin, beta-like 1	100136453	*Salmo salar*
		100009619	*Danio rerio*
	defbl2 defensin, beta-like 2	100009620	*Danio rerio*
	defbl3 defensin, beta-like 3	100009621	*Danio rerio*
	mbl-2 C-type MBL-2 protein	100136132	*Oncorhynchus mykiss*
	hbl3 hexose-binding lectin 3	100008009	*Danio rerio*
	mbl-1 C-type mannose-binding lectin	100136623	*Oncorhynchus mykiss*
Housekeeping Genes	Translation elongation factor-1α mRNA	68161044	*Ictalurus punctatus*
	18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene	836268556	*Pangasianodon hyphthalmus*
	β-actin mRNA	325152389	*Clarias batrachus*

Table 7.2. The list of identical immune related genes in *P. hypophthalmus* ESTs

GeneID	GeneName	RefSeq_ID	RefSeq_ length	EST_ID	EST_ length	Match_ length	e-value	Score	RefSeq_ name	RefSeq_ Description	RefSeq_ geneID	RefSeq_ Species
64691	a2 MHC class II integral membrane protein alpha chain 2	gi|18858227|ref| NP_571565.1|	243	HEADKIDNEY00000404 [3 - 737]	245	236	3.11E-65	643	mhc2a	major histocompatibility complex class II integral membrane alpha chain gene	30783	Danio rerio
64691	a2 MHC class II integral membrane protein alpha chain 2	gi|18858227|ref| NP_571565.1|	243	HEADKIDNEY00000405 [3 - 737]	245	236	3.11E-65	643	mhc2a	major histocompatibility complex class II integral membrane alpha chain gene	30783	Danio rerio
64691	a2 MHC class II integral membrane protein alpha chain 2	gi|52218920|ref| NP_001004534.1|	236	HEADKIDNEY00000933 [178 - 768]	197	197	7.92E-58	578	si:busm1-194e12.11	si:busm1-194e12.11	368993	Danio rerio
100136132	mbl-2 C-type MBL-2 protein	gi|292609496|ref| XP_002660413.1|	161	HEADKIDNEY00001665 [6 - 569]	188	159	1.09E-40	429	si:dkeyp-75b4.10	si:dkeyp-75b4.10	100320899	Danio rerio
64691	a2 MHC class II integral membrane protein alpha chain 2	gi|52218920|ref| NP_001004534.1|	236	INTESTINE00000221 [308 - 1045]	246	236	1.78E-61	611	si:busm1-194e12.11	si:busm1-194e12.11	368993	Danio rerio

Table 7.2(cont.). The list of identical immune related genes in *P. hypophthalmus* ESTs

GeneID	GeneName	RefSeq_ID	RefSeq_ length	EST_ID	EST_ length	Match_ length	e-value	Score	RefSeq_ name	RefSeq_ Description	RefSeq_ geneID	RefSeq_ Species
64691	a2 MHC class II integral membrane protein alpha chain 2	gi|55742394|ref| NP_001007206.1|	236	INTESTINE00000222 [308 - 1045]	246	236	8.05E-62	614	si:busm1-48c11.3	si:busm1-48c11.3	368614	Danio rerio
100305089	C3	gi|326664400|ref| XP_002660624.2|	1644	INTESTINE00000802 [1 - 771]	257	255	1.29E-80	776	LOC100331492	complement C3-H2-like	100331492	Danio rerio
64691	a2 MHC class II integral membrane protein alpha chain 2	gi|52218920|ref| NP_001004534.1|	236	INTESTINE00001787 [30 - 539]	170	159	6.60E-38	404	si:busm1-194e12.11	si:busm1-194e12.11	368993	Danio rerio
100305089	C3	gi|323422944|ref| NP_001008582.3|	1640	INTESTINE00001919 [1 - 507]	169	168	5.12E-53	534	zgc:103710	zgc:103710	494039	Danio rerio
100862742	factor B/C2A	gi|380036064|ref| NP_001244043.1|	749	LIVER00000028 [11 - 1858]	616	595	0	2473	LOC100862742	complement factor B/C2A	100862742	Ictalurus punctatus
100862742	factor B/C2A	gi|380036064|ref| NP_001244043.1|	749	LIVER00000029 [11 - 1495]	495	454	0	1967	LOC100862742	complement factor B/C2A	100862742	Ictalurus punctatus
100862742	factor B/C2A	gi|380036064|ref| NP_001244043.1|	749	LIVER00000030 [581 - 979]	133	117	3.32E-35	381	LOC100862742	complement factor B/C2A	100862742	Ictalurus punctatus
100862742	factor B/C2A	gi|380036064|ref| NP_001244043.1|	749	LIVER00000031 [81 - 530]	150	104	1.40E-30	341	LOC100862742	complement factor B/C2A	100862742	Ictalurus punctatus
100305089	C3	gi|363807314|ref| NP_571317.1|	1643	LIVER00000045 [25 - 4365]	1447	1421	0	4612	c3a	complement component c3a	321046	Danio rerio
100305089	C3	gi|363807314|ref| NP_571317.1|	1643	LIVER00000046 [25 - 4365]	1447	1423	0	4623	c3a	complement component c3a	321046	Danio rerio
100305089	C3	gi|363807314|ref| NP_571317.1|	1643	LIVER00000047 [25 - 4665]	1547	1508	0	4907	c3a	complement component c3a	321046	Danio rerio
100305089	C3	gi|363807314|ref| NP_571317.1|	1643	LIVER00000048 [25 - 4665]	1547	1510	0	4914	c3a	complement component c3a	321046	Danio rerio

Table 7.2(cont.). The list of identical immune related genes in *P. hypophthalmus* ESTs

GeneID	GeneName	RefSeq_ID	RefSeq_ length	EST_ID	EST_ length	Match_ length	e-value	Score	RefSeq_ name	RefSeq_ Description	RefSeq_ geneID	RefSeq_ Species
100305089	C3	gi|363807314|ref| NP_571317.1|	1643	LIVER00000049 [39 - 551]	171	102	8.01E-28	317	c3a	complement component c3a	321046	Danio rerio
100335020	transferrin	gi|318067980|ref| NP_001187249.1|	679	LIVER00000118 [911 - 2050]	380	648	8.42E-156	1427	LOC100335020	transferrin	100335020	Ictalurus punctatus
100335020	transferrin	gi|318067980|ref| NP_001187249.1|	679	LIVER00000119 [1 - 1041]	347	696	6.71E-145	1333	LOC100335020	transferrin	100335020	Ictalurus punctatus
100008009	hbl3 hexose-binding lectin 3	gi|68356570|ref| XP_695347.1|	253	LIVER00000432 [81 - 851]	257	148	5.72E-25	297	LOC566971	pulmonary surfactant-associated protein D-like	566971	Danio rerio
100135893	precerebellin-like protein	gi|169234619|ref| NP_001076456.2|	225	LIVER00000631 [3 - 581]	193	158	8.48E-41	431	cbln14	cerebellin 14	100007164	Danio rerio
570524	Serum amyloid P-component precursor (SAP)	gi|70778934|ref| NP_001020468.1|	222	LIVER00000908 [6 - 542]	179	156	1.61E-55	557	crp2	C-reactive protein 2	327615	Danio rerio
100136024	interleukin-1-beta	gi|317574215|ref| NP_001187148.1|	280	LIVER00000992 [3 - 503]	167	149	1.58E-53	539	il1b	interleukin 1, beta	100304696	Ictalurus punctatus