UNIVERSITI PUTRA MALAYSIA

PROFILING IMMUNE MARKERS FOR IDENTIFICATION OF LEUKOCYTES IN BROWN-MARBLED GROUPER Epinephelus fuscoguttatus FORSSKAL

CHONG CHOU MIN

IB 2014 7

PROFILING IMMUNE MARKERS FOR IDENTIFICATION OF LEUKOCYTES IN BROWN-MARBLED GROUPER *Epinephelus fuscoguttatus* FORSSKAL

By

CHONG CHOU MIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

August 2014

Specially dedicated to my beloved parents and both of my beloved late grandfathers

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PROFILING IMMUNE MARKERS FOR IDENTIFICATION OF LEUKOCYTES IN BROWN-MARBLED GROUPER *Epinephelus fuscoguttatus* (FORSSKAL, 1775)

By

CHONG CHOU MIN

August 2014

Chair : Maha Abdullah, PhD
Faculty : Institute of Bioscience

The high market value of grouper has encouraged a greater impetus for its development in aquaculture. Rapid expansion of mariculture of grouper however has been hampered by serious outbreaks of infectious diseases in the industry which poses a pressing need for disease prevention programs and supportive input from immunological studies on this premium fish. However, fundamental difficulties such as rapid blood clotting during blood collection, unsuitability of standard reagents for isolating leukocytes and paucity in monoclonal antibody (MAb) markers for identifying leukocyte subsets has hindered the progress of immunological studies in this field. The general aim of this study was to develop methodologies to isolate and identify leukocyte subpopulations in grouper. Various methodologies were used. Experimentation with different combinations of anticoagulants identified a new preparation which effectively prevented the remarkably rapid blood clotting capabilities of grouper (*Epinephelus fuscoguttatus*). This anticoagulant also prevented the blood coagulation in other tropical fish. Isopycnic density gradient showed that all of the grouper peripheral leukocytes possess heterogeneous densities and were hardly able to be purified using this method. Cytological assessment via microscopic observations of Romanowsky stained grouper blood cells revealed the five major cell components present, namely lymphocyte, thrombocyte, erythrocyte, monocyte, and granulocyte. Cytochemical stainings were also useful to characterise these cells. Putt's Eosin staining was found to differentiate grouper leukocytes into five clusters in flow cytometry and each was shown to correlate significantly with the aforementioned main cell types. Distinct flow cytometric light scattering characteristics of these subsets with eosin mirrored the biophysics of the cells in microscopic studies. The utilisation of flow cytometric eosin profile in cell subset identification is novel and applicable on other fish species. The profile was used to indicate granulocyte: lymphocyte ratio, whereby the index reflects inflammation status in fish groups treated with *Vibrio parahaemolyticus* relative to the control group. A panel of MAbs was developed against various blood cells of *E. fuscoguttatus* using the hybridoma fusion technique. Four (EF118, EF124, EF353 and EF512) were further characterised. With a combination of

immunochemistry assessments, flow cytometric analyses, and mitogen-induced leukocyte proliferation assays. EF118 was identified as a general marker for grouper lymphocytes, EF512 was identified a subset of lymphocyte, EF124 appeared to be specific for monocytes/macrophages, while EF353 stained mature blood cells (haematocytes). These markers were also used to profile changes in cellular compositions in grouper blood and spleen of controls and 4 hours acute infection with *V. parahaemolyticus*. Results showed modulation in percentage of EF118 and EF124 positive cells in these tissue/organ. Immunopanning with the EF118 monoclonal antibody was also tested to isolate specific cells from peripheral blood of grouper and successfully purified more than 95% of lymphocyte. With the successfully establishment of the methodologies and reagents in isolation and characterisation of leukocytes subsets in grouper blood and lymphoid organs, in addition to the development of MAB and non-MAb markers, immune responses of the grouper could be monitored against various environmental or biotic stressors and immunoprophylactic treatments that will lead to improvement of programs in aquaculture of brown-marbled grouper.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIPTAAN DAN PENCIRIAN PENANDA IMUN BAGI PENGENALAN LEUKOSIT DALAM IKAN KERAPU HARIMAU *Epinephelus fuscoguttatus* (FORSSKAL, 1775)

Oleh

CHONG CHOU MIN

Ogos 2014

Pengerusi : Maha Abdullah, PhD
Fakulti : Institute of Bioscience

Harga pasaran bagi ikan kerapu yang tinggi telah mendorong perkembangan akulkultur ikan tersebut di seluruh dunia. Namun begitu, pembanguan marikultur kerapu telah dilarang oleh penyebaran pelbagai penyakit berjangkit yang serius dalam industri tersebut. Senario ini merangsang dan mendorong pengajian immunologi dan pencegahan penyakit bagi ikan kerapu. Tetapi, maklumat mengenai sistem imun dalam ikan tropika yang sedia ada adalah terhad. Pembekuan darah yang cepat semasa proses pengumpulan darah dan kesukaran pengasingan leukosit adalah antara masalah yang dihadapi dalam pengajian imun ikan tropika. Kekurangan antibodi monoklonal untuk membezakan jenis leukosit adalah faktor lain yang menghalang kajian imunologi dalam bidang ini. Tujuan umum kajian ini adalah untuk mencipta kaedah berkesan bagi mengasingan dan mengenal pasti jenis leukosit ikan kerapu harimau. Pengujikajian pelbagai kombinasi antikoagulan telah mendorong penciptaan antikoagulan yang baru dan berkesan dalam pencegahan darah ikan kerapu harimau (*Epinephelus fuscoguttatus*) daripada membeku. Anticoagulan tersebut menyelesaikan isu pembekuan darah yang cepat yang dihadapi oleh ikan tropika. Pengemparan kadar zon yang berasalkan isopiknik cerun ketumpatan menunjukan bahawa semua jenis leukosit yang terdapat dalam darah perifera kerapu memiliki ketumpatan yang bertindih dan sama. Ini menyebakan sel-sel tersebut tidak dapat dipisahkan kepada subset individunya dengan mengunakan pendekatan kaedah pengemparan kadar zon semata-mata. Pemeriksaan sitologi menunjukan kehadiran lima jenis sel utama dalam darah perifera ikan kerapu, iaitu limfosit, trombosit, eritrosit, monosit dan granulosit. Teknik pewarnaan enzimatik telah digunakan untuk mengambarkan sifat sitokimia sel-sel ini. Putt's eosin didapati berjaya membezakan leukosit kerapu kepada dalam lima kelompok melalui analisis sitometri aliran. Setiap kelompat yang diperhati dalam simetri aliran didapati berkait rapat secara ketara dengan kelima-lima leukosit dalam darah. Prinsip asas kaedah baru ini adalah berdasarkan perbezaan afiniti terhadap eosin Y bagi sel-sel yang keterterapan sel membrannya telah diubahsuaikan oleh pengawet. Gambar rajah serakan cahaya sitometri aliran yang berbeza

mengambarkan ciri-ciri biofizik sebenar sel-sel leukosit tersebut. Pengunaan eosin profil dalam sitometri adalah inovasi yang baru untuk mengenal pasti subset sel dan kaedah ini didapati boleh diaplikasikan oleh ikan tropika yang lain. Profil eosin ini telah digunakan untuk menunjuk nisbah limfosit kepada granulosit dalam darah ikan. Nisbah ini adalah indeks yang menunjukkan status keradangan bagi sesuatu vertebrat. Pembezaan nisbah ini telah dikesan dalam kumpulan ikan yang telah dijangkiti dengan *Vibrio parahaemolyticus* berbanding dengan kumpulan kawalan. Empat antibodi monoklonal telah dihasilkan dengan teknik fusi hibridoma. Melalui penilaian imunokimia, analisasi sitometri aliran, dan ujian proliferasi limfosit, keempat-empat antibodi ini telah dikenal pasti sebagai penanda sel ikan kerapu. Mereka masing-masing merupakan penanda sel kepada limfosit, limfosit subset, monosit/ makrofaj, atau sel-sel darah matang. Penanda-penanda ini telah digunakan untuk mengaji ketukaran komposisi sel dalam darah dan limpa di kalangan kerapu yang disuntik *V. parahaemolyticus* berbanding dengan kumpulan kawalan. Aplikasi teknik panning dengan menggunakan piring petri yang dilapisi penanda limfosit (antibodi monoklonal EF118) telah berjaya mengasingkan limfosit dengan perolehan sel dan ketulenan pengasingan yang lebih tinggi berbanding dengan teknik pendekatan kaedah pengemparan kadar zon. Penciptaan antikoagulan dan penanda-penanda sel yang baru ini dijangka akan memberi rangsangan positif kepada pengajian imunologi ikan kerapu. Pelbagai teknik, pendekatan dan rekabentuk eksperimen baru dapat dicapai dengan mengaplikasikan perciptaan ini.

ACKNOWLEDGEMENTS

During the write up of this thesis, it brought me back to the days of research in PhD. There are a few people that I would like to give my appreciation. Firstly I would like to thank my supervisor Assoc. Prof. Dr. Maha Abdullah for the constant guidance, support and tolerance to my occasional absurd ideas. She was a light that draws me back to track whenever I strayed off. Despite her busy schedule, she always spares time to conduct our progress meeting and help smooth out the knots during our course of research.

Next in line is my co-supervisor Prof. Dr. Mariana Nor Shamsudin. I would like to thank her for sharing her research grant. Enabling me to purchase the much needed reagents and chemicals for the research. Even in her moments of sickness, she continuously cared and supported my research. I am forever grateful. I would also like to express my gratitude to another co-supervisor Prof. Dr. Fatimah Md Yusoff, who graciously taught me how to write a good journal and what it's like to be a researcher. The advice is priceless. Not forgetting Dr. Leslie Than Thian Lung, relentlessly guiding me on producing monoclonal antibody. Throughout the monoclonal antibody production, he has been indispensable in guidance and advice especially during the trying times.

I would also like to thank my parents and my sister. Although they do not fully comprehend the work I'm doing, they have been a source of continuous support. They respected my desire to continue with my studies and have never expected me to contribute to the home financially, despite social pressure. I would also like to thank my close friends who have been there for me, namely Tan Cher Kuang, Kelvin Tan, Lim Hui Ki, Ang Chai Ling, Yeo Kwok Shien, Chew Weiyun, Joey Ee Uli, Catherine Chieng, Hong Chia Yean, Yau Sook Kun and Tong Chih Kong. Ungrudgingly, some of them has been willing to ferry my fish around, putting up with unpleasant smell. Not forgetting my immunology lab mates and MARSlab staffs, seniors and juniors, who made my life in research colourful. They have always been there to give a helping hand.

I would like to thank Dr. Leong Pooi Pooi, Sammy Cheang and See Hui Shien who taught me on the technique of flow cytometry.

This research would not have been possible without the said people and every single one around me.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Maha Abdullah, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Mariana Nor Shamsudin, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Fatimah Md Yusoff, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Leslie Than Thian Lung, PhD
Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD.
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature:		Signature:	
Name of Chairman of Supervisory Committee:	Maha Abdullah, PhD	Name of Member of Supervisory Committee:	Mariana Nor Shamsudin, PhD
Signature:		Signature:	
Name of Member of Supervisory Committee:	Fatimah Md Yusoff, PhD	Name of Member of Supervisory Committee:	Leslie Than Thian Lung,PhD

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A_{450}	Optical density reading measured at 450 nm wavelength
APC	Allophycocyanin
ATCC	American Type Culture Collection
BCR	B-cell receptor
BG	ß-glucuronidase
BSA	Bovine serum albumin
CAF	Citrate-acetone formaldehyde
CFU	Colony-forming unit
CO_2	Carbon dioxide
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGC	Eosinophilic granular cell
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FL1	First fluorescence detector in flow cytometry
G:L ratio	Granulocyte to lymphocyte ratio
GIFT	Genetically improved farmed tilapia
HAT	Hypoxanthine, Aminopterin and Thymidine
HBSS	Hank's balanced salt solution
Hpf	Hours post-fertilisation
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horseradish peroxidase
i.m.	Intramascularly
i.p.	Intraperitoneally
ICE	Isotonic citrate EDTA
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgT	Immunoglobulin T
IUPAC	International Union of Pure and Applied Chemistry
l-15	Leibovitz-15
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MAb	Monoclonal antibody
MGG	May-Grünwald Giemsa staining
MHC	Major histocompatibility complex
MS222	Tricaine methane sulfonate
Na_2	Sodium
NBE	α-naphthyl butyrate esterase
NCC	Non-specific cytotoxic cell

NK cells	Natural killer cell
PAMPs	Pathogen associated molecular patterns
PAS	Periodic acid Schiff
PBL	Peripheral blood leukocyte
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	R-phycoerythrin
PEG	Polyethylene glycol
PHA	Phytohaemagglutinin
PMMA	Polymethylmethacrylate
ppt	Parts-per-trillion
PRP/R	Pattern recognition proteins or receptors
RNA	Ribonucleic acid
SIP	Stock isotonic Percoll
Sp.	Species
TCR	T-cell receptor
TLR	Toll-like receptor
TRITC	Tetramethylrhodamine isothiocyanate
USA	United states of America

CHAPTER 1

INTRODUCTION

The brown-marbled grouper (*Epinephelus fuscoguttatus)* of the Serranidae family or Tiger grouper as it is known in South-East Asia is a species of warmwater marine finfish (Unsworth *et al*., 2007). Groupers are the most rigorously exploited live fish group (Pierre *et al*., 2008) due to the remarkable quality of its flesh and high demand in the Asian market with an estimated cost ranging from USD 15–50 kg^{-1} with fluctuations in the price and increased demand during the festive seasons (Harikrishnan *et al*., 2010; Othman, 2008; Pierre *et al*., 2008; Parameswaran *et al*., 2007; Zhou *et al*., 2007; Antoro *et al*., 2006). Habitat loss and commercial fishery (Cornish, 2004 ; Zatcoff *et al*., 2004) however, have led to the development of mariculture of grouper in many countries including Malaysia, with a two-fold increase in the wholesale value of reared grouper from the year 2002 to 2004 (Othman, 2008).

Unfortunately, the mariculture industry is susceptible to diseases such as vibriosis which occurs at various stages of grouper cultivation (Sivaram *et al*., 2004) and serious infections which are potentially capable of wiping out entire fish stocks (Oh *et al*., 2012; Gong *et al*., 2011; Parameswaran *et al*., 2007; Chi *et al*., 2003). Immunology-based strategies such as vaccination and investigations on the susceptibility of infections may improve management and increase productivity of fish culture.

Insufficient knowledge is currently available on tropical warmwater fish compared to coldwater fish species (Bowater *et al*., 2012; Lin *et al*., 2005). One of the main differences between cold and warm water fish is the amount of time it takes for blood clotting to occur (Kawatsu, 1986; Feeney *et al*., 1972), which is more rapid in fish living in warmer temperature (Huss, 1993).

The rapid blood clotting mechanism in warm marine finfish is an evolutionary feature that prevents infections of open wounds which are more susceptible to pathogenic infections in warmer water systems. Apart from pathogenic infections, bleeding poses a lethal threat to fish as they possess a blood volume of relatively a fifth to a quarter of that of mammalian blood (Wolf, 1959), and the bleeding could also potentially attract aquatic predators with strong olfactory capabilities. Although the effective blood clotting has been beneficial for the warm water fish, it has hampered blood collection essential for downstream uses needed by fish farmers and researchers, such as sex determination assay, serological and health assessment, disease identification, assessment of vaccine, biomarker production as well as various endocrinology tests. Close to 60% of the world's aquaculture production is contributed by warm subtropical and tropical countries in Asia (De Silva and Soto, 2009). Thus, the development of an effective anticoagulant will significantly contribute to fundamental researches in disease prevention and management.

Like mammals, fish have various types of white blood cells, or leukocytes, which constitute a sophisticated defense mechanism called cellular immunity. These leukocytes encompass lymphocytes, monocytes, thrombocytes and granulocytes. The separation of blood cells into their respective cell lineages is often the first and crucial step for many immunological studies. Isolation of leukocytes into their respective cell lineages have been confined to the usage of buoyant centrifugation methods, for example, density gradient centrifugation as well as sedimentation (Rowley, 1990).

With the monoclonal antibody approach, great strides have been made in the identification, isolation, and characterisation of leukocyte subpopulations of some fish species. The availability of basic reagents and methodologies encourages the progress of immunological based studies including understanding mechanisms of immunity to infections and importance of abiotic/biotic stressors that may have immunomodulatory effect on boosting or suppressing immune response. Furthermore, MAbs enable comprehend evaluation of immune status in fish. Through this marker-assisted selective breeding approach, broodstock of strong immunity could be identified. With available supportive evidence for vaccine identification and production, along with selective breeding, establishment of culture programs for rearing of healthy and disease resistant fish can be conducted.

Despite immense applications of monoclonal antibodies, developed monoclonal antibodies tend to be limited to several cultured fish species, such as salmon, rainbow trout, carp, and catfish, due to complications and difficulties in MAb production. Since bony fish are the vertebrate group with the most abundant species and possess a wider diversity among the species of fish (Shigdar *et al.*, 2009), the applicability of these MAbs are limited to their corresponding species and there is no cross-reactivity with other fish species.

The lack of lineage specific antibodies has hindered progress in the study of the immune cells in many other fish species of high commercial values, including brown-marbled grouper. Without specific MAbs to recognise cell lineages, the classifications of leukocytes in these fish are based mainly on morphology, ultrastructure, and differential cytochemical staining (Katzenback and Belosevic, 2009; Shigdar *et al.*, 2009; Slierendrecht *et al.*, 1995).

In light of the paucity of MAbs, basic methods to study the immunology of grouper are limited. Neither cytochemical assessment of grouper leukocytes, nor investigation of the effectiveness of the density centrifugation approach on grouper leukocyte subtypes have been described in scientific literatures as of yet. No studies to date have reported on the flow cytometric profile of grouper or its related species.

Thus, the current project was carried out to answer and accentuate the aforementioned issues on brown-marbled grouper with the following objectives:

i. To develop an effective anticoagulant suitable for the rapid blood clotting nature of grouper blood

ii. To isolate various leukocyte subpopulations through physical-chemical methods.

iii. To characterise cytochemical staining and flow cytometric profiling of grouper leukocytes

iv. To establish monoclonal antibodies against the grouper's leukocyte subpopulations.

v. To demonstrate the applicability of the developed monoclonal antibodies on immunological study of grouper.

REFERENCES

ABELSON, J. N., SIMON, M. I. & CONN, P. M. 1999. *Confocal Microscopy*, Massachusetts: Academic Press

ADIAS, E. D. 2013. *Essentials of Blood Transfusion Science*, Indiana: AuthorHouse.

AFERO, F., MIAO, S. & PEREZ, A. A. 2010. Economic analysis of tiger grouper *Epinephelus fuscoguttatus* and humpback grouper *Cromileptes altivelis* commercial cage culture in Indonesia. *Aquaculture International,* 18**,** 725-739.

AINSWORTH, A. J. 1992. Fish granulocytes: morphology, distribution, and function. *Annual Review of Fish Diseases,* 2**,** 123-148.

AINSWORTH, A., DEXIANG, C. & GREENWAY, T. 1990. Characterization of monoclonal antibodies to channel catfish, *Ictalurus punctatus*, leucocytes. *Veterinary Immunology and Immunopathology,* 26**,** 81-92.

ALVAREZ-PELLITERO, P. 2008. Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. *Veterinary Immunology and Immunopathology,* 126**,** 171-198.

AN, H. S., KIM, J. W., LEE, J. W., KIM, S. K., LEE, B. I., KIM, D. J. & KIM, Y. C. 2012. Development and characterization of microsatellite markers for an endangered species, *Epinephelus bruneus*, to establish a conservation program. *Animal Cells and Systems,* 16**,** 50-56.

ANTORO, S., NA-NAKORN, U. & KOEDPRANG, W. 2006. Study of genetic diversity of orange-spotted grouper, *Epinephelus coioides*, from Thailand and Indonesia using microsatellite markers. *Marine Biotechnology,* 8, 17-26.

AQUAMAPS. 2010. Reviewed Native Distribution Map for *Epinephelus fuscoguttatus* (Brown-marbled grouper). Available: <www.aquamaps.org> [Accessed 14 May. 2013].

ARTHUR, J. & OGAWA, K. A brief overview of disease problems in the culture of marine finfishes in east and Southeast Asia. Aquaculture health management strategies for marine finfishes-Proceedings of a Workshop in Honolulu, Hawaii, 1995. 9-31.

ASENSIO, L., GONZÁLEZ, I., FERNÁNDEZ, A., RODRÍGUEZ, M. A., HERNÁNDEZ, P. E., GARCÍA, T. & MARTÍN, R. 2001. PCR-SSCP: A simple method for the authentication of grouper (*Epinephelus guaza*), wreck fish (*Polyprion americanus*), and Nile perch (*Lates niloticus*) fillets. *Journal of Agricultural and Food Chemistry,* 49**,** 1720-1723.

ASENSIO, L., GONZÁLEZ, I., ROJAS, M., GARCÍA, T. & MARTÍN, R. 2009. PCR-based methodology for the authentication of grouper (*Epinephelus marginatus*) in commercial fish fillets. *Food Control,* 20**,** 618-622.

BANBURA, J., SKWARSKA, J., BANBURA, M., GLADALSKI, M., HOLYSZ, M., KALINSKI, A., MARKOWSKI, M., WAWRZYNIAK, J. & ZIELINSKI, P. 2013. Spatial and Temporal Variation in Heterophil-

to-Lymphocyte Ratios of Nestling Passerine Birds: Comparison of Blue Tits and Great Tits. *PloS one,* 8, e74226.
BANCROFT, J. D. & GAMBLE, M. 2008. *Theory and Practice of Histological Techniques*, London: Churchill Livingstone.
BASSO, G., COCITO, M., SEMENZATO, G., PEZZUTTO, A. & ZANESCO, L. 1980. Cytochemical study of thymocytes and T lymphocytes. *British journal of haematology,* 44, 577-582.
BEELEN, R., HEIJDEN, T., BOOMS, G., VERDEGEM, M. & PAVANELLI, G. 1998. Blood values of young Brazilian catfish *Pseudoplatystoma corruscans* (Agassiz, 1829). *Acta Scientiarum,* 20.
BERNARD, A. M., FELDHEIM, K. A., RICHARDS, V. P., NEMETH, R. S. & SHIVJI, M. S. 2012. Development and characterization of fifteen novel microsatellite loci for the Nassau grouper (*Epinephelus striatus*) and their utility for cross-amplification on a suite of closely related species. *Conservation Genetics Resources,* 4**,** 983-986.
BLAXHALL, P. & DAISLEY, K. 1973. Routine haematological methods for use with fish blood. *Journal of Fish Biology,* 5, 771-781.
BOHLS, R. L., SMITH, R., FERRO, P. J., SILVY, N. J., LI, Z. & COLLISSON, E. W. 2006. The use of flow cytometry to discriminate avian lymphocytes from contaminating thrombocytes. *Developmental and Comparative Immunology,* 30, 843-850.
BONDAD-REANTASO, M. G., KANCHANAKHAN, S. & CHINABUT, S. Review of grouper diseases and health management strategies for grouper and other marine finfish diseases. Report of the Regional Workshop on Sustainable Seafarming and Grouper Aquaculture, Medan, Indonesia, 2000. 17-20.
BOWATER, R. O., FORBES-FAULKNER, J., ANDERSON, I. G., CONDON, K., ROBINSON, B., KONG, F., GILBERT, G. L., REYNOLDS, A., HYLAND, S., MCPHERSON, G., BRIEN, J. O. & BLYDE, D. 2012. Natural outbreak of *Streptococcus agalactiae* (GBS) infection in wild giant Queensland grouper, *Epinephelus lanceolatus* (Bloch), and other wild fish in northern Queensland, Australia. *Journal of Fish Diseases,* 35, 173-186.
BUNLIPATANON, P., SONGSEECHAN, N., KONGKEO, H., ABERY, N. W. & SILVA, S. S. 2012. Comparative efficacy of trash fish versus compounded commercial feeds in cage aquaculture of Asian seabass (*Lates calcarifer*)(Bloch) and tiger grouper (*Epinephelus fuscoguttatus*)(Forsskål). *Aquaculture Research*.
CAMPBELL, T. W. & ELLIS, C. K. 2013. *Avian and Exotic Animal Hematology and Cytology*, Wiley.
CATTON, W. 1951. Blood cell formation in certain teleost fishes. *Blood,* 6, 39-60.
CAXTON-MARTINS, A. 1979. Cytochemical studies of cell population in peripheral blood smears of two West African teleosts. *Journal of anatomy,* 128, 269.
CHANDLER, D. E. & ROBERSON, R. W. 2009. *Bioimaging: Current Concepts in Light and Electron Microscopy*, Massachusetts: Jones and Bartlett Publishers.
CHEESBROUGH, M. 2000. *District Laboratory Practice in Tropical Countries*, Cambridge: Cambridge University Press.

CHEN, S., ZHANG, J., CHEN, W., XU, D. & ZHOU, Y. 2011. Species identification of grouper and snapper in Taiwan Strait using polymerase chain reaction-restriction fragment length polymorphism analysis and lab-on-a-chip system. *Chinese Journal of Chromatography (Se Pu),* 29**,** 677-680.

CHENG, S. Y., CHEN, C. S. & CHEN, J. C. 2013. Salinity and temperature tolerance of brown-marbled grouper *Epinephelus fuscoguttatus*. *Fish Physiology and Biochemistry,* 39**,** 277-286.

CHI, S. C., SHIEH, J. R. & LIN, S. J. 2003. Genetic and antigenic analysis of betanodaviruses isolated from aquatic organisms in Taiwan. *Diseases of Aquatic Organisms,* 55, 221-228.

CHILMONCZYK, S. & MONGE, D. 1999. Flow cytometry as a tool for assessment of the fish cellular immune response to pathogens. Fish & Shellfish Immunology, 9, 319-333.

CHIU, S.-T., TSAI, R.-T., HSU, J.-P., LIU, C.-H. & CHENG, W. 2008. Dietary sodium alginate administration to enhance the non-specific immune responses, and disease resistance of the juvenile *grouper Epinephelus fuscoguttatus*. *Aquaculture,* 277**,** 66-72.

CHIU, T. H., SU, Y. C., PAI, J. Y. & CHANG, H. C. 2012. Molecular markers for detection and diagnosis of the giant grouper (*Epinephelus lanceolatus*). *Food Control,* 24**,** 29-37.

CHOU, H., HSU, C. & PENG, T. 1998. Isolation and characterization of a pathogenic iridovirus from cultured grouper (*Epinephelus sp*.) in Taiwan. *Fish Pathology (Japan)*.

COICO, R. & SUNSHINE, G. 2009. *Immunology: A Short Course*, Wiley.

COLIN, P., KOENIG, C. & LAROCHE, W. Development from egg to juvenile of the red grouper (*Epinephelus morio*)(Pisces: Serranidae) in the laboratory. Biology, Fisheries and Culture of Tropical Groupers and Snappers. ICLARM Conf. Proc, 1996. 399-414.

COLL, J. & DOMINGUEZ-JUNCAL, J. 1995. Applications of monoclonal antibodies in aquaculture. *Biotechnology advances,* 13**,** 45-73.

COLLER, H. A. & COLLER, B. S. 1983. Statistical analysis of repetitive subcloning by the limiting dilution technique with a view toward ensuring hybridoma monoclonality. *Hybridoma,* 2, 91-96.

COOK, M. T., MORRISON, R. N., WILKINSON, R., NOWAK, B. F., HAYBALL, P. J. & HAYBALL, J. D. 2001. A screen of mammalian antibodies on snapper (*Pagrus auratus*, Sparidae) peripheral blood leukocytes reveals cross reactivity of an anti-human CD3 antibody with a population of mIg− cells. *Developmental & Comparative Immunology,* 25**,** 553-559.

CORMACK, D. H. 2001. *Essential Histology*, Philadelphia: Lippincott Williams & Wilkins.

CORNISH, A. 2004 Epinephelus fuscoguttatus. Available: <www.iucnredlist.org> [Accessed 7 May 2013].

COX, R. A. 1989. *Immunology of the Fungal Diseases*, United Kingdom: Taylor & Francis.

CUESTA, A., ÁNGELES ESTEBAN, M. & MESEGUER, J. 2003. Identification of a FasL-like molecule in leucocytes of the teleost fish gilthead seabream *Sparus aurata* L.). *Developmental & Comparative Immunology,* 27**,** 21-27.

CUESTA, A., ESTEBAN, M. Á. & MESEGUER, J. 2005. Molecular characterization of the nonspecific cytotoxic cell receptor (NCCRP-1) demonstrates gilthead seabream NCC heterogeneity. *Developmental & Comparative Immunology,* 29**,** 637-650.

DANAYADON, Y., KRACHAIWONG, W., RUANGPAN, L. & DIREKBUSARAKOM, S. 1996. Causative agent and control measure of red boil disease in cultured grouper (*Epinephelus malabaricus*). *Warasan Kan Pramong,* 49.

DANILOVA, N., BUSSMANN, J., JEKOSCH, K. & STEINER, L. A. 2005. The immunoglobulin heavy-chain locus in zebrafish: Identification and expression of a previously unknown isotype, immunoglobulin Z. *Nature Immunology,* 6**,** 295-302.

DAVIDSON, G., ELLIS, A. & SECOMBES, C. 1994. A preliminary investigation into the phenomenon of oral tolerance in rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792). *Fish & Shellfish Immunology,* 4**,** 141-151.

DE INNOCENTIIS, S., SOLA, L., CATAUDELLA, S. & BENTZEN, P. 2001. Allozyme and microsatellite loci provide discordant estimates of population differentiation in the endangered dusky grouper (*Epinephelus marginatus*) within the Mediterranean Sea. *Molecular Ecology,* 10**,** 2163-2175.

DEPARTMENT OF FISHERIES MALAYSIA, D. O. F. 2011. Annual Fisheries Statistics. Available: <http://www.dof.gov.my/en/senarai-perangkaan-perikanan-2011> [Accessed 15 May 2013].

DIAZ, D., PRIETO, A., BARCENILLA, H., MONSERRAT, J., SÁNCHEZ, M. A., REYES, E. HERNANDEZ-FUENTES M. P., DE LA HERA A., ORFAO A. & ALVAREZ-MON M. 2006. Accurate apoptosis measurement requires quantification of loss of expression of surface antigens and cell fragmentation. *Cytometry A. ;69(4):240-8.*

DONG, Q. F., LIU, C. W., GUO, Y. S., LIU, L. & WU, Y. 2007. Microsatellite analysis of genetic diversity and phylogenetic relationship of nine species of grouper in genus *Epinephelus*. *Yi chuan = Hereditas / Zhongguo yi chuan xue hui bian ji,* 29**,** 837-843.

DOOLITTLE, R. F. & SURGENOR, D. M. 1962. Blood coagulation in fish. *American Journal of Physiology--Legacy Content,* 203**,** 964-970.

DOR, L., SHIRAK, A., GORSHKOV, S., RON, M. & HULATA, G. 2013. Development of genetic markers for the white grouper (*Epinephelus aeneus*). *Aquaculture*, In Press.

DOS SANTOS, N., TAVERNE, N., TAVERNE-THIELE, A. J., DE SOUSA, M. & ROMBOUT, J. H. 1997. Characterisation of monoclonal antibodies specific for sea bass (*Dicentrarchus labrax* L.) IgM indicates the existence of B cell subpopulations. *Fish & Shellfish Immunology,* 7**,** 175-191.

EDHOLM, E.-S., BENGTÉN, E., STAFFORD, J. L., SAHOO, M., TAYLOR, E. B., MILLER, N. W. & WILSON, M. 2010. Identification of two IgD+ B cell populations in channel catfish, Ictalurus punctatus. *The Journal of Immunology,* 185**,** 4082-4094.

ESTEBAN, M., MUÑOZ, J. & MESEGUER, J. 2000. Blood cells of sea bass (*Dicentrarchus labrax* L.). Flow cytometric and microscopic studies. *The Anatomical Record,* 258, 80-89.

EVANS, D. L. & JASO-FRIEDMANN, L. 1992. Nonspecific cytotoxic cells as effectors of immunity in fish. *Annual Review of Fish Diseases,* 2**,** 109-121.

EVANS, D. L., JASO-FRIEDMANN, L., SMITH, E., ST JOHN, A., KOREN, H. & HARRIS, D. 1988. Identification of a putative antigen receptor on fish nonspecific cytotoxic cells with monoclonal antibodies. *The Journal of Immunology,* 141**,** 324-332.

EVANS, D. L., KAUR, H., LEARY III, J., PRAVEEN, K. & JASO-FRIEDMANN, L. 2005. Molecular characterization of a novel pattern recognition protein from nonspecific cytotoxic cells: sequence analysis, phylogenetic comparisons and anti-microbial activity of a recombinant homologue. *Developmental & Comparative Immunology,* 29**,** 1049-1064.

FACTOR, J. R. 1995. *Biology of the Lobster: Homarus americanus*, Massachusetts: Academic Press.

FEENEY, R. E., VANDENHEEDE, J. & OSUGA, D. T. 1972. Macromolecules from cold-adapted antarctic fishes. *Die Naturwissenschaften,* 59, 22-29.

FICK, R. 2002. *IgE and Anti-IgE Therapy in Asthma and Allergic Disease*, United Kingdom: Taylor & Francis.

FISCHER, U. & KOELLNER, B. 2007. Cross-reactivity of human leukocyte differentiation antigen monoclonal antibodies on carp and rainbow trout cells. *Veterinary Immunology and Immunopathology,* 119**,** 142-155.

FISHMAN, W. 2012. *Metabolic Conjugation and Metabolic Hydrolysis*, Amsterdam: Elsevier Science.

FONSECA-CORONADO, S., DEL VALLE-ESPINOSA, E., PEDRAZA-SÁNCHEZ, S., & FLISSER, A. 2010. Proliferative Response of Hamster CD4+ T Cells after Different Mitogenic Stimulation. *Scand. J. Lab. Anim. Sci*, *37*(2).

FORSSKÅL, P. 1775. *Descriptiones animalium, avium, amphibiorum, piscium, insectorum, vermium*, ex officina Mölleri.

FROESE, R. A. D. P. 2011. *Epinephelus fuscoguttatus*. Available: <www.fishbase.org> [Accessed 11 May 2013].

GALINDO-VILLEGAS, J. & HOSOKAWA, H. 2004. Immunostimulants: towards temporary prevention of diseases in marine fish. *Advances en Nutricion. Acuicola VII Memorias del VII Simposium Internationale de Nutricion Acuícola***,** 16-19.

GAO, C., WANG, L., FAN, B., YANG, S., MENG, Z. & LIN, H. 2012. Isolation and Characterization of Microsatellite Markers From the Brown-marbled Grouper, *Epinephelus fuscoguttatus*. *Journal of the World Aquaculture Society,* 43**,** 442-446.

GILL, G. 2012. *Cytopreparation: Principles & Practice*, Heidelberg: Springer.

GODING, J. W. 1996. *Monoclonal Antibodies: Principles and Practice*, Massachusetts: Academic Press.

GOLBUU, Y. & FRIEDLANDER, A. M. 2011. Spatial and temporal characteristics of grouper spawning aggregations in marine

protected areas in Palau, western Micronesia. *Estuarine, Coastal and Shelf Science,* 92, 223-231.
GONG, J., HUANG, Y., HUANG, X., OUYANG, Z., GUO, M. & QIN, Q. 2011. Establishment and characterization of a new cell line derived from kidney of grouper, *Epinephelus akaara* (Temminck & Schlegel), susceptible to Singapore grouper iridovirus (SGIV). *Journal of Fish Diseases,* 34, 677-686.
GOVINDARAJU, G. S. & JAYASANKAR, P. 2004. Taxonomic relationship among seven species of groupers (genus *Epinephelus*; family serranidae) as revealed by RAPD fingerprinting. *Marine Biotechnology,* 6**,** 229-237.
GRABOWSKI, L. D., LAPATRA, S. E. & CAIN, K. D. 2004. Systemic and mucosal antibody response in tilapia, *Oreochromis niloticus* (L.), following immunization with *Flavobacterium columnare*. *Journal of Fish Diseases,* 27**,** 573-581.
GREENLEE, A. R., BROWN, R. A. & RISTOW, S. S. 1991. Nonspecific cytotoxic cells of rainbow trout (*Oncorhynchus mykiss*) kill YAC-1 targets by both necrotic and apoptic mechanisms. *Developmental & Comparative Immunology,* 15**,** 153-164.
GUDER, W. G., NARAYANAN, S., WISSER, H. & ZAWTA, B. 2009. *Diagnostic Samples: From the Patient to the Laboratory*, New Jersey: Wiley.
HANIF, A., BAKOPOULOS, V. & DIMITRIADIS, G. 2004. Maternal transfer of humoral specific and non-specific immune parameters to sea bream (*Sparus aurata*) larvae. *Fish & Shellfish Immunology,* 17**,** 411-435.
HANSEN, J. D., LANDIS, E. D. & PHILLIPS, R. B. 2005. Discovery of a unique Ig heavy-chain (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 6919-6924.
HARIKRISHNAN, R., BALASUNDARAM, C. & HEO, M.-S. 2010. Molecular studies, disease status and prophylactic measures in grouper aquaculture: Economic importance, diseases and immunology. *Aquaculture,* 309**,** 1-14.
HARIKRISHNAN, R., BALASUNDARAM, C. & HEO, M.-S. 2011. Fish health aspects in grouper aquaculture. *Aquaculture,* 320**,** 1-21.
HARRIS, E. N., EXNER, T., HUGHES, G. R. V. & ASHERSON, R. A. 1991. *Phospholipid-Binding Antibodies*, United Kingdom: Taylor & Francis.
HEEMSTRA, P. C., RANDALL, J. E., FOOD & NATIONS, A. O. O. T. U. 1993. *Groupers of the world (family Serranidae, subfamily Epinephelinae): an annotated and illustrated catalogue of the grouper, rockcod, hind, coral grouper and lyretail species known to date*, Food and Agriculture Organization of the United Nations.
HINE, P. 1992. The granulocytes of fish. *Fish & Shellfish Immunology,* 2**,** 79-98.
HOAR, W. S., RANDALL, D. J., IWAMA, G. & NAKANISHI, T. 1997. *The fish immune system: organism, pathogen, and environment*, Massachusetts: Academic Press.

HOGAN, R. J., TAYLOR, W. R., CUCHENS, M. A., NAFTEL, J. P., CLEM, L. W., MILLER, N. W. & CHINCHAR, V. G. 1999. Induction of target cell apoptosis by channel catfish cytotoxic cells. *Cellular immunology,* 195**,** 110-118.

HORIBATA, K. & HARRIS, A. W. 1970. Mouse myelomas and lymphomas in culture. *Experimental Cell Research,* 60**,** 61-77.

HOUGIE, C. 1972. Coagulation changes in healthy and sick pacific salmon. *Advances in Experimental Medicine and Biology,* 22**,** 89-102.

HUSS, H. H. 1993. *The effect of fish species, catching method, fishing ground and season,* Rome, FAO, FAO Fisheries Technical Paper.

IWAMA, G. K. & NAKANISHI, T. 1996. *The fish immune system: organism, pathogen, and environment*, Massachusetts: Academic Press

JANG, H.-N., WOO, J.-K., CHO, Y.-H., KYONG, S.-B. & CHOI, S.-H. 2004. Characterization of monoclonal antibodies against heavy and light chains of flounder (*Paralichthys olivaceus*) immunoglobulin. *Journal of biochemistry and molecular biology,* 37**,** 314-319.

JASO-FRIEDMANN, L., LEARY III, J. H. & EVANS, D. L. 2000. Role of nonspecific cytotoxic cells in the induction of programmed cell death of pathogenic protozoans: participation of the Fas ligand-Fas receptor system. *Experimental parasitology,* 96**,** 75-88.

JIANG, Y. & DOOLITTLE, R. F. 2003. The evolution of vertebrate blood coagulation as viewed from a comparison of puffer fish and sea squirt genomes. *Proceedings of the National Academy of Sciences,* 100**,** 7527-7532.

JONES, M. K. & OLIVER, J. D. 2009. Vibrio vulnificus: disease and pathogenesis. *Infection and immunity,* 77, 1723-1733.

KAPOOR, B. G. & KHANNA, B. 2004. *Ichthyology handbook*, Heidelberg: Springer.

KATZENBACK, B. A. & BELOSEVIC, M. 2009. Isolation and functional characterization of neutrophil-like cells, from goldfish (*Carassius auratus* L.) kidney. *Developmental & Comparative Immunology,* 33, 601-611.

KAWATSU, H. 1986. Clotting time of common carp blood. *Bulletin of the Japanese Society for the Science of Fish* 54, 591-595.

KELLEY, J. L., ROZEK, M. M., SUENRAM, C. A. & SCHWARTZ, C. J. 1987. Activation of human blood monocytes by adherence to tissue culture plastic surfaces. *Experimental and Molecular Pathology,* 46, 266-278.

KIRON, V. 2012. Fish immune system and its nutritional modulation for preventive health care. *Animal Feed Science and Technology,* 173**,** 111-133.

KOEDPRANG, W., NA-NAKORN, U., NAKAJIMA, M. & TANIGUCHI, N. 2007. Evaluation of genetic diversity of eight grouper species *Epinephelus spp*. based on microsatellite variations. *Fisheries Science,* 73**,** 227-236.

KÖHLER, G. & MILSTEIN, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature,* 256**,** 495-497.

KÖHLER, G. & MILSTEIN, C. 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *European journal of immunology,* 6**,** 511-519.

KÖHLER, G., HOWE, S. & MILSTEIN, C. 1976. Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines. *European journal of immunology,* 6**,** 292-295.

KÖLLNER, B., BLOHM, U., KOTTERBA, G. & FISCHER, U. 2001. A monoclonal antibody recognising a surface marker on rainbow trout (*Oncorhynchus mykiss*) monocytes. *Fish & Shellfish Immunology,* 11**,** 127-142.

KONDO, M., KONDO, K. & YUKINORI, T. 2011. Staining Properties of Monocyte Minor Granules in Sevenband Grouper, *Epinephelus septemfasciatus*. *The journal of the Shimonoseki University of Fisheries,* 60, 33-34.

KPL. 1999. *Technical Guide for ELISA* [Online]. KPL, Inc Available: [http://www.kpl.com/docs/techdocs/KPL%20ELISA%20Technical%20Guide.pdf] [Accessed 17 September 2013].

KUM, C. & SEKKIN, S. 2011. The Immune System Drugs in Fish: Immune Function, Immunoassay, Drugs. *In:* ARAL, F. & DOĞU, Z. (eds.) *Recent Advances in Fish Farms.* http://archimer. ifremer. fr/doc/00051/16203.: InTech.

KURODA, A., OKAMOTO, N. & FUKUDA, H. 2000. Characterization of monoclonal antibodies against antigens shared with neutrophils and macrophages in rainbow trout *Oncorhynchus mykiss*. *Gyobyo Kenkyu= Fish Pathology,* 35**,** 205-213.

LEONG, T. & WONG, S. 1990. Parasites of healthy and diseased juvenile grouper (*Epinephelus malabaricus* (Bloch and Schneider)) and seabass (*Lates calcarifer* (Bloch)) in floating cages in Penang, Malaysia. *Asian Fisheries Science,* 3**,** 319-327.

LEONG, T.-S. & WONG, S.-Y. 1988. A comparative study of the parasite fauna of wild and cultured grouper (*Epinephelus malabaricus* Bloch et Schneider) in Malaysia. *Aquaculture,* 68**,** 203-207.

LI, J., BARREDA, D. R., ZHANG, Y.-A., BOSHRA, H., GELMAN, A. E., LAPATRA, S., TORT, L. & SUNYER, J. O. 2006. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nature Immunology,* 7**,** 1116-1124.

LIESCHKE, G. J. & TREDE, N. S. 2009. Fish immunology. *Current Biology,* 19**,** R678-R682.

LIN, H. T., LIN, H. Y. & YANG, H. L. 2005. Histology and histochemical enzyme-staining patterns of major immune organs in *Epinephelus malab*aricus. *Journal of Fish Biology,* 66, 729-740.

LLOYD-EVANS, P., BARROW, S. E., HILL, D. J., BOWDEN, L. A., RAINGER, G. E., KNIGHT, J. & ROWLEY, A. F. 1994. Eicosanoid generation and effects on the aggregation of thrombocytes from the rainbow trout, *Oncorhynchus mykiss*. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* 1215**,** 291-299.

LO, L. C. & YUE, G. H. 2008. Microsatellites for broodstock management of the Tiger grouper, *Epinephelus fuscoguttatus*. *Animal Genetics,* 39**,** 90-91.

LOCKEY, R. F., SAMUEL C. BUKANTZ, R. F. L. J. B., BUKANTZ, S. C. & BOUSQUET, J. 1998. *Allergens and Allergen Immunotherapy, Second Edition*, Taylor & Francis.

LUER, C. A., WALSH, C. J. & BODINE, A. B. 2004. The immune system of sharks, skates and rays. *Biology of Sharks and Their Relatives*, 369-395.

MACHIN, G. A. & HALPER, J. P. 1980. Cytochemically demonstrable B-glucuronidase activity in normal and neoplastic human lymphoid cells. *Blood,* 56, 1111-1119.

MAGGIO, T., ANDALORO, F. & ARCULEO, M. 2006. Genetic population structure of *Epinephelus marginatus* (Pisces, Serranidae) revealed by two molecular markers. *Italian Journal of Zoology,* 73**,** 275-283.

MAGNADOTTIR, B. 2010. Immunological control of fish diseases. *Marine Biotechnology,* 12**,** 361-379.

MAGNADÓTTIR, B., LANGE, S., GUDMUNDSDOTTIR, S., BØGWALD, J. & DALMO, R. 2005. Ontogeny of humoral immune parameters in fish. *Fish & Shellfish Immunology,* 19**,** 429-439.

MAINWARING, G. & ROWLEY, A. F. 1985. Separation of leucocytes in the dogfish (*Scyliorhinus canicula*) using density gradient centrifugation and differential adhesion to glass coverslips. *Cell and Tissue Research,* 241, 283-290.

MAKI, J. L. & DICKERSON, H. W. 2003. Systemic and cutaneous mucus antibody responses of channel catfish immunized against the protozoan parasite *Ichthyophthirius multifiliis*. *Clinical and Diagnostic Laboratory Immunology,* 10**,** 876-881.

MALE, D. K., BROSTOFF, J., ROITT, I. M. & ROTH, D. B. 2006. *Immunology*, Missouri: Mosby Elsevier.

MANZ, A., PAMME, N., IOSSIFIDIS, D. & LOSSIFIDIS, D. 2004. *Bioanalytical chemistry*, Singapore: World Scientific.

MATSUMOTO, H., IIJIMA, N. & KAYAMA, M. 1989. The prostaglandin synthesis in marine fish thrombocyte. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 93, 397-402.

MAWDESLEY-THOMAS, L. E., BURRIS, K. W. & KNUCKLES, J. L. 1974. *Diseases of Fish*, New York: Ardent Media.

MAZIA, D., SCHATTEN, G. & SALE, W. 1975. Adhesion of cells to surfaces coated with polylysine. Applications to electron microscopy. *The Journal of cell biology,* 66, 198-200.

MILSTEIN, O., HAGIN, D., LASK, A., REICH-ZELIGER, S., SHEZEN, E., OPHIR, E., EIDELSTEIN, Y., AFIK, R., ANTEBI, Y. E., DUSTIN, M. L. & REISNER, Y. 2011. CTLs respond with activation and granule secretion when serving as targets for T-cell recognition. *Blood,* 117**,** 1042-1052.

MIYADAI, T., OOTANI, M., TAHARA, D., AOKI, M. & SAITOH, K. 2004. Monoclonal antibodies recognising serum immunoglobulins and surface immunoglobulin-positive cells of puffer fish, torafugu (*Takifugu rubripes*). *Fish & Shellfish Immunology,* 17**,** 211-222.

MOKHTAR, M. A., NORMAH, M. N., KUMAR, S. V. & BAHARUM, S. N. 2011. Characterization of 10 novel microsatellite loci for the brown marbled grouper, *Epinephelus fuscoguttatus* (Serranidae). *Genetics and molecular research : GMR,* 10**,** 885-888.

MONTET-ABOU, K., DAIRE, J. L., HYACINTHE, J. N., JORGE-COSTA, M., GROSDEMANGE, K., MACH, F., PETRI-FINK, A., HOFMANN, H.,

MOREL, D. R., VALLÉE, J. P. & MONTET, X. 2010. In vivo labelling of resting monocytes in the reticuloendothelial system with fluorescent iron oxide nanoparticles prior to injury reveals that they are mobilized to infarcted myocardium. *European Heart Journal,* 31, 1410-1420.

MOORE, H. F. 1919. *Groupers: Fishes You Should Try*, Washington, D.C: U.S. Government Printing Office.

MUKAI, Y., LIM, L. S., LU, K. C., RASHID, M. K. A. & SAAD, S. 2012. Light Intensity Requirements for Feeding Behaviour by the Brown-marbled Grouper, *Epinephelus fuscoguttatus*. *Sains Malaysiana,* 41**,** 1193-1196.

MULERO, I., GARCÍA-AYALA, A., MESEGUER, J. & MULERO, V. 2007. Maternal transfer of immunity and ontogeny of autologous immunocompetence of fish: A minireview. *Aquaculture,* 268**,** 244-250.

MUTHS, D. & BOURJEA, J. 2011. Characterization of thirteen new polymorphic microsatellite markers from the honeycomb grouper *Epinephelus merra*. *Conservation Genetics Resources,* 3**,** 629-631.

NAGASAWA, K. & CRUZ-LACIERDA, E. R. 2004. *Diseases of cultured groupers*, Southeast Asian Fisheries Development Center, Aquaculture Department Iloilo, Philippines.

NAKAYASU, C., YOSHITOMI, T., OYAMATSU, T., OKAMOTO, N. & IKEDA, Y. 1997. Separation of carp (*Cyprinus carpio* L.) thrombocytes by using a monoclonal antibody, and their aggregation by collagen. *Veterinary Immunology and Immunopathology,* 57**,** 337-346.

NASH, G., ANDERSON, I. G., SHARIFF, M. & SHAMSUDIN, M. N. 1987. Bacteriosis associated with epizootic in the giant sea perch, *Lates calcarifer*, and the estuarine grouper, *Epinephelus tauvina*, cage cultured in Malaysia. *Aquaculture,* 67**,** 105-111.

NISHIMURA, H., IKEMOTO, M., KAWAI, K. & KUSUDA, R. 1997. Cross-reactivity of anti-yellowtail thymic lymphocyte monoclonal antibody (YeT-2) with lymphocytes from other fish species. *Archives of histology and cytology,* 60**,** 113-119.

NURHIDAYU, A., INA-SALWANY, M., DAUD, H. M. & HARMIN, S. 2012. Isolation, screening and characterization of potential probiotics from farmed tiger grouper (*Epinephelus fuscoguttatus*). *Afr. J. Microbiol. Res,* 6**,** 1924-1933.

OCHOA, A. 2002. *Mechanisms of Tumor Escape from the Immune Response*, Taylor & Francis.

OH, B. S., OH, D. J., JUNG, M. M. & JUNG, Y. H. 2012. Complete mitochondrial genome of the longtooth grouper *Epinephelus bruneus* (Perciformes, Serranidae). *Mitochondrial DNA,* 23**,** 137-138.

OH, M. J., TAKAMI, I., NISHIZAWA, T., KIM, W. S., KIM, C. S., KIM, S. R. & PARK, M. A. 2012. Field tests of Poly(I:C) Immunization with nervous necrosis virus (NNV) in sevenband grouper, *Epinephelus septemfasciatus* (Thunberg). *Journal of Fish Diseases,* 35, 187-191.

ORCHARD, G. & NATION, B. 2011. *Histopathology*, Oxford: OUP Oxford.

OSEKO, N., PALANISAMY, V., KUA, B. & THYE, C. Occurrence of lymphocystis disease in cultured tiger grouper, *Epinephelus fuscoguttatus* in Malaysia. Aquatic Animal Health for Sustainability", Book of Abstracts, OP 40, Fourth Symposium on Diseases in Asian Aquaculture, 1999.

OTHMAN, M. F. 2008. *Challenges Ahead in Meeting Aquaculture Production in Malaysia under the Third National Agrciultural Policy, NAP 3 (1998-2010)* Department of Fisheries, Ministry of Agriculture and Agro-based Industry Malaysia.

ØVERLAND, H. S., PETTERSEN, E. F., RØNNESETH, A. & WERGELAND, H. I. 2010. Phagocytosis by B-cells and neutrophils in Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.). Fish & Shellfish Immunology, 28, 193-204.

OWEN, J. C., CORNELIUS, E., ARSNOE, D. & GARVIN, M. 2013. Leukocyte Response to Eastern Equine Encephalomyelitis Virus in a Wild Passerine Bird. *Avian Diseases,* 57, 744-749.

PARAMESWARAN, V., AHMED, V. P. I., SHUKLA, R., BHONDE, R. R. & HAMEED, A. S. S. 2007. Development and characterization of two new cell lines from milkfish (*Chanos chanos*) and grouper (*Epinephelus coioides*) for virus isolation. *Marine Biotechnology,* 9, 281-291.

PARENRENGI, A., SHAMSUDIN, L., ISMAIL, P. & AMIN, N. M. 2001. A study on DNA polymorphic markers in the grouper, *Epinephelus merra*. *Asia Pac. J. Mol. Biol. Biotechnol.,* 9**,** 52-59.

PASTORET, P. P., GRIEBEL, P., BAZIN, H. & GOVAERTS, A. 1998. *Handbook of Vertebrate Immunology*, Massachusetts: Academic Press.

PATTERSON-DELAFIELD, J., MARTINEZ, R. J. & LEHRER, R. I. 1980. Microbicidal cationic proteins in rabbit alveolar macrophages: a potential host defense mechanism. *Infection and immunity,* 30, 180-192.

PEARS, R. J., CHOAT, J. H., MAPSTONE, B. D. & BEGG, G. A. 2007. Reproductive biology of a large, aggregation-spawning serranid, *Epinephelus fuscoguttatus* (Forsskål): Management implications. *Journal of Fish Biology,* 71**,** 795-817.

PET, J. S., MOUS, P. J., RHODES, K. & GREEN, A. 2006. Introduction to monitoring of spawning aggregations of three grouper species from the Indo-Pacific: A manual for field practitioners.

PETIT-BERTRON, A. F., FITTING, C., CAVAILLON, J. M. & ADIB-CONQUY, M. 2003. Adherence influences monocyte responsiveness to interleukin-10. *Journal of Leukocyte Biology,* 73, 145-154.

PETRIE-HANSON, L. & AINSWORTH, A. J. 2000. Differential cytochemical staining characteristics of channel catfish leukocytes identify cell populations in lymphoid organs. *Veterinary Immunology and Immunopathology,* 73, 129-144.

PIERRE, S., GAILLARD, S., PRÉVOT-D'ALVISE, N., AUBERT, J., ROSTAING-CAPAILLON, O., LEUNG-TACK, D. & GRILLASCA, J.-P. 2008. Grouper aquaculture: Asian success and Mediterranean trials. *Aquatic Conservation: Marine and Freshwater Ecosystems,* 18**,** 297-308.

PIGANELLI, J. D., ZHANG, J. A., CHRISTENSEN, J. M. & KAATTARI, S. L. 1994. Enteric coated microspheres as an oral method for antigen delivery to salmonids. *Fish & Shellfish Immunology,* 4**,** 179-188.

PURCELL, M. K., BROMAGE, E. S., SILVA, J., HANSEN, J. D., BADIL, S. M., WOODSON, J. C. & HERSHBERGER, P. K. 2012. Production and characterization of monoclonal antibodies to IgM of Pacific herring (*Clupea pallasii*). *Fish & Shellfish Immunology,* 33**,** 552-558.

RAMÍREZ, M. A., PATRICIA-ACEVEDO, J., PLANAS, S., CARLIN, J. L., FUNK, S. M. & MCMILLAN, W. O. 2006. New microsatellite resources for groupers (Serranidae). *Molecular Ecology Notes,* 6**,** 813-817.

RANDALL, J. E. & TAYLOR, L. R. 1988. *Review of the Indo-Pacific Fishes of the Serranid Genus Liopropoma: With Descriptions of Seven New Species*, Bernice Pauahi Bishop Museum.

RANDELLI, E., BUONOCORE, F. & SCAPIGLIATI, G. 2008. Cell markers and determinants in fish immunology. *Fish & Shellfish Immunology,* 25**,** 326-340.

RATHORE, G., KUMAR, G., SOOD, N., KAPOOR, D. & LAKRA, W. S. 2008. Development of monoclonal antibodies to rohu (*Labeo rohita*) immunoglobulins for use in immunoassays. *Fish and Shellfish Immunology,* 25**,** 761-774.

REITE, O. B. & EVENSEN, Ø. 2006. Inflammatory cells of teleostean fish: a review focusing on mast cells/eosinophilic granule cells and rodlet cells. *Fish & Shellfish Immunology,* 20**,** 192-208.

RENSHAW, M. A., NEMETH, R. S. & GOLD, J. R. 2011. Isolation and characterization of microsatellite markers from yellowfin grouper, *Mycteroperca venenosa*. *Conservation Genetics Resources,* 3**,** 341-344.

RENSHAW, M. A., NEMETH, R. S. & GOLD, J. R. 2012. Isolation of microsatellite markers from tiger grouper (*Mycteroperca tigris*) and characterization in yellowfin grouper (*Mycteroperca venenosa*), coney (*Cephalopholis fulva*), and red hind (*Epinephelus guttatus*). *Conservation Genetics Resources,* 4**,** 1049-1054.

RENSHAW, S. A. & TREDE, N. S. 2012. A model 450 million years in the making: zebrafish and vertebrate immunity. *Disease models & mechanisms,* 5**,** 38-47.

RHODES, K. L., MCILWAIN, J., JOSEPH, E. & NEMETH, R. S. 2012. Reproductive movement, residency and fisheries vulnerability of brown-marbled grouper, *Epinephelus fuscoguttatus* (Forsskål, 1775). *Coral Reefs,* 31**,** 443-453.

RICHARDSON, L. R. & GOLD, J. R. 1993. Mitochondrial DNA variation in red grouper (*Epinephelus morio*) and greater amberjack (*Seriola dumerili*) from the Gulf of Mexico. *ICES Journal of Marine Science,* 50**,** 53-62.

RICHARDSON, L. R. & GOLD, J. R. 1997. Mitochondrial DNA diversity in and population structure of red grouper, *Epinephelus morio*, from the Gulf of Mexico. *Fishery Bulletin,* 95**,** 174-179.

RIEGER, A. M. & BARREDA, D. R. 2011. Antimicrobial mechanisms of fish leukocytes. *Developmental & Comparative Immunology,* 35**,** 1238-1245.

RISTOW, S., EVANS, D. L. & JASO-FRIEDMANN, L. 2000. Analyzing nonspecific cytotoxic cells in fish. *Natural Killer Cell Protocols.* Springer.

RIVERA, M. A. J., GRAHAM, G. C. & RODERICK, G. K. 2003. Isolation and characterization of nine microsatellite loci from the Hawaiian grouper *Epinephelus quernus* (Serranidae) for population genetic analyses. *Marine Biotechnology,* 5**,** 126-129.

ROBINSON, J., AUMEERUDDY, R., JORGENSEN, T. L. & OHMAN, M. C. 2008. Dynamics of camouflage (*Epinephelus polyphekadion*) and brown marbled grouper (*Epinephelus fuscoguttatus*) spawning aggregations at a remote reef site, Seychelles. *Bulletin of Marine Science,* 83**,** 415-431.

RODRIGUES, K. F., SHIGEHARU, S. & CH'NG, C. L. 2011. Microsatellite markers for the identification of commercially important groupers *Epinephelus lanceolatus*, *Cromileptes altivelis* and *Epinephelus fuscoguttatus*. *Pertanika Journal of Tropical Agricultural Science,* 34**,** 311-315.

ROMANO, N., PICCHIETTI, S., TAVERNE-THIELE, J., TAVERNE, N., ABELLI, L., MASTROLIA, L., VERBURG-VAN KEMENADE, B. & ROMBOUT, J. 1998. Distribution of macrophages during fish development: an immunohistochemical study in carp (*Cyprinus carpio*, L.). *Anatomy and embryology,* 198**,** 31-41.

ROMBOUT, J. & BERG, A. 1989. Immunological importance of the second gut segment of carp. I. Uptake and processing of antigens by epithelial cells and macrophages. *Journal of Fish Biology,* 35**,** 13-22.

ROMBOUT, J., JOOSTEN, P., ENGELSMA, M., VOS, A., TAVERNE, N. & TAVERNE-THIELE, J. 1998. Indications for a distinct putative T cell population in mucosal tissue of carp (*Cyprinus carpio* L.). *Developmental & Comparative Immunology,* 22**,** 63-77.

ROMERO, P. 2002. An etymological dictionary of taxonomy. Madrid. Unpublished.

RØNNESETH, A., WERGELAND, H. I. & PETTERSEN, E. F. 2007. Neutrophils and B-cells in Atlantic cod (*Gadus morhua* L.). *Fish & Shellfish Immunology,* 23, 493-503.

ROWLEY, A. F. 1996. The evolution of inflammatory mediators. *Mediators of inflammation,* 5**,** 3-13.

SABNIS, R. W. 2010. *Handbook of Biological Dyes and Stains: Synthesis and Industrial Applications*, New Jersey: Wiley.

SADOVY DE MITCHESON, Y., CRAIG, M. T., BERTONCINI, A. A., CARPENTER, K. E., CHEUNG, W. W. L., CHOAT, J. H., CORNISH, A. S., FENNESSY, S. T., FERREIRA, B. P., HEEMSTRA, P. C., LIU, M., MYERS, R. F., POLLARD, D. A., RHODES, K. L., ROCHA, L. A., RUSSELL, B. C., SAMOILYS, M. A. & SANCIANGCO, J. 2013. Fishing groupers towards extinction: A global assessment of threats and extinction risks in a billion dollar fishery. *Fish and Fisheries,* 14**,** 119-136.

SADOVY, Y., EKLUND, A. M. & OFFICE, U. S. N. M. F. S. S. P. 1999. *Synopsis of Biological Data on the Nassau Grouper, Epinephelus* Striatus (Bloch, 1792), and the Jewfish, *E. Itajara (Lichtenstein, 1822)*, U.S. Department of Commerce, National Oceanic and

Atmospheric Administration, National Marine Fisheries Service, Scientific Publications Office.
SALINAS, I., MESEGUER, J. & ESTEBAN, M. Á. 2007. Assessment of different protocols for the isolation and purification of gut associated lymphoid cells from the gilthead seabream (*Sparus aurata* L.). *Biological Procedures Online,* 9**,** 43-55.
SALINAS, I., ZHANG, Y.-A. & SUNYER, J. O. 2011. Mucosal immunoglobulins and B cells of teleost fish. *Developmental & Comparative Immunology,* 35**,** 1346-1365.
SÁNCHEZ, C., LOPEZ-FIERRO, P., ZAPATA, A. & DOMINGUEZ, J. 1993. Characterisation of monoclonal antibodies against heavy and light chains of trout immunoglobulin. *Fish & Shellfish Immunology,* 3**,** 237-251.
SANTARÉM, M. & FIGUERAS, A. 1995. Leucocyte numbers and phagocytic activity in turbot *Scophthalmus maximus* following immunization with *Vibrio damsela* and *Pasteurella piscicida* O-antigen bacterins. *Diseases of Aquatic Organisms,* 23, 213-220.
SARGEANT, F. 1996. *The Reef Fishing Book: A Complete Anglers Guide*, Florida: Larsen's Outdoor Pub.
SASAKI, Y., MAITA, M. & OKAMOTO, N. 2002. Rainbow trout neutrophils are responsible for non-specific cytotoxicity. *Fish & Shellfish Immunology,* 12**,** 243-252.
SATCHELL, G. H. 1991. *Physiology and Form of Fish Circulation*, Cambridge: Cambridge University Press.
SCAPIGLIATI, G. 2013. Functional aspects of fish lymphocytes. *Developmental & Comparative Immunology,* 41**,** 200-208.
SCAPIGLIATI, G., FOCHETTI, R., TIBERI, M. & MAZZINI, M. 2006. Morphological and flow cytometric characterization of leukocytes from the notothenioid teleosts *Dissostichus eleginoides*, *Notothenia coriiceps*, and *Trematomus hansoni*. *Polar Biology,* 29, 872-877.
SCAPIGLIATI, G., MAZZINI, M., MASTROLIA, L., ROMANO, N. & ABELLI, L. 1995. Production and characterisation of a monoclonal antibody against the thymocytes of the sea bass *Dicentrarchus labrax* (L.) (Teleostea, Percicthydae). *Fish & Shellfish Immunology,* 5, 393-405.
SCAPIGLIATI, G., ROMANO, N., PICCHIETTI, S., MAZZINI, M., MASTROLIA, L., SCALIA, D. & ABELLI, L. 1996. Monoclonal antibodies against sea bass *Dicentrarchus labrax* (L.) immunoglobulins: immunolocalisation of immunoglobulin-bearing cells and applicability in immunoassays. *Fish & Shellfish Immunology,* 6**,** 383-401.
SECOMBES, C. J., VAN GRONINGEN, J. J., VAN MUISWINKEL, W. B. & EGBERTS, E. 1983. Ontogeny of the immune system in carp (*Cyprinus carpio* L.) the appearance of antigenic determinants on lymphoid cells detected by mouse anti-carp thymocyte monoclonal antibodies. *Developmental & Comparative Immunology,* 7**,** 455-464.
SEPULCRE, M., PELEGRÍN, P., MULERO, V. & MESEGUER, J. 2002. Characterisation of gilthead seabream acidophilic granulocytes by

a monoclonal antibody unequivocally points to their involvement in fish phagocytic response. *Cell and Tissue Research,* 308**,** 97-102.
SHEN, L., STUGE, T. B., ZHOU, H., KHAYAT, M., BARKER, K. S., QUINIOU, S., WILSON, M., BENGTÉN, E., CHINCHAR, V. G. & CLEM, L. W. 2002. Channel catfish cytotoxic cells: a mini-review. *Developmental & Comparative Immunology,* 26**,** 141-149.
SHEPRO, D., BELAMARICH, F., MERK, F. & CHAO, F. 1969. Changes in thrombocyte ultrastructure during clot retraction. *Journal of Cell Science,* 4**,** 763-779.
SHIGDAR, S., HARFORD, A. & WARD, A. C. 2009. Cytochemical characterisation of the leucocytes and thrombocytes from Murray cod (*Maccullochella peelii peelii*, Mitchell). *Fish & Shellfish Immunology,* 26, 731-736.
SHIMA, S., MATSUOKA, H., IWAMOTO, T. & SAKAI, H. 1984. Antimicrobial action of epsilon-poly-L-lysine. *The Journal of antibiotics,* 37, 1449.
SIMA, P. & VETVICKA, V. 1990. *Evolution of Immune Reactions*, United Kingdom: Taylor & Francis.
SIVARAM, V., BABU, M. M., IMMANUEL, G., MURUGADASS, S., CITARASU, T. & MARIAN, M. P. 2004. Growth and immune response of juvenile greasy groupers (*Epinephelus tauvina*) fed with herbal antibacterial active principle supplemented diets against Vibrio harveyi infections. *Aquaculture,* 237, 9-20.
SLIERENDRECHT, W., LORENZEN, N., GLAMANN, J., KOCH, C. & ROMBOUT, J. 1995. Immunocytochemical analysis of a monoclonal antibody specific for rainbow trout (*Oncorhynchus mykiss*) granulocytes and thrombocytes. *Veterinary Immunology and Immunopathology,* 46**,** 349-360.
SOOD, N., CHAUDHARY, D. K., RATHORE, G., SINGH, A. & LAKRA, W. 2011. Monoclonal antibodies to snakehead, *Channa striata* immunoglobulins: Detection and quantification of immunoglobulin-positive cells in blood and lymphoid organs. *Fish & Shellfish Immunology,* 30**,** 569-575.
SOOD, N., CHAUDHARY, D. K., SINGH, A. & RATHORE, G. 2012. Monoclonal antibody to serum immunoglobulins of *Clarias batrachus* and its application in immunoassays. *Gene,* 511**,** 411-419.
SOOD, R. 2006. *Textbook of medical laboratory technology*, New Delhi: Jaypee Brothers Medical Publishers.
STAFFORD, J. L., MCLAUCHLAN, P. E., SECOMBES, C. J., ELLIS, A. E. & BELOSEVIC, M. 2001. Generation of primary monocyte-like cultures from rainbow trout head kidney leukocytes. *Developmental and Comparative Immunology,* 25, 447-459.
STENVIK, J. & JØRGENSEN, T. Ø. 2000. Immunoglobulin D (IgD) of Atlantic cod has a unique structure. *Immunogenetics,* 51**,** 452-461.
STOSIK, M., DEPTUIA, W., TRAVNICEK, M. & BALDY-CHUDZIK, K. 2001. Phagocytic and bactericidal activity of blood thrombocytes in carps (*Cyprinus carpio*). *VETERINARNI MEDICINA-PRAHA-,* 47**,** 21-25.
STOSIK, M., DEPTULA, W., WIKTOROWICZ, K., TRAVNICEK, M. & BALDY-CHUDZIK, K. 2001. Qualitative and quantitative cytometric

analysis of peripheral blood leukocytes in carps (*Cyprinus carpio*). *VETERINARNI MEDICINA-PRAHA-,* 46, 149-152.
STRASBURG, D. W. & SCHULTZ, L. P. 1953. *Th*e Blenniid Fish Genera Cirripectus and Exallias with Description of Two New Species from the Tropical Pacific. Journal of the Washington Academy of Sciences, 43(4), 128-136.
STYCZYNSKA-JUREWICZ, E. & SOCIETY, E. M. 1979. *Cultivation of Fish Fry and Its Live Food*, Belgium: European Mariculture Society.
SUGAMA, K., RIMMER, M. A., ISMI, S., KOESHARYANI, I., SUWIRYA, K., GIRI, N. A., ALAVA, V. R. & RESEARCH, A. C. F. I. A. 2012. *Hatchery Management of Tiger Grouper (Epinephelus Fuscoguttatus): A Best-practice Manual*, Canberra: Australian Centre for International Agricultural Research.
SUJATHA, K., ISWARYA DEEPTI, V. A. & SHRIKANYA, K. V. L. 2011. Allozyme electrophoretic studies in four species of groupers (Pisces: Serranidae) represented in the commercial fishery of Visakhapatnam - India. *Indian Journal of Marine Sciences,* 40**,** 365-371.
SWAIN, P. & NAYAK, S. 2009. Role of maternally derived immunity in fish. *Fish & Shellfish Immunology,* 27**,** 89-99.
TANSEY, E. & CATTERALL, P. 1994. Monoclonal antibodies: a witness seminar in contemporary medical history. *Medical history,* 38**,** 322.
TAVARES-DIAS, M. & BARCELLOS, J. F. M. 2005. Peripheral blood cells of the armored catfish *Hoplosternum littorale* Hancock, 1828: a morphological and cytochemical study. *Brazilian Journal of Morphological Sciences,* 22, 215-220.
TAVARES-DIAS, M. & MORAES, F. R. D. 2007. Leukocyte and thrombocyte reference values for channel catfish (*Ictalurus punctatus* Raf), with an assessment of morphologic, cytochemical, and ultrastructural features. *Veterinary Clinical Pathology,* 36, 49-54.
TAVARES-DIAS, M. & OLIVEIRA, S. R. 2009. A review of the blood coagulation system of fish. *Revista Brasileira de Biociências,* 7.
THRALL, M. A., WEISER, G., ALLISON, R. & CAMPBELL, T. W. 2012. *Veterinary Hematology and Clinical Chemistry*, New Jersey: Wiley.
THUVANDER, A., FOSSUM, C. & LORENZEN, N. 1990. Monoclonal antibodies to salmonid immunoglobulin: characterization and applicability in immunoassays. *Developmental & Comparative Immunology,* 14**,** 415-423.
TODD, I. & SPICKETT, G. 2011. *Lecture Notes: Immunology*, New Jersey: Wiley.
TOMANA, M., PARTON, A. & BARNES, D. W. 2008. An improved method for separation of leucocytes from peripheral blood of the little skate (*Leucoraja erinacea*). *Fish and Shellfish Immunology,* 25, 188-190.
TRIPATHI, N. K., LATIMER, K. S. & BURNLEY, V. V. 2004. Hematologic reference intervals for koi (*Cyprinus carpio*), including blood cell morphology, cytochemistry, and ultrastructure. *Veterinary Clinical Pathology,* 33, 74-83.
TUMBOL, R. A., BAIANO, J. C. F. & BARNES, A. C. 2009. Differing cell population structure reflects differing activity of Percoll-separated

pronephros and peritoneal leucocytes from barramundi (*Lates calcarifer*). *Aquaculture,* 292, 180-188.
TUPPER, M. & SHERIFF, N. 2008. Capture-based aquaculture of groupers. *Capture-Based Aquaculture. Global Overview*, 217-253.
UEDA, I. K., EGAMI, M. I., SASSO, W. D. S. & MATUSHIMA, E. R. 2001. Cytochemical aspects of the peripheral blood cells of *Oreochromis* (Tilapia) *niloticus*.(Linnaeus, 1758)(Cichlidae, Teleostei)-Part II. *Braz J Vet Res Anim Sci,* 38, 273-277.
UNSWORTH, R. F., POWELL, A., HUKOM, F. & SMITH, D. 2007. The ecology of Indo-Pacific grouper (Serranidae) species and the effects of a small scale no take area on grouper assemblage, abundance and size frequency distribution. *Marine Biology,* 152, 243-254.
UPADHYAY, S. K., JUN, W., SU, Y. Q., DING, S. X. & CHATURVEDI, S. 2006. Genetic diversity of yellow grouper (*Epinephelus awoara*) determined by random amplified polymorphic DNA (RAPD) analysis. *Fishery Bulletin,* 104, 638-642.
URIBE, C., FOLCH, H., ENRIQUEZ, R. & MORAN, G. 2011. Innate and adaptive immunity in teleost fish: a review. *Vet Med,* 56, 486-503.
VAN DER HEIJDEN, M., ROOIJAKKERS, J., BOOMS, G., ROMBOUT, J. & BOON, J. 1995. Production, characterisation and applicability of monoclonal antibodies to European eel (*Anguilla anguilla* L., 1758) immunoglobulin. *Veterinary Immunology and Immunopathology,* 45, 151-164.
VAN RIJT, L. S., KUIPERS, H., VOS, N., HIJDRA, D., HOOGSTEDEN, H. C. & LAMBRECHT, B. N. 2004. A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma. *Journal of Immunological Methods,* 288, 111-121.
VERBURG-VAN KEMENADE, B. M. L., GROENEVELD, A., VAN RENS, B. T. T. M. & ROMBOUT, J. H. W. M. 1994. Characterization of macrophages and neutrophilic granulocytes from the pronephros of carp (*Cyprinus carpio*). *J Exp Biol,* 187, 143-158.
WALSH, C. J. & LUER, C. A. 2004. Elasmobranch hematology: Identification of cell types and practical applications. *Elasmobranch Husbandry Manual: Captive Care of Sharks, Rays, and Their Relatives*, 307-323.
WANG, B., ZHANG, Z., WANG, Y., ZOU, Z., WANG, G., WANG, S., JIA, X. & LIN, P. 2009. Molecular cloning and characterization of macrophage migration inhibitory factor from small abalone *Haliotis diversicolor supertexta*. *Fish & Shellfish Immunology,* 27, 57-64.
WANG, L., MENG, Z., LIU, X., ZHANG, Y. & LIN, H. 2011. Genetic diversity and differentiation of the orange-spotted grouper (*Epinephelus coioides*) between and within cultured stocks and wild populations inferred from microsatellite DNA analysis. *International Journal of Molecular Sciences,* 12, 4378-4394.
WANG, S., DU, J., WANG, J. & DING, S. 2007. Identification of *Epinephelus malabaricus* and *Epinephelus coioides* using DNA markers. *Acta Oceanologica Sinica,* 26, 122-129.

WATANABE, M., SHIMIZU, T., KAMARUDIN, A. S. B., KUNIYOSHI, H., OHARA, K., TAKAGI, M. & UMINO, T. 2011. Ten novel polymorphic microsatellite loci of Red-spotted grouper (*Epinephelus akaara*) revealed from full-sib progeny and unrelated individuals. *Conservation Genetics Resources,* 3**,** 613-616.

WEBER, K., YORK, M. R., SPRINGER, T. A. & KLICKSTEIN, L. B. 1997. Characterization of lymphocyte function-associated antigen 1 (LFA-1)-deficient T cell lines: the alphaL and beta2 subunits are interdependent for cell surface expression. *The Journal of Immunology,* 158**,** 273-279.

WEBSTER, G. A., BOWLES, M. J., KARIM, M. S., WOOD, R. F. M. & POCKLEY, A. G. 1995. Flow cytometric analysis of peripheral blood lymphocyte subset light scatter characteristics as a means of monitoring the development of rat small bowel allograft rejection. *Clinical and Experimental Immunology,* 100, 536-542.

WHYTE, S. K. 2007. The innate immune response of finfish–a review of current knowledge. *Fish & Shellfish Immunology,* 23**,** 1127-1151.

WILSON, M., BENGTÉN, E., MILLER, N. W., CLEM, L. W., DU PASQUIER, L. & WARR, G. W. 1997. A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. *Proceedings of the National Academy of Sciences of the United States of America,* 94**,** 4593-4597.

WITESKA, M. & WARGOCKA, W. 2011. Disodium EDTA used as anticoagulant causes hemolysis in common carp blood. *Turk. J. Vet. Anim. Sci,* 35, 99-104.

WOLF, K. 1959. Plasmoptysis and gelation of erythrocytes in coagulation of blood of freshwater bony fishes. *Blood,* 14, 1339-1344.

WYSOCKI, L. & SATO, V. 1978. “Panning" for lymphocytes: a method for cell selection. *Proceedings of the National Academy of Sciences,* 75**,** 2844-2848.

XU, Z., CHEN, C. F., MAO, Z. J. & ZHU, W. Y. 2009. Detection of serum and mucosal antibody production and antibody secreting cells (ASCs) in large yellow croaker (*Pseudosciaena crocea*) following vaccination with Vibrio harveyi via different routes. *Aquaculture,* 287**,** 243-247.

YANG, S., WANG, L., ZHANG, Y., LIU, X. C., LIN, H. R. & MENG, Z. N. 2011. Development and characterization of 32 microsatellite loci in the giant grouper *Epinephelus lanceolatus* (Serranidae). *Genetics and molecular research : GMR,* 10**,** 4006-4011.

ZATCOFF, M. S., BALL, A. O. & CHAPMAN, R. W. 2002. Characterization of polymorphic microsatellite loci from black grouper, *Mycteroperca bonaci* (Teleostei: Serranidae). *Molecular Ecology Notes,* 2**,** 217-219.

ZATCOFF, M. S., BALL, A. O. & SEDBERRY, G. R. 2004. Population genetic analysis of red grouper, *Epinephelus morio*, and scamp, *Mycteroperca phenax*, from the southeastern U.S. Atlantic and Gulf of Mexico. *Marine Biology,* 144, 769-777.

ZENG, H. S., DING, S. X., WANG, J. & SU, Y. Q. 2008. Characterization of eight polymorphic microsatellite loci for the giant grouper

(*Epinephelus lanceolatus* Bloch). *Molecular Ecology Resources,* 8**,** 805-807.

ZHANG, Y.-A., SALINAS, I., LI, J., PARRA, D., BJORK, S., XU, Z., LAPATRA, S. E., BARTHOLOMEW, J. & SUNYER, J. O. 2010. IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nature Immunology,* 11**,** 827-835.

ZHAO, L., SHAO, C., LIAO, X. & CHEN, S. 2009a. Isolation and characterization of polymorphic microsatellite loci from a dinucleotide-enriched genomic library of seven-band grouper (*Epinephelus septemfasciatus*) and cross-species amplification. *Conservation Genetics,* 10**,** 627-629.

ZHAO, L., SHAO, C., LIAO, X., MA, H., ZHU, X. & CHEN, S. 2009b. Twelve novel polymorphic microsatellite loci for the Yellow grouper (*Epinephelus awoara*) and cross-species amplifications. *Conservation Genetics,* 10**,** 743-745.

ZHOU, G. Z., LI, Z. Q., YUAN, X. P. & ZHANG, Q. Y. 2007. Establishment, characterization, and virus susceptibility of a new marine cell line from red spotted grouper (*Epinephelus akaara*). *Marine Biotechnology,* 9, 370-376.

ZHU, Z. Y. & YUE, G. H. 2008. The complete mitochondrial genome of red grouper *Plectropomus leopardus* and its applications in identification of grouper species. *Aquaculture,* 276**,** 44-49.

ZHU, Z. Y., LO, L. C., LIN, G., XU, Y. X. & YUE, G. H. 2005. Isolation and characterization of polymorphic microsatellites from red coral grouper (*Plectropomus maculatus*). *Molecular Ecology Notes,* 5**,** 579-581.

ZINKL, J., COX, W. & KONO, C. S. 1991. Morphology and cytochemistry of leucocytes and thrombocytes of six species of fish. *Comparative Haematology International,* 1, 187-195.