Influence of moderate hypoxia on vaccine efficacy in Nile tilapia Oreochromis niloticus against Vibrio anguillarum and Streptococcus agalactiae

Doctoral Dissertation

INFLUENCE OF MODERATE HYPOXIA ON VACCINE EFFICACY IN NILE TILAPIA *Oreochromis niloticus* AGAINST *Vibrio anguillarum* AND *Streptococcus agalactiae.*

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This dissertation submitted to the Graduate School of Marine Science and Technology at Tokyo University of Marine Science and Technology as a partial fulfilment of the Degree of Doctor of Philosophy in Marine Sciences.

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Declaration

I certify that this thesis consists of with my original work that has not published previously by another person. In addition, I declare that materials used in this thesis were not submitted to any other degree or diploma under my name in this university or other institution.

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II. List of Tables

III. List of Figures

IV. Index

2.3. Results

3.3. Results

Abstract

Hypoxia is known as key factor which promotes disease outbreaks in fish by modulating their immunity. A considerable number of scientific studies have examined hypoxia-mediated, compromised immunity and increased susceptibility to infectious diseases in fish. However, until now, no studies have investigated the effects of hypoxia or moderate hypoxia on fish vaccine efficacy. This could likely be an important limiting factor in our ability to predict vaccine efficacy. Therefore, this study carried out with the purpose of revealing the influence of moderate hypoxia on vaccine efficacy in Nile tilapia. To accomplish this goal, immunological basis of protection offered via vaccination and influence of moderate hypoxia (55 ±5% dissolved oxygen saturation) on those mechanisms were examined in Nile tilapia following vaccination with formalin inactivated *Vibrio anguillarum* and *Streptococcus agalactiae.*

In the first part of this research, vaccine efficacy against *Vibrio anguillarum* was examined using serum antibody titer as surrogate marker. In addition, several haematological and immunological parameters were also examined. The fish were acclimatized either to moderate hypoxic or normoxic (85±5%DO saturation) conditions for 2 weeks and immunized with formalin inactivated *V. anguillarum* ($5x10^9$ CFU/ml). When Nile tilapia raised and vaccinated under normoxic condition, serum antibody titer was significantly higher than that of moderate hypoxic fish at all the detected time points. The peaked antibody titer in normoxic group was observed at 14^{th} dpv (days post vaccination) while moderate hypoxic group rose to a peak at 21^{st} or 28^{th} dpv indicating that not only suppressed but also delayed antibody response under moderate hypoxic condition. In addition, absolute lymphocyte count in blood was also significantly lowered in moderate hypoxic group compared to normoxic group at 14^{th} dpv. Serum bactericidal activities were measured and it was found to be significantly higher in normoxic

group compared with moderate hypoxic group at 7th and 14th dpv. Serum killing of *V. anguillarum* appear to be mainly via antibody dependent classical complement pathway. Following vaccination, fish were transferred between normoxic and moderate hypoxic groups at 0 hour or 7th dpv in order to examined the importance of first week of vaccination on antibody production in fish and results revealed that even though continuous supply of higher oxygen is necessary to gain maximum antibody response, first week following vaccination can be considered as critical where important immune regulatory pathways are activated. This view was further supported by results obtained from gene expression experiments where transcription level of all the detected immune related genes in spleen (IgM, IL-1β, TCR-β, MHC-IIβ) except B cell activating factor were significantly lowered following exposure to moderate hypoxia during first week post vaccination. Plasma chemistry analytes and electrolytes were shown non-significant variation between normoxic and moderate hypoxic groups throughout the study. In contrast, packed cell volume exhibited significant alteration attributed to hypoxia indicating that Nile tilapia demonstrating an adaptive response toward moderate hypoxia. Overall this study explained that moderate hypoxia negatively affects on vaccine efficacy in vaccinated Nile tilapia by lowering antibody production, serum killing and immune related gene expression. Furthermore, studies carried out to find the possible counter measure to enhance the vaccine efficacy in moderate hypoxic fish revealed that booster vaccination might be useful where no difference was found in antibody titer and serum bactericidal activities between normoxic and moderate hypoxic fish. However, it should be noteworthy that antibody mediated other defensive mechanisms that we didn't study here may not be recovered as antibody titer hence those should be subjected for further studies.

The second part of this study was conducted in order to understand the pathogen clearance mechanisms in vaccinated Nile tilapia following experimental challenge with *S. agalactiae* and influence of moderate hypoxia on those mechanisms. At first, fish were acclimated to moderate hypoxic or normoxic conditions and vaccinated with formalin inactivated *S. agalactiae* pellet ($5x10^{10}$ CFU/ml) via intra-peritoneal (IP) injection. Antibody titer was measured at 0, 7^{th}, 15^{th} and 30^{th} day post vaccination (dpv) and serum bactericidal activities and serum lysozyme activities were also detected. At 30^{th} dpv, fish were challenge with live *S. agalactiae* ($1.3x10^{7}$ CFU/fish) via IP injection and mortalities were recorded daily. The tissue samples and blood collected at 1^{st}, 3^{rd}, 5^{th}, 7^{th} and 15^{th} day post challenge were analysed for viable bacteria count. In addition, several *In vitro* studies carried out with head kidney leukocytes (HKLs) to reveal dissolved oxygen (DO) dependency and antibody dependency of their cellular functions. Serum antibody titer was significantly higher in normoxic vaccinated group compared to the moderate hypoxic vaccinated group at 15^{th} and 30^{th} dpv. *S. agalactiae* appear to be resistance for serum killing even when presence of specific antibodies. The cumulative mortality in vaccinated normoxic fish were significantly lower (5.5%) compared to the moderate hypoxic vaccinated fish (20%) and control groups reflecting pre challenge antibody titer is correspondence with protection against *S. agalactiae*. Lowest cumulative mortality among control groups were found to be in normoxic control fish (45.4%) while highest was in moderate hypoxic fish (74.5%) indicating DO dependency of the pathogen clearance in these fish. Highest pathogen burden was found to be in moderate hypoxic control fish and lowest was detected in normoxic vaccinated fish at all the detected time points in brain, head kidney and blood. Furthermore, blood of normoxic vaccinated fish was free from *S. agalactiae* at all detected time point while moderate hypoxic fish took more than 5 days for total clearance of pathogen in their blood. Pathogen burden in tissues and blood appear to be directly correlate with survival and rapid clearance of bacteria in blood

seems to be important for the survival of the fish. Highest bacteria burden observed in moderate hypoxic control group might link with the compromised innate immune clearance mechanisms under moderate hypoxic condition. This idea was confirmed by the results obtained *in vitro* from studies where phagocytosis and intracellular reactive oxygen species (ROS) production exhibited oxygen dependent variations. Furthermore, presence of specific antibodies in the opsonising serum and the amount of antibodies increased the phagocytosis, ROS production and lowered intracellular survival of *S. agalactiae* in the HKLs. Therefore, it is clear that higher cumulative mortality in moderate hypoxic fish even after vaccination not only linked to lower antibody production but also to the lowered phagocytes function under moderate hypoxic conditions. Overall this study highlighted that mechanisms of vaccine protection against *S. agalactiae* mainly via antibody dependent phagocytic pathways and efficacy of this mechanism depends upon optimum DO and amount of specific antibodies presence in the serum.

In conclusion, large body of experimental evidences in current study emphasized that moderate hypoxia negatively affects on antibody production and other immune mechanisms in vaccinated fish those that involve in pathogen clearance hence lower the vaccine efficacy.

CHAPTER 1

General introduction

1.0 General introduction

1.1 Importance of aquaculture

Today, millions of people in our planet suffering from malnutrition especially with protein deficiencies which is identified as main cause for child mortality in developing countries [1]. On the other hand, human population is keep growing day by day and expected to grow over 9 billion by 2050 [2]. To feed this huge human population and to mitigate existing malnutrition problems we must find the way to increase the global food production while protecting our natural resources in land and hydrosphere for the future generation. At this point, fisheries and aquaculture identified as one of the most appalling solutions to provide high quality protein and to improve human health in our planet for today and tomorrow.

However most of our wild fisheries or capture based fisheries known to reached its maximum level where very low potential to increase the fish production via this sector [3]. Therefore, wild fisheries seem to be unable to keep up with growing demand for fish and related products. In contrast, during last 50 years, aquaculture has undergone huge expansion and growth hence it is appearing to reach to the level of capture fisheries production in near future [3]. Expanded aquaculture production in the world have provided high quality low cost proteins, employments to the poorer and lift up living status and serve as main income source for many developing nations. With this huge expansions of aquaculture, today 2.9 billion people fulfil 20 percent of their animal protein requirement via fish and related products [3]

1.2 Constrains for further development of aquaculture industry

To keep up with ever growing demand, many aquaculture operations around the world tend to intensify their production systems. Those intensive aquaculture operations characterised with higher stocking density, poor water qualities, excess use of food, disinfectants, antimicrobial drugs and chemical etc. [4]. Even though the productivity is higher in intensive farms, frequently occurring infectious disease outbreaks were identified as major limiting factor for its sustainability. The infectious disease organisms such as virus, bacteria, fungi, parasites those that appear to be less problematic in wild fish populations became huge problem under farming conditions due to stressful environments in farms [5].

On the other hand, high stocking densities often increase host contacts rate hence infectious organisms can spread across the farms with no time. These conditions appear to be perfect for the many opportunistic pathogens hence today many aquaculture operations are frequently experiencing series of infectious disease outbreaks annually. The annual economic loss caused by disease in aquaculture sector was estimated to as billions of U.S. dollar [6]. Today success of the many aquaculture practises totally or partially depends upon prevention or control of infectious diseases. Therefore, farmers all around the world utilising enormous amount of antibiotics, pesticides, disinfectants and other chemicals to prevent infectious diseases and stay profitable in the future [4]. Antibiotics usage alone created huge damage to the environment as well as became one of the biggest human health concern due to development of antibiotics resistance bacteria strains in many aquaculture facilities [7]. Recent research founding's confirmed the possibilities of antibiotic resistance gene transfer events between fish pathogenic bacteria and human isolates and these findings stressed that antibiotics are adverse ecological as well as public health hazard

[8, 9]. Today, there are enormous amount of chemicals and biological pollutant out there in our aquatic environment yet we have no idea about how big the issue they created on food safety as well as on human health. Antibiotics-independent counter measures against infectious diseases in aquatic animals should be advanced to develop and spread.

1.3 Vaccines in aquaculture industry

Health of our planet is our own health hence following recognition of the damage we are doing through the use of antibiotics and drugs in our aquaculture operations, scientist tend to find alternative methods. As a result, during last few decades' vaccines and other immune stimulants became important role players and they were proved as potential alternatives to reduce infectious disease burden in aquaculture industry. Therefore, we can keep antibiotic usage at minimum level in the industry. Due to their environmental friendly nature, vaccines appeared as most important prophylactic measure we have today to combat infectious diseases, especially for bacterial diseases [10]. Even though fish vaccines are still in primitive stage compared to the human vaccines, they have shown potential to turn our aquaculture operations in to responsible and sustainable industry in the future. Enormous dedication of fish biologist together with industrial partners on vaccine development in aquaculture industry brings its to the state where some of the aquaculture operations such as Norwegian aquaculture able to reduce its antibiotic usage from 47 tonnes to around 1 tonne annually [11, 12]. This numbers will provide silent answer to the question that how important the fish vaccine in today's aquaculture industry. All most all salmon farms giving vaccines to their small fish before they transfer from fresh water to salt water rearing systems [13]. Today there are many commercial vaccines available as

successful prophylactic for many devastating infectious diseases in fin fish aquaculture industry such as for vibriosis, furunculosis, and streptococcosis [10].

1.4 Vibriosis caused by *Vibrio anguillarum*

Vibriosis caused by *Vibrio anguillarum* is known as considerable threat in intensive farming of commercially important fish species such as rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*), turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax),* winter flounder (*Pseudopleuronectes americanus*), Japanese Eel (*Anguilla japonica),* Channel cat fish, *(Ictalurus punctatus),* Ayu (*Plecoglossus altivelis*), Salmon (*Salmo salar)* etc. [14, 15]. *V. anguillarum* is gram negative, flagellated, small curve shape bacterium grows on nutrient medium supplemented with 1-2% NaCl and form round, entire, raised yellow colour colonies on TCBS agar [16]. Wider geographical distribution and wider host range make it difficult to control this pathogen in farms specially the open water aquaculture farms hence frequently cause server disease outbreaks and considerable economic loss. Poor water qualities such high water temperature and high salinity reported as favourable conditions for disease out brakes especially during late summer [15, 17, 18]. The main clinical signs associated with Vibriosis has been identified as red patches or petechial haemorrhages on ventral side of the body, darkening skin, skin ulcerations, anorexia, reddish margins around mouth and base of the fins etc. [18, 19]. Production of cytolytic toxins called haemolysin identified as one of the main virulence factors of *V. anguillarum* [20]. In addition, efficient iron sequestering system, haemagglutination activity and ability to resist serum bactericidal activities identified as possible virulence factors of this pathogen [18, 21]. Commercial vaccines have developed, available in farm level and confirmed that they are able to provide considerable protection. The protection offered via vaccination is confirmed to be based on optimum antibody level in the serum. [22, 23]

1.5 Streptococcosis caused by *Streptococcus agalactiae*

Streptococcosis caused by *S. agalactiae* considered as one of the deadly bacterial disease in tilapia aquaculture around the world which causes mass mortalities even up to 100% in some farms [24]. *S. agalactiae* is gram positive, beta haemolytic, encapsulated, catalase negative, non-motile cocci usually exist as small chains [25]. This bacterium is the main cause of meningoencephalitis in tilapia and have been isolated from more than 10 fish species all-around the world such as rainbow trout, mullet *(Mugil cephalus*), giant Queensland grouper *(Epinephelus lanceolatus),* trout, seabream (*Sparus aurata*), yellowtail, cat fish, croaker (*Micropogonias undulatus*), killifish (*Nothobranchius rachovii*) [24, 25, 26, 27]. Not only fish but also several mammalians known to infect with this bacteria including human. *S. agalactiae* causes neonatal meningitis, septicaemia and enhanced mortality in immunocompromised adults [28]. Recent study found that *S. agalactiae* isolated from tilapia able to kill mouse, suggesting that fish isolates able cross the interspecies barrier hence they are no more non infective to mammalian.

The clinical signs associated with Streptococcosis described as erratic swimming associated with brain damage, loss of appetite, lethargy, C shape body curve, uni or bilateral exophthalmia, eye haemorrhages, abscesses at the base of the pectoral fins, eye opacity, protruding anus etc. [24, 29]. Streptococcosis in tilapia farms known to promotes by high stocking densities, higher temperature, lower dissolved oxygen [30]. Thus, certainly suggesting that optimum farming conditions might be effective to reduce the potential disease outbreaks. This ubiquitous bacterium evolved several advance strategies to avoid many immune mechanisms. The capsule and capsule components such as BibA, sialic acids known to avoid complement deposition hence complement activation against this pathogen which is considered

as major virulence factor of this pathogen [31]. Furthermore, this bacterium has shown ability to survive in macrophages when internalised via non opsonic pathways hence can avoid other immune mechanisms. Those virulence factors make them one of the most dangerous pathogens in fish farms. Even though this bacterium has several strategies to overcome immune response, specific antibodies produced following vaccination proven to be effective to inhibit pathogen survival and distribution in the host hence vaccine appear to be promising prophylactic measure against this pathogen. However, specially designed Streptococcosis vaccine against this pathogen in aquaculture such as "Aquavac Strep Sa" is still in experimental level and will be available in commercial level near future.

1.6 Vaccine Immunology

Most of the commercial vaccines in the aquaculture industry are inactivated vaccines consist of with whole pathogen or pathogen associated molecules known to carry antigenic properties such as extracellular products, cell wall components or capsular polysaccharides. These antigens can induce innate and adaptive immune systems of fish. This induction triggers series of immunological mechanisms to produce pathogen specific molecules called antibodies and to keep immunological memory. Therefore, vaccines are giving remarkable long lasting protection to the fish against target pathogen [32].

To gain the better understanding on protection offered via vaccines, and to optimise existing vaccination strategies and to develop effective vaccines in the future, knowledge on underlying mechanisms in vaccine immunology is necessary. Proper understanding of vaccine immunology is the key to the future progress in fish vaccinology.

Fish immune system can be divided in to two sub groups as innate and adaptive even though both group work together for effective elimination of pathogen [32, 33]. Among these two sub systems, innate immune system act as first line defender against infectious organisms and consist of with three major defensive weapons those are known as physical, humoral and cellular barriers [33, 34]. Anatomic barriers such as fish skin, scale, mucous and gut epithelial layer act as physical barriers to prevent pathogen entry to the host. The antimicrobial peptides, enzymes, natural antibodies, inflammatory mediators, cytokines, chemokines, presence in mucous and blood act as innate humoral defenders against colonisation and survival of infectious organisms [35]. The complement component in blood also known as one of the most important defenders in humoral arm of the innate immune system which can activate via three different pathways known as classical, alternative and lectin pathways. Classical complement pathway initiates via antigen antibody complex while other two can trigger independent from antigen antibody complex [36, 37]. These three complement pathways merge at the middle of their activation pathways and led to form membrane attack complex which have ability to lyse microbes by making pours on their surface [37, 38]. On the other hand, complement deposition on particular pathogen can act as important opsonic material which can increase pathogen engulfment via effector cells in innate immune system [39]. Cell mediated innate immune response is the last and most important defensive weapon in innate immune system which consist of with several cell types such as macrophages, monocytes, neutrophils, eosinophils nonspecific cytotoxic cells etc. [40]. Some of those cells contains specific surface molecules called pattern recognition receptors (PRRS), those that conserved over thousands of years during evolutionary process to recognise pathogen specific molecular patterns [40, 41]. Those receptors can recognise the pathogen specific molecular patterns such as lipopolysaccharides, peptidoglycan, double stranded DNA, CpG, bacteria associated structures such as flagella etc. [40]. This recognition is not specific for

particular pathogen. Following recognition of pathogen by PRRS, it triggers series of signalling pathways those led to internalise, kill, process and present pathogen or pathogenic molecules to the other cell types in immune systems [40]. In addition, activated PRRS trigger cytokine release, antimicrobial peptide production and complement activation those that important to induce several arms of immune system to work toward one goal, to eliminate the target pathogen. The professional phagocytic cells in innate immune system such as monocytes and macrophages are important role players whom can engulf and kill the pathogen via phagocytosis process. In addition, they have specific ability to process and present pathogenic molecules to the cells in adaptive immune system as membrane bound component [42]. This pathogen presentation is carried out by specific group of receptors called major histocompatibility complexes (MHC) [43]. Extracellular antigens such as bacteria, parasites and their toxins known to present by MHC class II receptors while intracellular pathogen associated molecules by MHC class I receptors [43]. Therefore, antigen presenting cells in innate immune system known to make cross road, help linking innate and adaptive arms of the immune system to raise effective and powerful reaction against pathogen [33, 41, 43].

Adaptive immune system consists off with two main subgroups known as B cell and antibody based humoral immunity and T cell mediated cellular immunity [33, 43]. Action of adaptive immune system is pathogen specific and able mount immunological memory hence response will be quick and effective at the phase of repeated exposure [44]. In adaptive humoral arm, B cell is the cell type produces specific antibodies against target pathogen [42]. In addition, B cells itself have ability to recognise extracellular pathogen or toxins such as LPS and activate without help of antigen presenting cells [34]. Activated B cells proliferates and differentiate in to plasma B cells which can secrete specific antibodies against target pathogen. In addition to direct

activation, B cell can activate via activated T cells [40]. Antigen presenting on professional phagocytes can induce T cells and activated T cells activates B cells result in more effective response where B cells can produce plasma cells those can secrete best fitting specific antibodies against target pathogen or pathogenic molecules [34, 41]. Furthermore, another sub population of B cells produced following activation of naive B cell will serve as memory B cells which important for the rapid and direct recognition of pathogen during repeated exposure hence immune response will be more effective and fast than first time [44].

The main purpose of vaccines is to induce adaptive immune system by different antigens such as inactivated whole cell, live attenuated pathogen, toxoid, DNA molecules, to produce pathogen specific antibodies and to induce memory B cells production in order to keep memory about particular pathogen or pathogen associated molecule without a disease. Regardless of activation pathways, pathogen specific antibody produced following vaccination consider as hallmark of vaccine efficacy and several studies done on fish species explained positive correlation between amount of specific antibodies and percent survival following challenge with respective pathogen [45, 46, 47, 48]. Specific antibodies directed against particular pathogen have been shown wide range of actions such as promote phagocytosis and intracellular killing of pathogen, neutralization of pathogen or pathogen derived toxins, induce antibody mediated cellular cytotoxicity, trigger complement cascade to lyse the pathogen etc. [49]. Antibodies have ability to bind and block pathogenic structures which interferes pathogen attachment to the host cells [49]. Furthermore, antibodies have shown the capability of aggregating pathogen in blood or mucous thus inhibit motility of motile pathogens such as *Vibrio* species [50]. Moreover, antibody known to activate classical complement pathway to form membrane attack complexes which can lyse the pathogen by forming pores on their surface [37]. In addition, specific antibody directed

against some pathogen considered as most important opsonic component which facilitates effective engulfment and killing of pathogen specially the pathogen who have developed strategies to escape complement mediated immune mechanisms such as *Streptococcus* sp [30, 39, 50]. Therefore, optimum antibody production as well as proper functioning of other antibody mediated effector mechanisms in immune system are equally important in order to gain maximum protection offered via vaccination. However, today, in aquaculture industry other than screening of production of specific antibodies following vaccination and percent survival following challenge, little concern has shown on mode of action of the antibodies toward different group of pathogens hence vaccine immunology is generally poorly understood.

1.7 Importance of environmental parameters on fish immunity

Nevertheless, vaccines are integral part of today's aquaculture, continuous research on vaccines, vaccinations programs, vaccine efficacy under several environmental conditions are important to optimise vaccination programs in order to gain the maximum benefits offered by vaccination. Fish are living in aquatic environment where many of environmental changes such as dissolved oxygen, temperature, pH, salinity, photoperiod are profoundly affecting on their morphology, behaviour, physiology, biology including immunity [51, 52]. A considerable number of scientific studies have shown environmental parameters can modulate fish immunity [53, 54, 55, 56, 57]. Due to vaccines provide protection via induction of fish immune system, if environmental parameters modulate fish immunity which in turn affect vaccine based protection hence not only the immunogenicity of the vaccine, but also the effect of different environmental factors on vaccine efficacy should be a future direction of vaccine research in aquaculture industry. Some of the environmental parameters such as temperature and nitrates already

identified as potential modulators on vaccine efficacy in fish [58, 59, 60]. Therefore, progress of the future fish vaccinology will strongly depend upon identification of co correlation between vaccine induced immunity and role of environmental modulator on this immunity. At this point in my study concerned on one of the most important water quality parameters, dissolved oxygen.

1.8 Dissolved oxygen and hypoxia

Weather it is terrestrial or aquatic, oxygen is the most vital element for all most all form of life. Oxygen is the final electron acceptor in cellular respiration process in which energy generates in form of ATP, essential for all most all biological functions hence functioning of the organism [61]. On the other hand, oxygen is essential for various cellular reactions mediated with oxidases, peroxidases etc. [62]. In view of those, adequate amount of oxygen in water as well as in body is important for wellbeing of the aquatic animal including fish. In aquatic environment, dissolved oxygen is the major limiting factor due to water contain low oxygen amount compared to the air [63]. On the other hand, today, oxygen level in intensive aquaculture systems became far too low than naturally available amount due to high stocking density, huge feeding, accumulation and decomposition of fish waste, higher bacteria load, nutrient enrichment mediated algal blooms, low water flow rate etc. [64,65]. Limitation of oxygen in water or hypoxia apparently creates the similar internal environment in fish hence all most all functions including growth, reproduction, endocrine system, behaviour locomotion, metabolisms and immunity may adversely affect [66]. Fish in natural environments can avoid the hypoxic environments by migrating but fish in aquaculture systems have no opportunity to do so. If hypoxia is stronger, it can have profound effect on behaviour of fish such as decrease feeding, gulping air at the water surface, increased gill ventilation, decreased activity etc. [66, 67]. On the

other hand, if hypoxia persist in aquatic environment, fish may show several physiological and biochemical adaptations such as increase blood haemoglobin level, mobilisation of energy reserves, reduce metabolic activities and turn into anaerobic metabolisms which lead to accumulate toxic end products such as lactic acids in fish body [67]. Furthermore, hypoxia identified as a condition induce secretion of stress hormones known as corticosteroids which have ability to regulate metabolism, growth and immunity there by affect on fish disease resistance [67]. If hypoxic condition continue, fish may face severe energy crisis and metabolic depression that will ultimately affect almost all body functions including effector mechanisms in immune system [68, 69,70].

Several studies have shown that, hypoxia affected on fish immunity by modulating several effector mechanisms thereby increased susceptibility to infectious organisms. Some of those studies and research findings were summarised in the Table 1.

Table 1 Summary of the influence of hypoxia on fish immunity and disease susceptibility

Author and year	Fish	Finding
Scapigliati *et al*. 1999 [71].	Sea bass	Hyper oxygenation increased natural immunoglobulin level
Choi *et al*. 2007 [70].	Nile tilapia	Acute hypoxia compromised immunity of fish
Welker *et al*. 2007 [55].	Channel cat fish	Sub lethal hypoxia compromised immune response including antibody production and increased susceptibility to Enteric septicemia.
Cecchini and Saroglia, 2002 [53].	Sea bass	Specific antibody response reduced after hypoxia exposure.
Boleza *et al*. 2001 [72].		Hypercapnic hypoxia compromised bactericidal activity of fish anterior kidney cells.
Fukuda *et al*. 1997 [73].	Yellowtail	Hypoxia increased mortality following challenge with *Enterococcus seriolicida*
Kvamme *et al.* 2013 [74].	Atlantic salmon	Chronic hypoxia modulated expression of important immune related genes
Douxfils *et al*. 2012 [75].	Eurasian perch	Lysozyme activities were decreased following repeated hypoxia
Niklasson *et al*. 2011 [76].	Atlantic salmon	Do level as low as 50% clearly affects the intestinal mucosal immune system and result is chronic inflammation.
Singh *et al.* 2016 [77].	*Catla catla*	Lower oxygen stress decreased lysozyme, respiratory burst and nitric oxide synthase.

Bunch and Bejerano, 1997 [78].	Nile tilapia	Low oxygen level increased mortality in hybrid tilapia due to *Streptococcous* sp. Infection.
Cuesta *et al*. 2003 [79].	Gilthead seabream	Hypoxia inhibited head kidney natural cytotoxic cell activities.
Evan *et al*. 2003 [68].	Nile tilapia	Sub lethal dissolved oxygen increased susceptibility to *Streptococcus agalactiae*.
Cuesta *et al*. 2004 [80].	Seabream	Hypoxia did not affect on natural IgM level

Aim of the Study

As explained above, vaccines offer protection to the fish via induction of series of effector mechanisms in immune system. Hypoxia highlighted as potential immune modulator in fish. At this point we can assume that hypoxia might be a potential modulator on vaccine efficacy. However up to date, there is no study carried out on influence of hypoxia on vaccine efficacy in fish hence it can be important limiting factor for optimisation of future vaccination programs in aquaculture industry. In view of these knowledge gap current study was carried out with following purposes

1. To find the influence of moderate hypoxia on vaccine efficacy in Nile tilapia (*Oreochromis niloticus*) against *Vibrio anguillarum.*
2. To reveal the influence of moderate hypoxia on pathogen clearance mechanisms and susceptibility of vaccinated Nile tilapia following experimental challenge with *S. agalactiae.*

In my study, serum antibody titer, cumulative mortality, serum bactericidal activities, bacteria burden in tissue and blood, antibody mediated cellular innate immune mechanisms such as phagocytosis were used as direct and indirect indicators to measure vaccine efficacy. The broad spectrum of research data generates via this study expected to provide comprehensive picture of hypoxia on vaccine efficacy. Instead of lethal hypoxic condition which can recognise by observing behaviour of fish, I decided to use moderate hypoxic condition (around 55% DO saturation in water) in my research due to it has no visible impact on morphology or behaviour of Nile tilapia. Such a condition will difficult to recognise by simple observations hence it can retain in aquaculture systems without noticing by farmers. On the other hand, moderate hypoxia found

to be not affecting on fish appetite hence other than dissolved oxygen, other changes in fish such as nutritional deficiencies will not interfere with research outcome.

Nile tilapia (*Oreochromis niloticus*) was selected as experimental fish due to special features such as one of the largest cultured fish species in our planet, well adapted for domestication, ability to survive in poor water conditions, short production cycle, higher reproductively, rapid growth rate, known to resist many bacterial, parasitic, fungal and viral diseases compared with other farmed fish species [81].

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CHAPTER 2

Influence of moderate hypoxia on vaccine efficacy against *Vibrio anguillarum* in *Oreochromis niloticus* (Nile tilapia).

Abstract

Hypoxia is known as a potential immunomodulator in fish. This study therefore assesses the impact of chronic, moderate hypoxia on vaccine efficacy in Nile tilapia, *Oreochromis niloticus*. Serum antibody titer was used as a surrogate marker to detect vaccine efficacy. The fish were acclimatized to either moderate hypoxia (55±5% DO) or normoxia (85±5%DO) and immunized with formalin inactivated *Vibrio anguillarum*. Significantly, a higher antibody titer was found in normoxic fish than in moderate hypoxia. The normoxic group titer peaked at 14^{th} dpv (days post vaccination) while the moderate hypoxic group peaked at 21^{st} or 28^{th} dpv. The absolute blood lymphocyte counts and serum bactericidal activities against *V. anguillarum* were significantly higher in normoxic fish. Serum killing of *V. anguillarum* appeared to be mainly via antibody-dependent classical complement pathway. Furthermore, the first week following vaccination appears critical for antibody production. This view was further supported by results obtained from gene expression assay, where the transcription level of all the detected immune related genes (IgM, IL-1β, TCR-β, MHC-IIβ), except B cell activating factor, were significantly suppressed following exposure to moderate hypoxia. Booster vaccination and high dose vaccination significantly increased antibody production in moderate hypoxic fish. Furthermore, following booster vaccination, differences in serum killing between normoxic and hypoxic fish were disappeared. The overall results highlight that even though moderate hypoxia is not easily detectable in Nile tilapia, it negatively affects antibody production by suppressing and delaying antibody response, ultimately affecting vaccine efficacy.

Key words: Nile tilapia, dissolved oxygen, vaccination, antibody titer, bacterium

2.1. Introduction

Intensive fish culture operations often result in enhanced outbreaks of infectious diseases. Huge economic losses from mortality or reduced product quality of infected fish affect sustainability of the industry worldwide [1]. To overcome this challenge, preventive and control measures are practiced and vaccination has proven the most promising and environmentally-friendly management strategy for many existing infectious diseases. Vaccines induce specific immune system in fish to produce specific antibodies, memory B and T cell components against target pathogens or pathogen-associated molecules, and thus offer long-lasting protection [2,3,4]. As in mammals, vaccine effectiveness in fish is regulated by several factors, including vaccine-derived factors, administration route, endogenous factors within the fish and exogenous factors, such as nutrition, marginal or stressful environmental conditions [5,6,7].

Optimum environmental factors in aquaculture systems play an important role in health of cultured organisms. Dissolved oxygen (DO) is one of the most critical aquatic environmental factors, since water contains relatively less oxygen compared to the atmosphere [8]. Hypoxia is the condition where oxygen concentration in water is measurably low [9] and it seems to be frequent episode in aquaculture systems. There are many factors promoting hypoxia: decomposition, accumulation of organic matter through faeces and unconsumed food and high stocking density all result in increased biological oxygen demand within the system. Despite the fact that physiological threshold oxygen level varies considerably among fish species [10], if hypoxia is strong enough to alter homeostasis, energy reserves are diverted to maintain homeostasis. Ultimately, growth, reproduction, immunity and other energy demanding activities may be adversely affected. [11,12,13].

Numerous studies have proven that hypoxia is a key factor promoting disease outbreaks in aquatic environments [14,15,16,17]. Compared with natural environments, the potential negative impact of hypoxia in aquaculture system is significant, since avoidance/escape is impossible. In the channel catfish, *Ictalurus punctatus* increased susceptibility and mortality to enteric septicaemia is positively correlated with acute hypoxia, and hypoxia likely compromised the immunity of the fish [13]. Similarly, Nile tilapia, *Oreochromis niloticus* exposed to hypoxia exhibited higher mortality following experimental challenge with *Streptococcus agalactiae* [16]. Small and Bilodeau (2005) [18] also noted higher mortality in experimentally challenged channel catfish, *I. punctatus* under low DO. Furthermore, overall compromised immunity in *O. niloticus* was observed shortly after exposure to acute hypoxia [19]. Many studies examine acute, short-term hypoxia exposure, but chronic exposure more likely represents the reality in aquatic systems. Chronic hypoxia has been identified as the factor driving either reduced or delayed immune related gene expression in the Atlantic salmon, *Salmo salar* [20].

A considerable number of scientific studies have examined hypoxia-mediated, compromised immunity and increased disease susceptibility of fish. However, until now, no studies have investigated the effects of hypoxia or moderate hypoxia on fish vaccine efficacy. This could likely be an important limiting factor in our ability to predict vaccine efficacy. Since vaccination induces energy demanding processes, such as immune cell proliferation, differentiation and antibody production, it is worth to assess the impact of hypoxia on vaccine efficacy. Current study is carried out, therefore to investigate the impact of moderate hypoxia (55±5% DO saturation) on vaccine efficacy in *O. niloticus* vaccinated against *Vibrio anguillarum*. Rather than focusing on a single experiment, this paper presents a synthesis of data accumulated from a series of individual experiments addressing immunological and hematological studies. The broad spectrum of research

data shown herein will allow a more thorough understanding of the impact of moderate hypoxia on vaccination processes.

2.2 Materials and methods

2.2.1 Experimental fish and rearing conditions

Apparently healthy Nile tilapia naive to *V. anguillarum* infection weighing 146±32g were obtained from the Aquaculture laboratory, Tokyo University of Marine Science and Technology, Japan. The fish were randomly assigned to twelve, 60 L glass tanks (12-15 fish per tank) connected with a recirculation system. Water temperature, pH, ammonia, nitrate and nitrite were 24±0.5°C, 7.2±0.2, less than 2mg/L, less than 10mg/L and less than 0.5mg/L, respectively. The fish were fed with commercial dry feed (Nippai, Kanagawa, Japan) twice daily until satiation and feeding was discontinued 24h before sampling and restored following sampling. Photoperiod was held constant at 12h dark and 12 h light per day. Oxygen concentration in the water was adjusted according to the experimental set up of each experiment. Normoxic (85±5%DO saturation) and moderate hypoxic (55±5%DO saturation) environments in glass tanks were created by manipulating aeration and directly injecting N_2 gas to the tanks through aerators connected with a flow meter (Kofloc, Kyoto, Japan). Dissolved oxygen and temperature in each tank were measured using an oxygen meter (Mettler Toledo, Switzerland) 3 times per day and all other parameters were recorded daily. Water flow rate in each tank was adjusted to 1 L /min to avoid any other change~~s~~ in water quality (except for dissolved oxygen). Water in the storage tank and settling tank of the recirculation system was replaced 3 times per week. Prior to the vaccination, fish were allowed to acclimate either to normoxic or moderate hypoxic condition for two weeks. All the fish handling and treatment were done according to the ethical principles proposed in administrative guidelines offered by the Head of Science and Minister of Education in Japan.

2.2.2. Section 1

2.2.2.1 Vaccine preparation and vaccination

V. anguillarum (Strain 775) originally isolated from rainbow trout, *Oncorhynchus mykiss*was used for the immunization experiment. Prior to vaccine preparation, isolated bacteria were confirmed as *V. anguillarum* by using PCR with specifically designed primers for *V. anguillarum* [21], observing colony characteristic on thiosulfate citrate bile salts sucrose agar (TCBS, Eiken chemical Japan) and detecting biochemical properties of the bacteria. A primary stock of *V. anguillarum* was prepared in tryptic soy broth (TSB, BD, USA) supplemented with NaCl to 1.5% and stored in -80°C with 10% glycerol as 1ml aliquots.

Two days before the vaccination experiment, frozen stock was thawed and bacteria was mass cultured in 500 ml TSB supplemented with 1.5% NaCl for 24h with mild shaking in a water bath at 30°C. Following 24h culture, the bacteria suspension was centrifuged at 3000 rpm for 20 min and the pellet was re-suspended in 1x phosphate buffered saline (PBS, Sigma). The resulted suspension was tested for purity by culturing on tryptic soy agar (TSA, BD, USA) and TCBS agar. The bacteria concentration was adjusted as$5.5x10^9$colony forming units (CFU)/ mL for formalin inactivation. Bacteria inactivation was carried out by adding 3% formaldehyde to the bacteria suspension, then mixed and stored overnight at 4°C. The following day, the formalin–killed bacteria suspension was washed 3 times with PBS and divided into two groups. One was re-suspended in 1xPBS approximately to a $5x10^9$CFU/mL for vaccination and the other portion was re-suspended in 1xPBS to an absorbance of 0.5 OD at 600nm for use in agglutination assay. Both suspensions were stored at 4°C until use. Inactivation was confirmed by plating 100µl of resulted suspensions on TCA supplemented with 1.5% NaCl at 30°C for 48h.

On the day of immunization, fish from moderate hypoxic and normoxic groups were placed in a holding tank and 100µl of prepared vaccine ($5x10^8$CFU/fish) was administrated via intra-peritoneal (IP) injection, while a control group received a similar amount of sterile 1xPBS. Immediately after vaccination, fish were returned to their initial experimental tank or to other experimental tanks according to the experimental plan.

2.2.2.2. Experimental plan and sample collection.

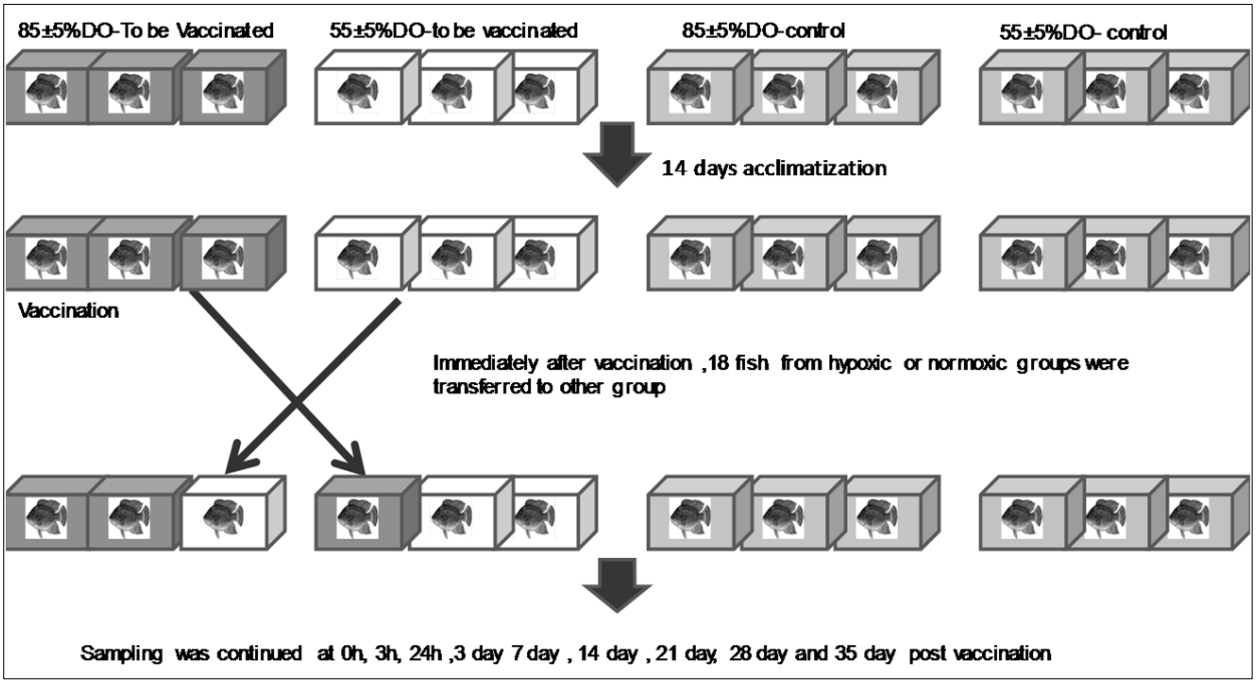

Figure 2.1.0 Diagrammatic view of experimental design. Fish were acclimatized either to moderate hypoxia or normoxia for 14 days. Fish in "to be vaccinated tanks" were vaccinated via IP injection of inactivated *V. anguillarum* (100µl inactivated bacteria suspension per fish equivalent to $5x10^8$ cells) while control groups received a similar amount of sterile PBS. Immediately after vaccination, 18 fish in the moderate hypoxic vaccinated group were transferred to normoxic conditions and the same number in the normoxic vaccinated group were transferred to the moderate hypoxic condition. Fish were sampled at 0h, 3h, 24h, 3rd ,7th 14th, 21st, 28th ,35th dpv.

In experiment one, fish were acclimatized for 2 weeks under normoxic or moderate hypoxic conditions. As shown in Fig.1, the experiment initially consisted of four treatments groups in triplicates: 1. normoxic control fish: 2. moderate hypoxic control fish: 3. normoxic fish to be vaccinated: 4. moderate hypoxic fish to be vaccinated. Immediately after vaccination, baseline (0 h) blood samples were aseptically collected from four fish in each group. The fish were anaesthetized in 2-Phenoxyethanol (300 µl/L) (Wako, Japan), bled from caudal vein with 24 G needle fitted with 2.5ml heparinized (500 Units/ml) syringe, and then into a sterilized 2ml Eppendorf tube. Before clotting, Packed cell volume (PCV) was determined and the blood was then allowed to clot for 1h at room temperature. Coagulated blood samples were centrifuged at 3000g for 15 min at 4°C, serum was collected, aliquoted and stored at -20°C until use in an agglutination assay. Immediately after vaccination, 18 fish from the vaccinated moderate hypoxic group were transferred to the normoxic condition (treatment 5) and a similar number in the vaccinated normoxic group were transferred to the moderate hypoxic condition (treatment 6). Hereafter, treatment groups were n=6, including moderate hypoxia to normoxia and normoxia to moderate hypoxia transfer groups (Fig.2.1.0). Samplings were continued at 7^{th}, 14^{th}, 21^{st}, 28^{th} and 35^{th} dpv in all groups, with 4 fish from each group at each sampling date.

In experiment two, the experimental set-up and sampling methodology were similar to experiment one, except fish transfer from moderate hypoxia to normoxia and normoxia to moderate hypoxia was done at 7 dpv. In addition to blood, spleen samples were also collected at 0h, 3h, 24h, 3^{rd} and 7^{th} day following vaccination and transferred to RNAlater (Ambion, USA), kept for 24h under 4°C and subsequently stored at -20°C until RNA extraction. Serum samples were used to detect antibody titer, plasma chemistry, serum bactericidal activities, serum lysozyme activities and for the assay of complement based serum killing of *V. anguillarum*.

2.2.2.3 Serum antibody titer by agglutination assay

Serum antibody titer was measured as described by Klesius *et al.* (2000) [22] with some modifications. Briefly, serum collected at each post-vaccination sampling was allowed to thaw on ice and 50 µl of each sample was doubly diluted in a similar amount of PBS across 96 round-bottom well plate from well 1-12. Each sample was assayed in triplicates and PBS used as a negative control while anti- *V. anguillarum* serum raised in rabbit was used as a positive control. To each well, 50µl of formalin inactivated *V. anguillarum* suspension was added, mixed well and incubated overnight at 25°C. The plates were covered with paraffin film to avoid evaporation and the following day, examined visually and under light microscope at 40 x magnification. Highest serum dilution appeared as a circular diffused button with fuzzy edges taken as agglutination titer of respective serum, while negative wells appeared as a circular compact button at the bottom of the well. The antibody titer was expressed as two-fold serum dilution.

2.2.2.4. Serum bactericidal assay

The bactericidal assay was a modification of the bacteriostatic assay explained by Crosbie and Nowak, (2004) [23]. Bactericidal activity of the serum was performed in 96 round-bottom well plate and each sample was assayed in triplicate. The bacteria were cultured overnight in TSB supplemented with 1.5% NaCl, and 150µl of the resulting suspension was inoculated into a newly prepared TSB with 1.5% NaCl, incubated for 2.5h in a shaking water bath at 130 rpm, 30°C to get a logarithmic phase bacteria culture. The serum was collected as before and 50µl of serum was mixed well with 50µl of freshly prepared live *V. anguillarum* ($1x10^6$cells/mL) suspension in TSB.

The samples were incubated for 2.5 h at room temperature (~25°C) with mild shaking. As a negative control, TSB was incubated with heat inactivated normal serum (56°C for 30min) collected from Nile tilapia and bacteria incubated with PBS served as a positive control.

After incubation, 15 µl of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT (5 mg/mL) (Sigma Aldrich, St. Louis, USA) was added to each well, mixed by pipetting and incubated for 15 min at room temperature. Resulted formazan was dissolved by adding 50 µl of dimethyl sulfoxide (Sigma Aldrich, USA). Optical density (OD) in each well was read at 570 nm. Antimicrobial activity of serum was determined by the following formula and results were given as % inhibition of *V. anguillarum* relative to the positive control.

$$\% \text{ inhibition of } V.\ anguillarum = \frac{(\text{OD of positive control} - \text{OD of the sample})}{\text{OD of positive control}} X100$$

To clarify the role of specific antibody in serum killing of *V. anguillarum*, immune and non-immune serum obtained at 14 dpv adsorbed with formalin killed *V. anguillarum.* Briefly, the bacteria suspension (0.5 OD at 600nm) was dispensed into a 1.5 mL Eppendorf tube at 100µl per each and centrifuged at 4000 rpm for 15 min at 4°C. The resulted supernatant was discarded; the pellet was re-suspended in 100µl of immune serum, incubated for 1h at room temperature while shaking in order to adsorb the potential antibody directed against *V. anguillarum* in the serum. After adsorption, serum was centrifuged at 4000 rpm for 15 min at 4°C and the supernatant was filtered through a 0.22 µm syringe filter (Millipore). The adsorbed serum was used for bactericidal assay, as explained previously.

2.22.5 Serum complement based anti- *V. anguillarum* activities

The experiment was conducted to verify the role of antibody on complement based antimicrobial activities in Nile tilapia. Immune serum obtained following two weeks' post-vaccination and normal serum obtained from healthy Nile tilapia was used for the experiment. All the immune serum was heat inactivated by heating at 56°C for 30min. Normal serum (NS) was divided into four groups, and one group was incubated with 10mM (final concentration) EGTA (Ethylene glycol tetra acetic acid) plus 10mM of $MgCl_2$ to chelate Ca^{2+}, which is an essential factor for classical complement activities in the serum. The second group of NS received 10mM (final concentration) of EDTA, (Ethylene diamine tetra acetic acid), which is able to chelate both Ca^{2+} and Mg^{2+}, hence inactivating both classical and alternative complement activities. The third NS was heat inactivated as before to inactivate complement and other enzymatic activities. The final NS was not subject to any modification. Logarithmic-phase *V. anguillarum* culture was prepared as noted and cell concentration adjusted to $1x10^6$ CFU/mL. The bacteria suspension was dispensed into a 96 round-bottom well plate and incubated with heat inactivated immune serum (H.I.S) (50 µl bacteria suspension+10 µl heat inactivated immune serum) with mild shaking for 1h at room temperature. Each inactivated immune serum +bacteria sample was incubated in 15 wells, and at the end of the incubation period triplicates of well received 100 µl of NS +EGTA +Mg^{2+} or NS+EDTA or heat inactivated NS or untreated NS or PBS. The resulting combinations were further incubated for 2 h at room temperature (~25°C). Antimicrobial activities were detected by adding 15µl of MTT (as explained in section 2.3) and results given as percentage inhibition of bacteria in each treatment group compared to the NS+ PBS.

2.2.2.6 Serum lysozyme assay

Serum lysozyme activity was measured with the turbidometric method described by Sankaran and Gurani (1972) [24], using hen egg white lysozyme (Sigma Aldrich, USA) as a standard. Briefly, immediately before the assay, lyophilized *Micrococcus lysodeikticus* cells (Sigma Aldrich, USA) were suspended in 1xPBS (pH 6.0) at a concentration of 0.25 mg cells/mL. Immune and non-immune serum (10µl per well) obtained from Nile tilapia were placed in a 96 round-bottom well plate and 200µl of prepared *Micrococcus lysodeikticus* cell suspension was added to each well and mixed by pipetting. Initial absorbance and absorbance after 15 min incubation at room temperature were measured at 450nm using a micro plate reader (Multiskan FC, Thermo). The concentration series of hen egg white lysozyme (0-20 µg/mL) was prepared in 1xPBS (pH 6.0) and used as a standard solution to construct a standard curve. Lysozyme activity in each sample was measured as reduction in absorbance units and expressed as lysozyme concentration (µg/mL).

2.2.2.7 Total RNA extraction and RT-PCR analysis for gene expression

Total RNA was extracted from Nile tilapia spleen using Isogen II (Molecular research, Japan) according to manufacturer's protocol. RNA samples were quantified using e-spect spectrophotometer (BM equipment, Japan) and quality was detected by gel electrophoresis. Extracted RNA was normalized with Milli-Q water, and 2µg of each RNA sample was used for cDNA synthesis using a high capacity cDNA reverse transcription kit (Applied Biosystems, USA) following manufacturer's instructions. The reaction mix contained 4.2µl Milli-Q water, 2 µl 10x RT buffer, 0.8 µl dNTP mix, 2 µl 10xRT random primers, 1 µl multiscribe reverse transcriptase

enzyme and 10µl (2µg) of diluted RNA sample. The reaction condition was 37°C for 60 min and 95°C for 5 min. The cDNA samples were used for RT-PCR in order to examine the expression of immune related genes such as major histocompatibility complex II β (MHC class II β), Interleukin one β (IL-1β), T cell receptor β (TCR β), Immunoglobulin M (IgM) and B cell activating factor (BCAF). RT- PCR was performed in a Mini option real time PCR system (Bio Rad) by using Fast SYBR green super mix (Bio Rad, Singapore). The β actin was used as the control in all amplifications and for all the primers, the melting curve was analyzed to verify that primers did not form any primer dimmers. Each gene used in this study, amplicon size, and gene bank accession numbers are listed in Table 1. The cycling conditions were as follows: one cycle of initial denaturation at 95°C for 2 min and 35 cycles of 95°C for 30s, 55°C for 30s, 72°C for 30s and final elongation was performed at 72°C for 3min. All amplifications were conducted as triplicate of wells and one well contains 10µl of SYBER green, 5µl of Milli-q water, 1µl of forward and reverse primers and 3µl of cDNA template.

Relative quantification of each gene expression was calculated according to $2^{-\Delta\Delta ct}$ method using the threshold cycle value (Ct) of each sample. The β-actin was used as an internal control and sample collected at 0h post injections served as calibrator.

Table 2.1 Primers used for RT-PCR analysis.

Primer Names	Sequence (5´to 3´)	Accession No.	Amplicon size (bp)
IgM	F/AGGCACAACGGTCACTGTCA R/GCAAGGCAGCCAAGAGTGAC	KJ676389.1	108
β-actin	F/GCGGAATCCACGAAACCACC R/CTGTCAGCGATGCCAGGGTA	KJ126772.1	121
MHC II β	F/GAGGAACAAGCTCGCCATCG R/AGTCGTGCTCTGACCTCGAG	JN967618.1	106
TCR β	F/CCGTGAAGCCATTGAACCGC R/AACCAGAGGCCACACAGACG	HM162889.1	113
IL-1β	F/GACAAGCCAACCCTCCACCT R/GTTGGGGTAGCGGACAGACA	KJ574402.1	147
BCAF	F/ACAACCCAGCGCAGACAGAT R/ACAGGTGGACACTCGGGTTG	KF741364.1	81

Abbreviations- Immunoglobulin M (IgM), Actin Beta (β-actin), Major histo-compatibility complex II beta (MHC II β), T-cell receptor beta (TCR β), Interleukin 1-beta (IL-1β), B-cell activating factor (BCAF)

2.2.2.8. Plasma chemistry analytes and electrolytes

Serum samples stored at -20°C were allowed to thaw on ice and 200µl of each sample was analyzed for biochemical parameters such as total cholesterol (TCHO), triglycerides (TG), non-esterified fatty acids(NEFA), total protein (TP), glucose (GLU), sodium(Na), potassium (K), and chloride (Cl) using an automated chemical analyzer (Hitachi 7020, Japan). All the necessary chemicals for analysis were supplied by Wako (Osaka, Japan).

2.2.2.9. Packed cell volume and absolute blood lymphocyte count

Blood was with drawn into a heparinized tube as described from 12 fish acclimatized for moderate hypoxic or normoxic conditions. Blood from each fish was used to determine the packed cell volume (PCV)after centrifugation at 12,000g for 5 min. PCV value was detected using a haematocrit reader (Tommy Seiko, Japan).

Heparinized blood samples collected from vaccinated fish at 14^{th} and 21^{st} dpv were used for total white blood cell count with C- chip hemocytometer (Improved neubauer type hemocytometer, Digital Bio, Japan) and blood smears were prepared, fixed in methanol, stained with May-Grünwald-Giemsa stain solution according to the manufacturer's instruction (Wako chemical, Japan) to get the differential blood cell count. Percentage lymphocyte count in smears and absolute lymphocyte count in each blood sample was determined with following formulas.

Percentage lymphocyte count $= (Number\ of\ lymphocytes\ in\ 1000\ white\ blood\ cells\ /$ 1000 white blood cells $)X100$

Absolute lymphocyte count ($\times 10^3$/ µl) = Total white blood cell count in hemocytometer x percentage lymphocyte count in blood smear (expressed as decimal)

2.3 Section two

2.2.3.1 Booster vaccination

Vaccine preparation and vaccination in this study was conducted as explained in chapter 2, section 1 (2.2.1). At 14 days' post vaccination, booster dose was given in same manner and fish were sampled up to 35 days' post vaccination to collect the blood as explained before (Fig. 2.3.1). Collected blood samples were used to separate serum and agglutination assay and serum anti *V. anguillarum* assay were conducted as described in chapter 2 section 1 (2.2.3 and 2.2.3).

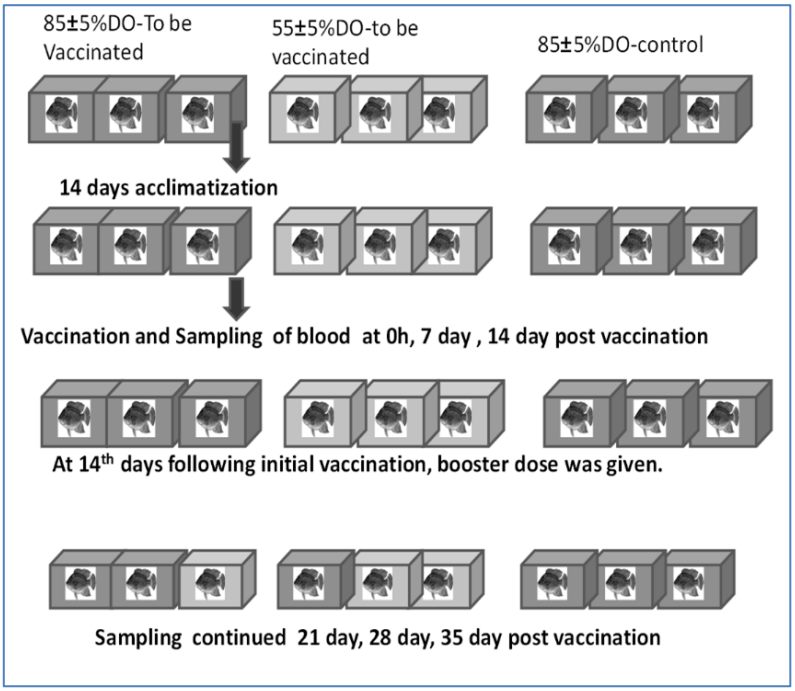

Figure 2.2.0 Diagrammatic view of experimental design of booster vaccination. Fish were acclimatized either to moderate hypoxia or normoxia for 14 days. Fish in "to be vaccinated tanks" were vaccinated via IP injection of inactivated *V. anguillarum* (100µl inactivated bacteria suspension per fish equivalent to $5x10^8$ cells) while control groups received a similar amount of sterile PBS. Fish were sampled at 0h, 7^{th} 14^{th} dpv. Following 14^{th} days post vaccination, booster dose was given and sampling continued up to 35^{th} days post vaccination.

2.2.3.2 High dose vaccination

In this experiment two vaccine doses were used as low dose (1×10^9 CFU/ ml) and high dose (1×10^{12} CFU/ ml). Following acclimatization to each oxygen condition, fish were vaccinated with low dose or high dose vaccine hence each oxygen condition had three experimental set up as high dose vaccinated group, low dose vaccinated group and control. Sampling was done up to 35^{th} days post vaccination and collected serum samples were subjected for agglutination assay to measure antibody titer.

2.2.4 Statistics

All other experimental data were analyzed using t-test or one-way ANOVA, and multiple comparisons were made using Turkey's multiple range test. XLSTAT software was used to perform all the analysis. Significance was set at $p<0.05$ in all the experiments.

2.3. Results

2.3.1 Section 1

Following exposure to moderate hypoxia, fish were not showing any marked alterations in behavior such as surface breathing, increased gill ventilation.

2.3.1.1 Serum antibody titer

In vaccination experiment 1 (Fig.2.1.1), specific antibody production against *V. anguillarum* was markedly impaired ($p < 0.05$) by moderate hypoxia compared to the normoxic group starting from 7^{th} dpv up to 35^{th} dpv. These results reflect the oxygen-dependent nature of antibody production. The antibody production in normoxia to moderate hypoxia transferred fish was significantly suppressed compared to the normoxic fish ($p < 0.05$) at all the detected time points. In contrast, antibody titer in the reperfusion group (moderate hypoxia to normoxia transferred group) was markedly restored to the level of normoxic antibody titer after 7^{th} dpv where no difference in antibody titer was detected between normoxic and moderate hypoxic to normoxic transferred groups. The first antibody response was detectable at 7^{th} dpv. The highest antibody titer in normoxic and moderate hypoxic to normoxic transfer groups was recorded at 14^{th} dpv while moderate hypoxic group and normoxic to moderate hypoxic transferred group fishes reached peak antibody titer at 28^{th} dpv, indicating suppressed as well as delayed antibody response under moderate hypoxic condition.

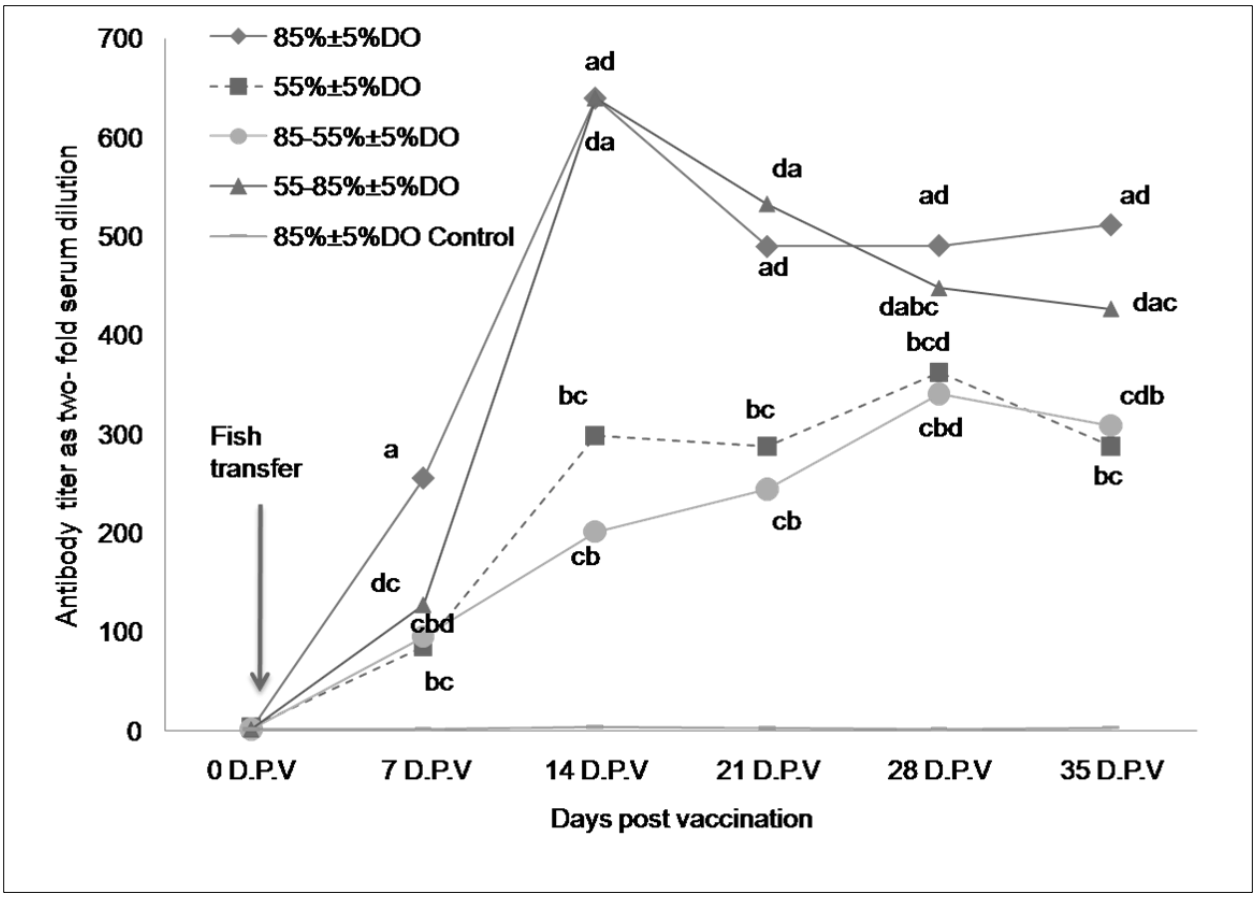

Figure 2.1.1 Serum antibody titer of Nile tilapia in response to dissolved oxygen concentrations at different post-vaccination time points. Fish were vaccinated with *V. anguillarum* and serum antibody titer was determined with an agglutination test. Fish transfer between vaccinated groups was done immediately after vaccination (0 h) as shown by the arrow. Antibody response in control (PBS injected) is also shown and antibody titer was given as two-fold serum dilution. Each value represents mean (n=4) and error bars are omitted for clarity. Different letters at the same time point indicate significant differences ($p<0.05$) between immunized groups.

In experiment two, the intent was to determine the critical time-frame following vaccination where hypoxia might have profound impacts on antibody production. We transferred the vaccinated fish in normoxic conditions to moderate hypoxic conditions, and moderate hypoxic conditions to normoxic conditions following 7th dpv. In this experiment, we observed similar trends

in antibody production as in experiment one. The antibody titer was significantly ($p < 0.05$) lower in the moderate hypoxic group compared to the normoxic group at all the detected post- vaccination time points (Fig. 2.1.2). Following transfer from moderate hypoxic to normoxic conditions at 7th dpv, antibody production increased ($p < 0.05$) compared to the moderate hypoxic group at 21st and 28th dpv, even though the titer was somewhat below the normoxic antibody titer.

By contrast, compared to normoxic fish, a significant reduction ($p < 0.05$) of antibody titer was observed in the group transferred from normoxic to moderate hypoxic conditions following vaccination. Fig. 2.1.2 shows the variation of antibody titer between groups and peaking time points. All the vaccinated groups demonstrated significantly higher ($p < 0.05$) antibody titer compared to respective controls, receiving doses of sterile PBS.

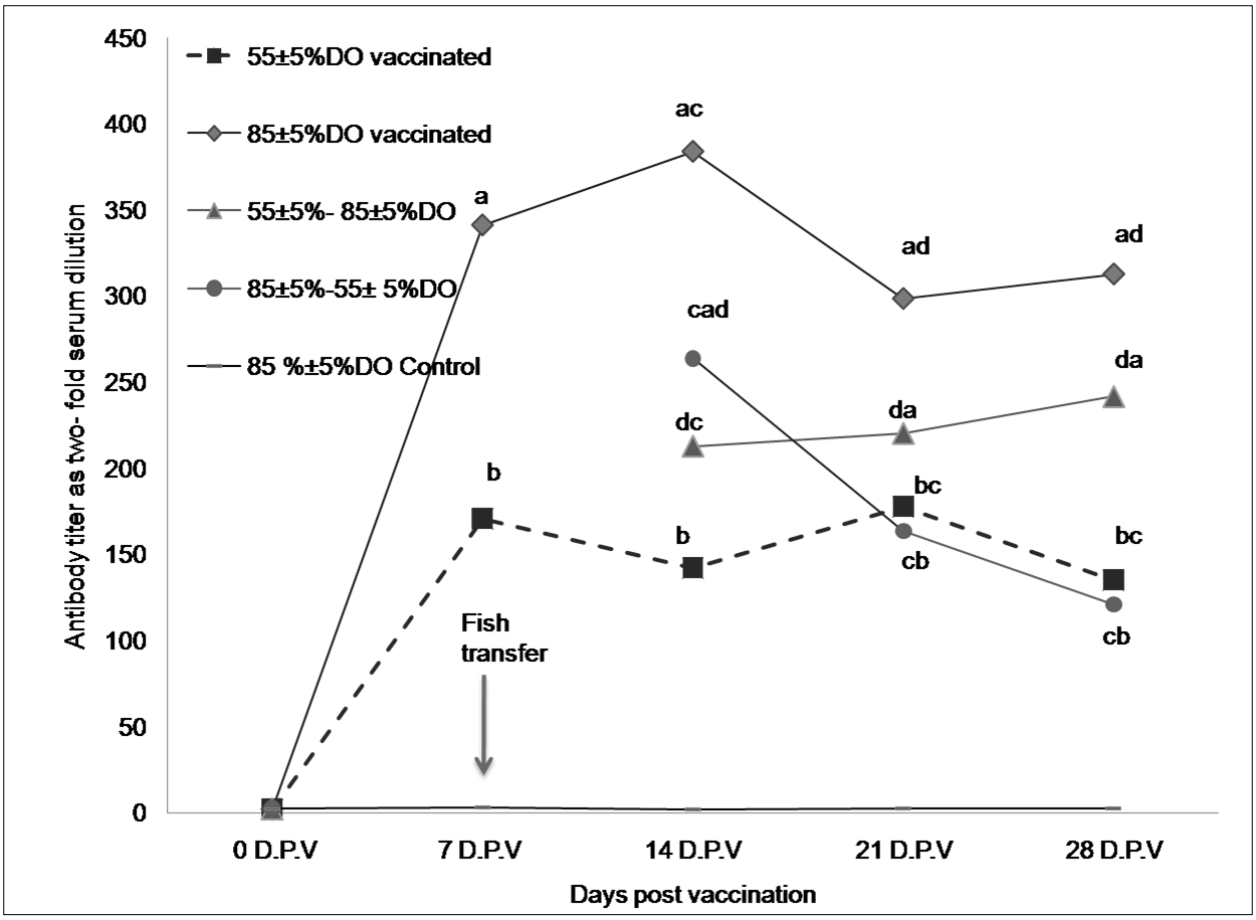

Figure 2.1.2 Serum antibody titer of Nile tilapia in response to dissolved oxygen concentrations at different post-vaccination time points (Experiment 2). Fish were vaccinated with *V. anguillarum* and serum antibody titer was determined with an agglutination test. Fish transfer was done following 7th dpv as shown by the arrow. Antibody response in control (PBS injected) is also shown and antibody titer was given as two-fold serum dilution. Each value represents mean (n=4) and error bars are omitted for clarity. The vaccinated groups in the same time point with different letters indicate significant differences ($p<0.05$) between immunized groups.

2.3.1.2 Serum bactericidal activities

Anti-*V. anguillarum* activities of the serum obtained from vaccinated moderate hypoxic fish, normoxic fish and non-vaccinated control fish at different post-vaccinated time points (Fig. 2.1.3). Following 7th dpv, the percentage inhibition of *V. anguillarum* in normoxic serum was ~41%, and it was significantly ($p< 0.05$) higher compared to the moderate hypoxic serum, which illustrated only 24% inhibition. At 14th dpv, bactericidal activity in the serum of immunized normoxic and moderate hypoxic groups increased compared to the 7th dpv. However, the percentage inhibition in immunized normoxic serum was still significantly ($p< 0.05$) higher than that of immunized moderate hypoxic serum. Antibody produced against *V. anguillarum* was precipitated in both moderate hypoxic and normoxic immune serum by incubating the serum with formalin killed *V. anguillarum*, with the resulting serum subjected to bactericidal assay as before. After precipitation of antibodies, serum bactericidal activities in both normoxic and moderate hypoxic immunized fish significantly decreased to the level where no difference was detectable between immunized and control groups. This indicates that serum bactericidal activity might be driven predominantly by the presence of anti-*V. anguillarum* antibody in the serum.

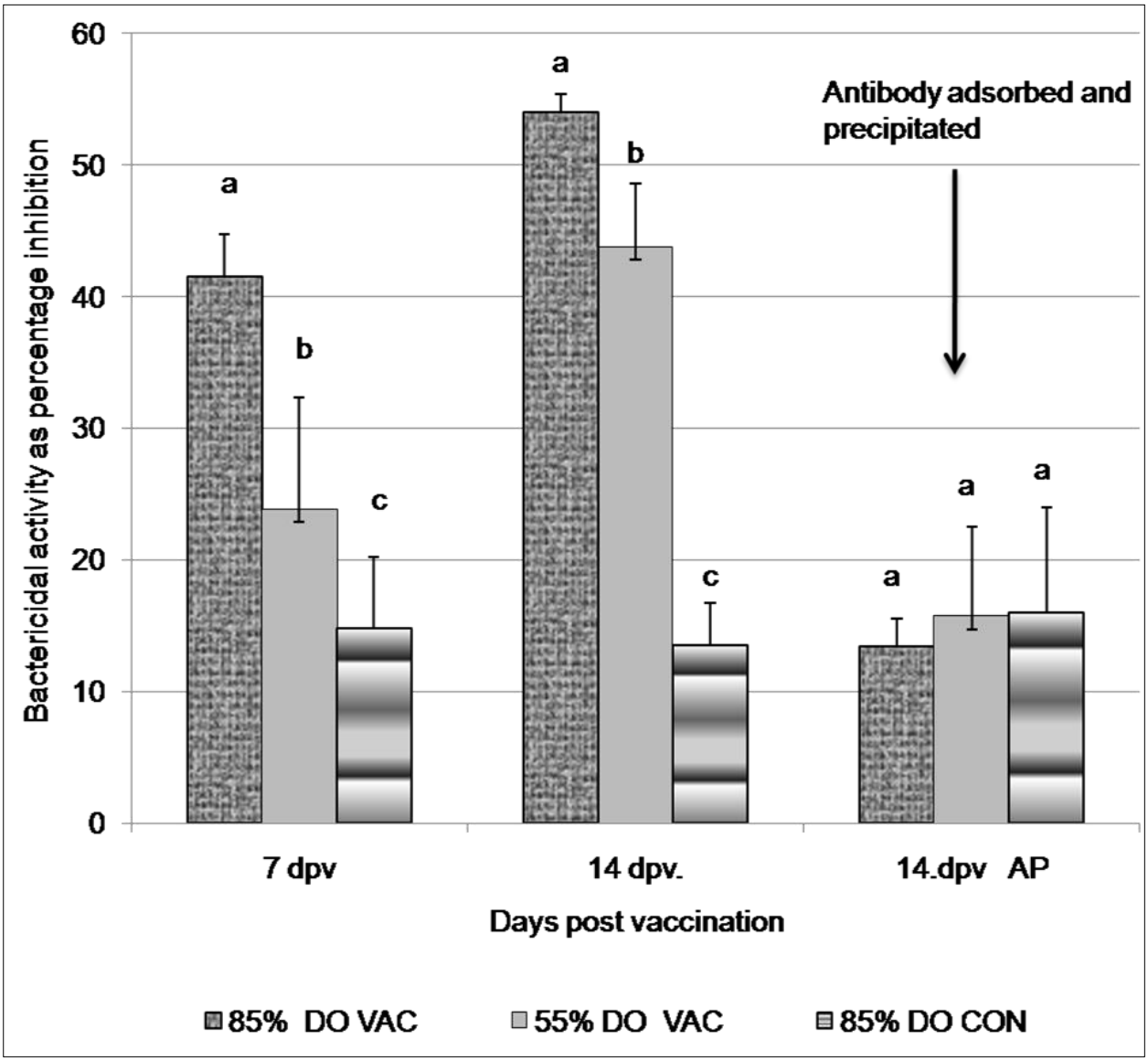

Figure 2.1.3. Serum bactericidal activity (SBA) as % inhibition of *V. anguillarum* in Nile tilapia kept under moderate hypoxic or normoxic conditions following vaccination. Fish were vaccinated with *V. anguillarum* and SBA was measured at 7th and 14th dpv. The SBA in serum collected at 14th dpv was analyzed twice, with or without antibody adsorption and precipitation (AP). Each value represents the mean±SD (n=4). Significant differences ($p<0.05$) between groups at each time point are indicated by different letters

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2.3.1.3 Serum complements based anti- *V. anguillarum* activities

As shown in Fig. 2.1.4, when heat inactivated immune serum (H.I.I.S) coated *V. anguillarum* was incubated with EGTA + MgCl2 treated normal serum, bactericidal activity was significantly lower ($p < 0.05$) compared with untreated normal serum, indicating that serum killing was mediated by a classical complement pathway. On the other hand, serum killing in EDTA-treated normal serum was also significantly lower ($p < 0.05$) than that of untreated normal serum, but no difference in serum killing between the EGTA + MgCl2 treated group and the EDTA treated group was found. This suggests that the alternative complement pathway in serum killing *V. anguillarum* is insignificant. When normal serum is subjected to heat inactivation to abolish complement activities and other enzymatic activities, bacteria are not inhibited.

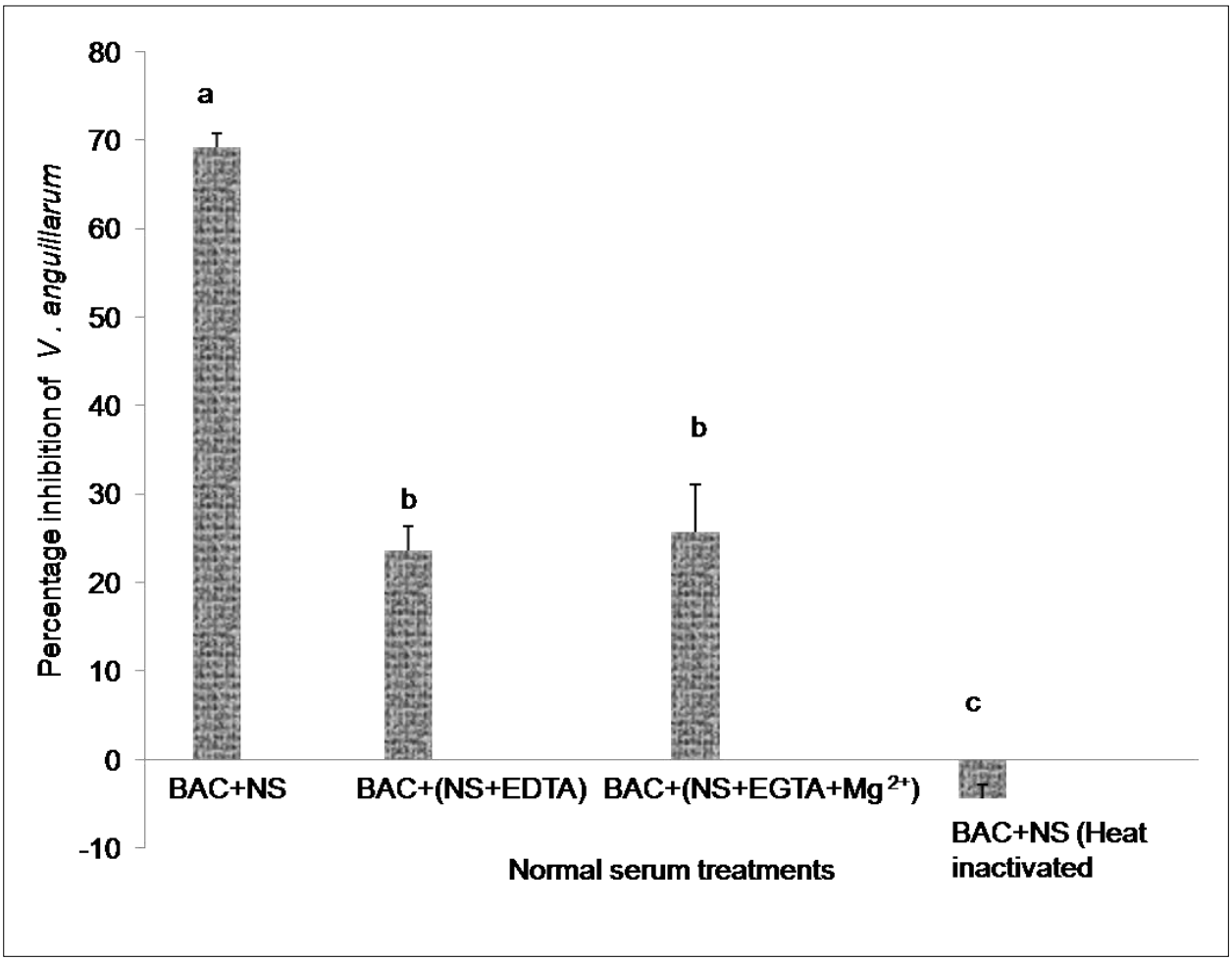

Figure 2.1.4. Complement mediated killing of *V. anguillarum* by immune serum collected from vaccinated Nile tilapia. Immune serum was subjected to heat inactivation and incubated with live bacteria. After incubation with heat inactivated immune serum (H.I.I.S), bacteria were further incubated with normal serum (NS) which was treated with EDTA or EGTA+Mg^{2+} or heat or kept as pure form. Antimicrobial activity was measured with MTT. Significant differences ($p< 0.05$) between groups indicated by different letters above the bar. Bactericidal activity of serum was calculated as the decrease in number of viable *V. anguillarum* compared to the control (H.I.I. S+Bac+PBS). Each value represents the mean±SD (n=4).

2.3.1.4 Serum lysozyme activity

Serum lysozyme activity in immunized normoxic and moderate hypoxic fish remained unchanged for the entire study period (Fig 2.1.5). This suggests that differences observed in serum killing of *V. anguillarum* between vaccinated normoxic and moderate hypoxic groups was unlikely caused by differences in serum lysozyme activity.

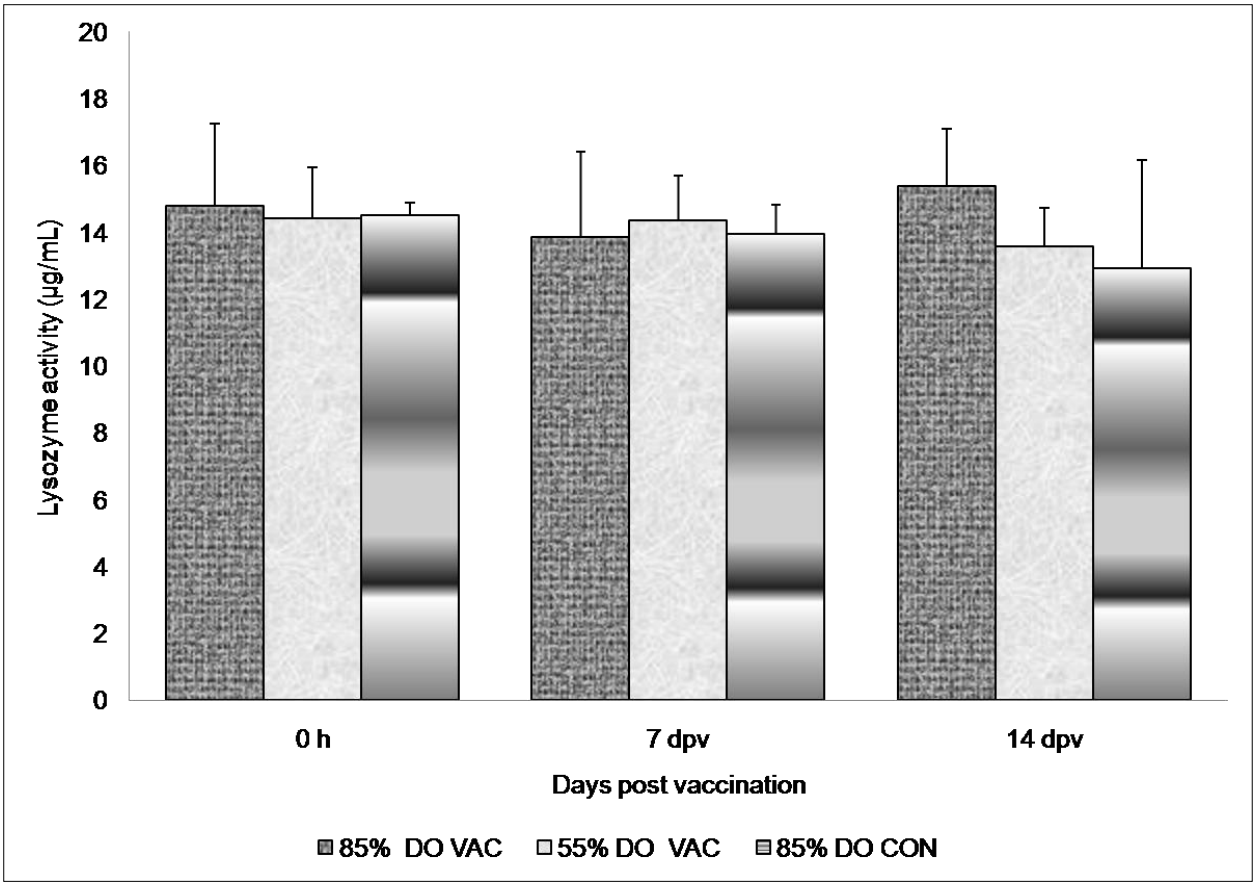

Figure 2.1.5. Serum lysozyme activity in Nile tilapia (n=4). Immune and non-immune serum collected from fish was incubated with *Micrococcus lysodeikticus* for 15 min and reduction of absorbance at 450nm was measured. Results expressed as lysozyme concentration (µg/mL). Each value is mean±SD.

2.3.1.5 Expression of immune –related genes in the spleen

Following vaccination, 4 of 5 genes examined (TCR- β, MHC class II β, IL-1β and IgM) were significantly ($p< 0.05$) up-regulated in fish held under normoxic conditions compared to moderate hypoxic conditions (Fig.2.1.6). However, no significant difference in B cell activating factor expression was seen in these two groups, as expression exhibited great variation at the individual level (Fig.2.1.7). The MHC class II β (Fig. 2.1.6) in normoxic fish was significantly up-regulated ($p<0.05$) at 24 hand 3rd dpv compared to the moderate hypoxic group and returned to the initial level following 7th dpv. The highest expression level was detected in IL-1β (Fig. 2.1.6) at 3 h post-vaccination (~ 65-fold increase) in the normoxic group and it was significantly higher ($p< 0.05$) compared to the moderate hypoxic group. The significant up-regulation ($p< 0.05$) of the transcription level of TCR β (Fig. 2.1.6) in the normoxic group was observed following 3rd and 7th dpv compared to the moderate hypoxic group. The IgM gene (Fig. 2.1.6) was significantly up-regulated ($p< 0.05$) in 3rd dpv in the normoxic group, but transcription rapidly decreased to the initial level at 7th dpv.

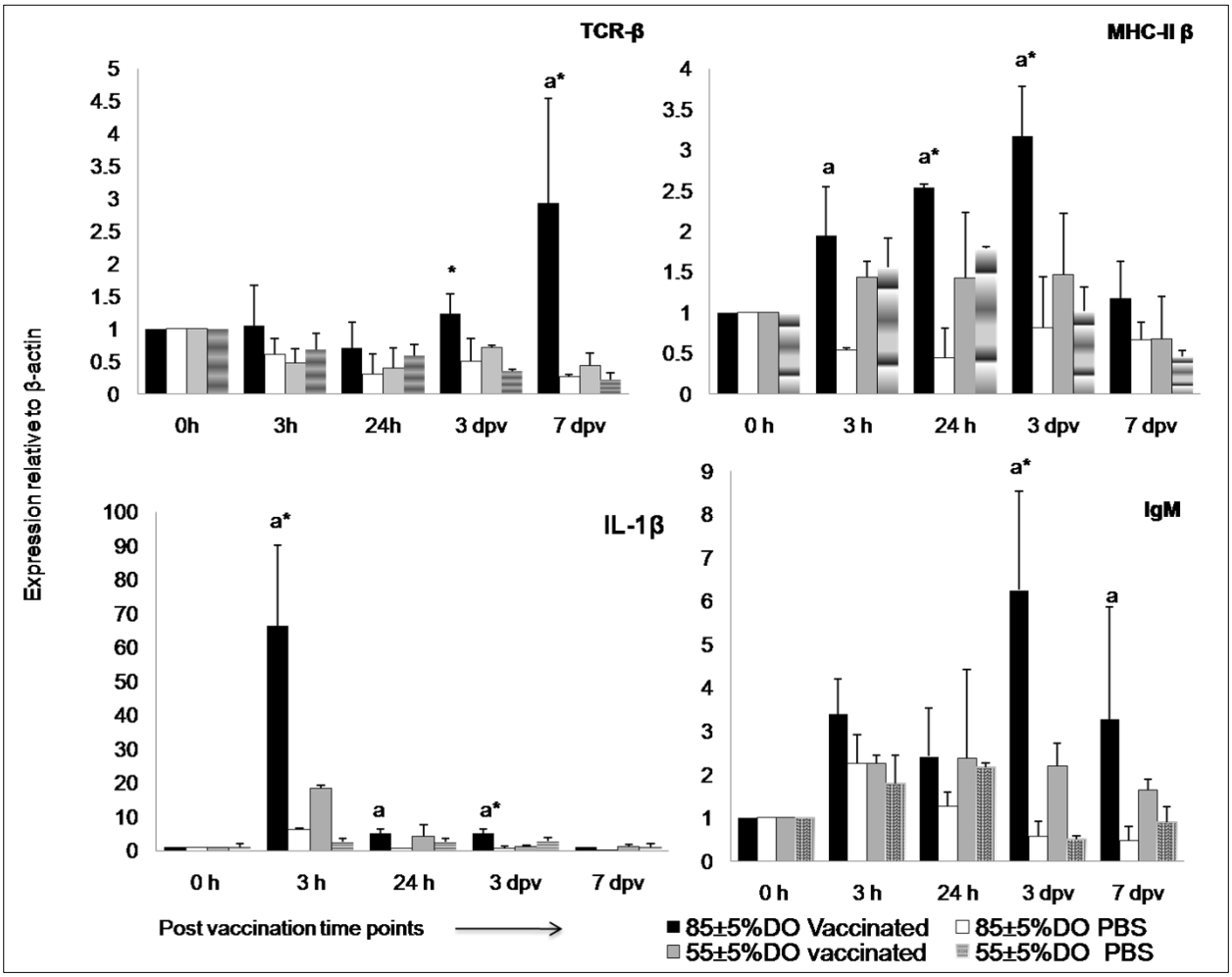

Figure 2.1.6 The relative expression of TCR β, MHC II β, IL -1β and IgM in the spleen of Nile tilapia following immunization with *V. anguillarum* or PBS injection at normoxic and moderate hypoxic conditions. Bars represent mean±SD. Significant up-regulation (*p< 0.05) relative to the moderate hypoxic group indicated by asterisk above the bar and significant up-regulation (^{a}p< 0.05) relative to the PBS injected group indicated by letter "a" (n=4)

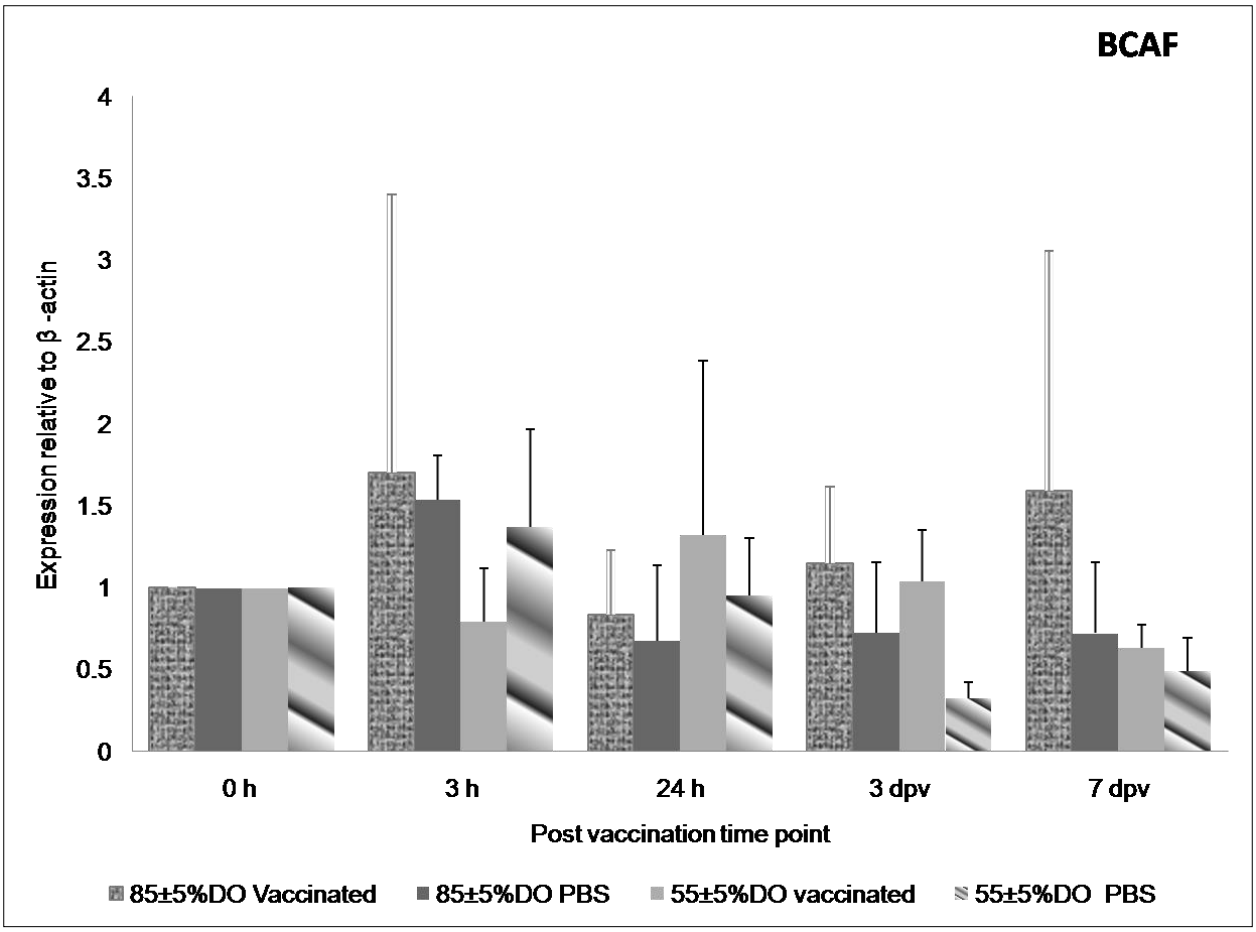

Figure 2.1.7. The relative expression of B cell activating factor in Nile tilapia spleen following immunization with *V. anguillarum* or PBS injection at normoxic and moderate hypoxic conditions. Bars represent mean±SD (n=4).

2.3.1.6 Plasma chemistry analytes, plasma electrolytes, packed cell volume and absolute blood lymphocyte count

No significant differences could be detected in any plasma chemistry analytes or electrolytes between normoxic and moderate hypoxic groups throughout the study (Table 2.2). It should be noted that the individual concentrations of all the tested parameters resulted in a much broader range. Significantly higher ($p < 0.05$) PCV was detected in fish reared under moderate hypoxic

conditions compared to normoxic. The absolute blood lymphocyte count was significantly lower ($p< 0.05$) at 14^{th} dpv under moderate hypoxic condition compared to normoxic, even though at 21^{st} dpv, those differences were statistically non-significant.

Table 2.2.

Plasma chemistry analytes, plasma electrolytes, packed cell volume and absolute blood lymphocyte count of vaccinated or control Nile tilapia shown as mean±SD for each parameter. Different superscript letters at same time point for each parameter indicates significant ($p<0.05$) difference

Parameter	DO level	Treatment	0h	7 dpv	14 dpv	21 dpv
Glucose (mg/dL)	85±5%	control	61.68±17.76	48.87±27.10	59.34±16.37	49.14±8.20
	55±5%	control	70.56±40.15	59.91±26.38	65.06±32.39	52.38±34.04
TCHO (mg/dL)	85±5%	control	213.56±48.21	215.83±55.01	316.48±32.03	329.52±32.03
	55±5%	control	234.53±175.9	293.39±71.05	228.25±116.51	364.28±116.51
TG(mg/dL)	85±5%	control	255.33±11.15	158.66±93.98	480.33±335.28	528.00±311.76
	55±5%	control	237.33±302.87	310.00±90.07	385.00±456.12	498.00±396.99
TP(g/dL)	85±5%	control	3.15±0.24	2.82±0.07	3.22±0.03	3.54±0.14
	55±5%	control	2.99±0.43	3.02±0.24	3.13±0.09	3.34±0.08
NEFA (mmol/L)	85±5%	control	0.23±0.05	0.33±0.05	0.26±0.04	0.25±0.08
	55±5%	control	0.29±0.07	0.23±0.02	0.2±0.10	0.26±0.06
Na (mEq/L)	85±5%	control	162.4±1.76	158.5±3.36	163.07±4.12	166.6±5.47
	55±5%	control	157.7±7.94	159.47±4.87	155.2±12.01	162.3±0.97
Cl (mEq/L)	85±5%	control	144.53±0.49	141.77±2.45	146.2±1.56	148.4±4.01
	55±5%	control	142±4.42	143.06±1.63	139.37±6.68	143.65±0.99
K (mEq/L)	85±5%	control	5.13±1.34	7.58±4.14	5.83±5.82	2.39±1.73
	55±5%	control	7.32±4.75	9.25±4.12	9.12±3.76	3.42±3.73
PCV(%)	85±5%	control	29.65±1.8 [a]	ND	ND	ND
	55±5%	control	33.64±3.9 [b]	ND	ND	ND
ALC($\times 10^3$/µl)	85±5%	VAC	ND	ND	17.87±1.48[a]	17.41±0.81
	55±5%	VAC	ND	ND	13.06±2.07[b]	15.41±1.44

Abbreviations-Total cholesterol(TCHO), Glucose (GLU), Triglycerides (TG), Total protein (TP), Non- esterified fatty acid (NEFA), Sodium (Na), Chloride (Cl), Potassium(K), Packed cell volume (PCV), Absolute blood lymphocyte count (ALC), Not detected (ND), Vaccinated (VAC)

2.3.2.1 Serum antibody titer following booster vaccination and high dose vaccination

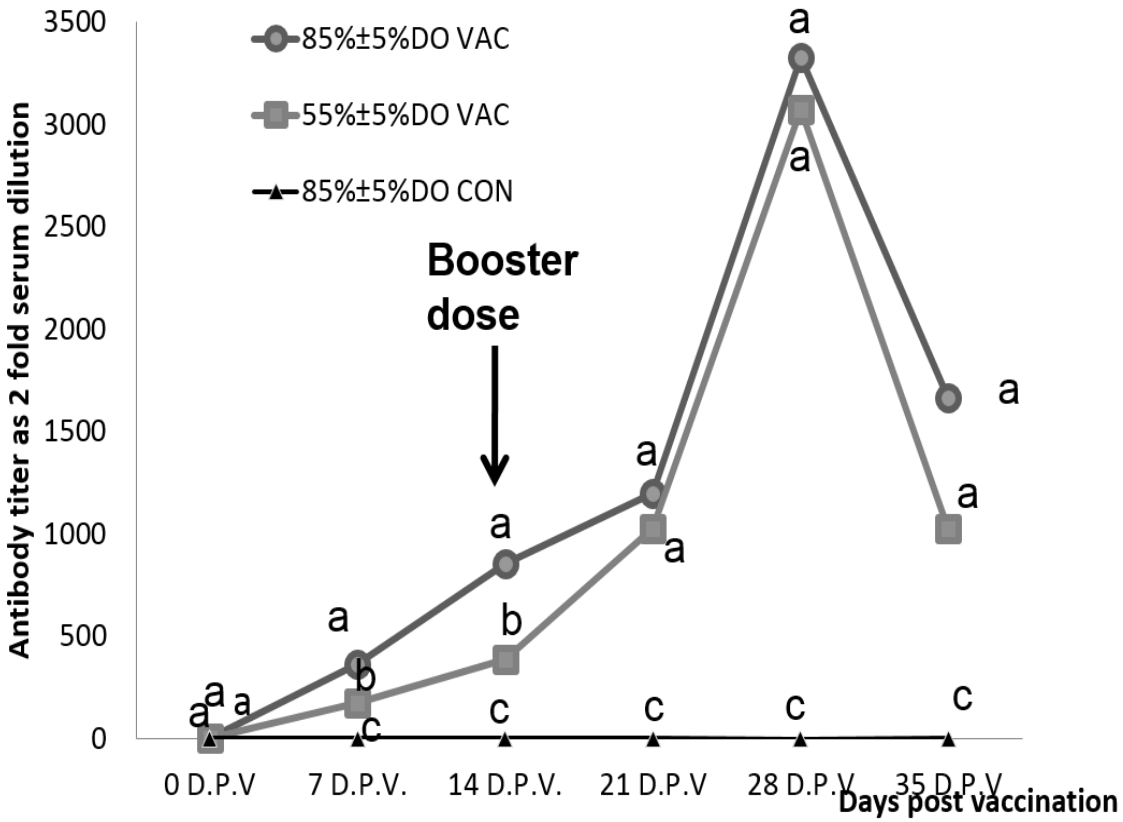

Figure 2.2.1. Serum agglutination titer of Nile tilapia following booster vaccination. Following acclimatization to each oxygen level fish were vaccinated with *V. anguillarum* and sampled at 0h, 7th dpv and 14th dpv. At 14 dpv, fish were received booster dose and sampling done up to 35th dpv. Serum antibody titer was determined with an agglutination test. Antibody response in control (PBS injected) is also shown and antibody titer was given as two-fold serum dilution. Each value represents mean (n=4) and error bars are omitted for clarity. Different letters at the same time point indicate significant differences ($p<0.05$) between groups.

The purpose of this experiment was to evaluate the possible counter measures to enhance the vaccine efficacy in moderate hypoxic Nile tilapia. In booster vaccination, following initial vaccine dose, antibody titer in moderate hypoxia group was significantly ($p< 0.05$) lower compared to the

normoxic group. Surprisingly, following booster dose, those differences could not be detected between vaccinated groups. In this experiment, we observed steep increase in antibody production following booster vaccination and this might be indicating presence of immunological memory (Fig. 2.2.1). These results are suggesting that booster dose is possible counter measure to enhance antibody production in Nile tilapia reared in moderate hypoxic condition.

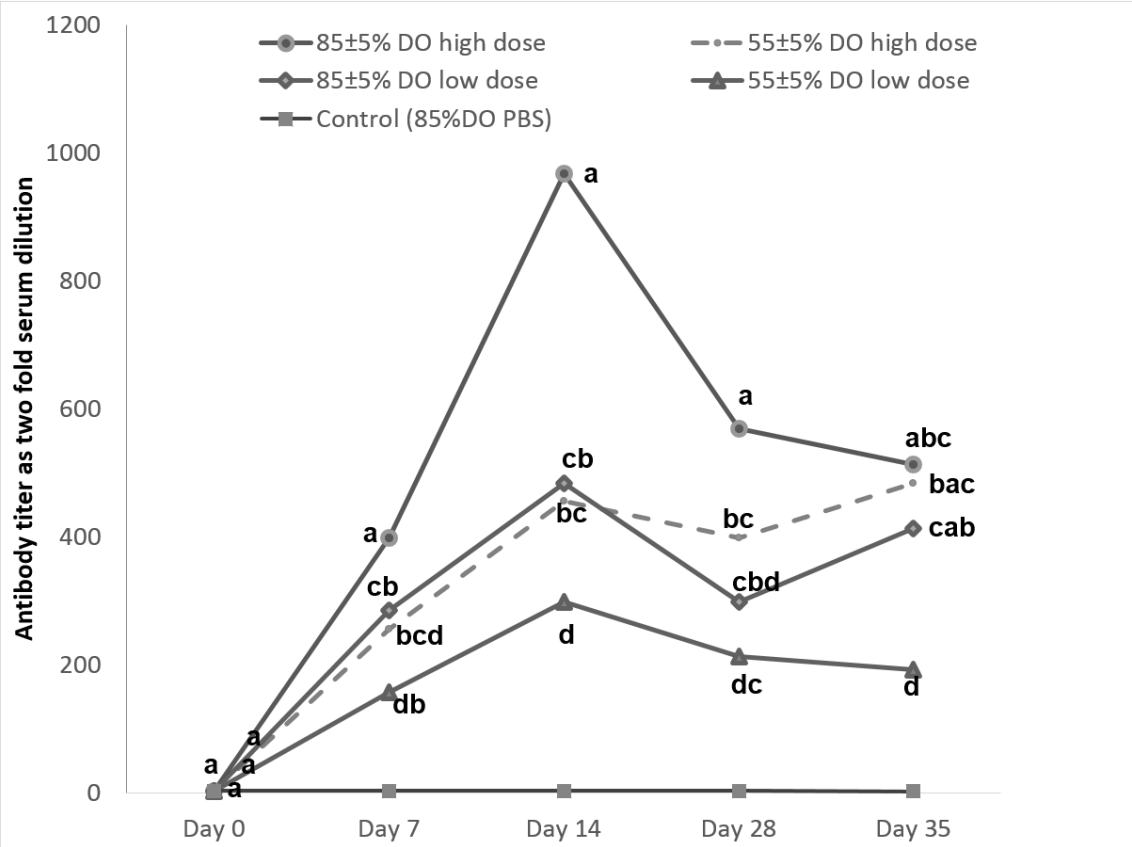

Figure 2.2.2. Serum agglutination titer of Nile tilapia following high dose and low dose vaccination. Following acclimatization to each oxygen level fish were vaccinated with high dose or low dose vaccine prepared with inactivated *V. anguillarum* and sampling done at 0h, 7th dpv 14th dpv 28th dpv and 35th dpv. Serum antibody titer was determined with an agglutination test. Antibody response in control (PBS injected) is also shown and antibody titer was given as two-fold serum dilution. Each value represents mean (n=4) and error bars are omitted for clarity. Different letters at the same time point indicate significant differences ($p<0.05$) between vaccinated groups.

In the high dose vaccination experiment, fish were given low dose vaccine or high dose vaccine and kept at each oxygen conditions up to 35th days post vaccination. Antibody titer was measured at each sampling date and variations in antibody production are shown in figure 2.2.2. This result clearly shows that antibody production in Nile tilapia is dose dependent at both oxygen conditions. The highest antibody production ($p< 0.05$) throughout the study period was seen in the normoxic fish vaccinated with high dose vaccine and lowest was observed in moderate hypoxic fish received low dose vaccine. Except 35th days post vaccination, antibody production in moderate hypoxic high dose vaccinated fish were significantly lower compared to the normoxic high dose vaccinated fish. Interestingly antibody titer between normoxic low dose vaccinated fish and moderate hypoxic high dose vaccinated fish appear to be not difference hence we can suggest that high dose vaccine is appear to be lowering the negative impact of moderate hypoxia on antibody production in Nile tilapia.

However, it should noteworthy that even though antibody production in moderate hypoxic fish improved following high dose vaccination, it is not as effective as booster vaccination where we observed antibody production has no difference between normoxic and moderate hypoxic groups.

2.3.2.2. Serum bactericidal activity following booster vaccination

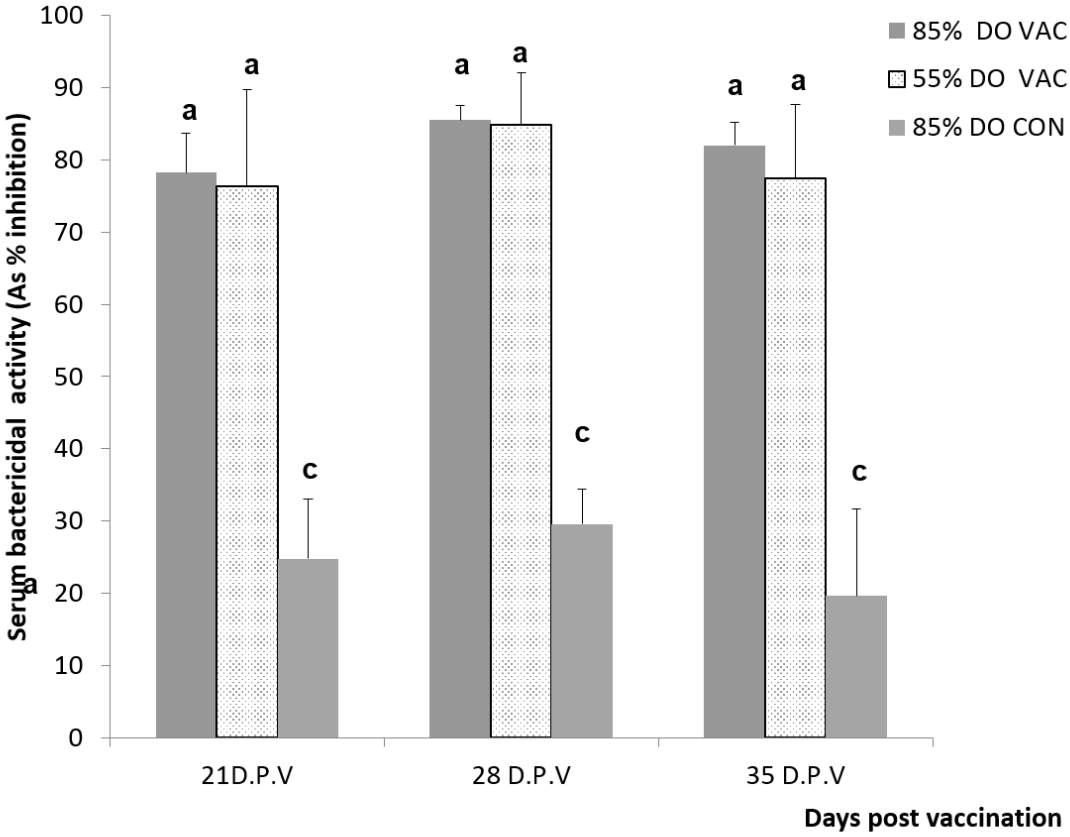

Figure 2.2.3 Serum Bactericidal activity (S.B.A) as % inhibition of *V. anguillarum* in Nile tilapia (n=4 at each group at each sampling day) kept under moderate hypoxic or normoxic conditions following booster vaccination. Fish were given booster dose at 14th days following initial vaccination and SBA was measured at 21st ,28th and 35th dpv. The significant differences between groups at each time point indicated by different letters ($P < 0.05$). SBA was calculated as the decrease in number of viable *Vibrio anguillarum* compared to the control.

The serum bactericidal activity between normoxic and moderate hypoxic vaccinated groups found to be not different at all the detected time points following administration of booster dose.

2.4. Discussion

In this study, significant changes in behavior attributed to hypoxia were not observed in moderate hypoxic fish. In addition, significant alteration of plasma chemistry analytes, such as increase in blood glucose, non-esterified fatty acid, triglycerides or decreased plasma electrolytes (Na, K, Cl), were not detected in moderate hypoxia as compared with normoxic fish. In contrast, compared with normoxic fish, PCV was significantly higher in moderate hypoxic fish, highlighting that moderate hypoxia results in an adaptive response to lower oxygen levels. Therefore, it is perhaps not surprising that under moderate hypoxia, fish exhibit no severe energy mobilization response.

The main objective of our study was to detect vaccine efficacy under normoxic and moderate hypoxic conditions. Vaccination targets a specific immune system to produce antigen-specific antibodies, and thus antibody titer is a promising indicator in assessing vaccine efficacy. The results clearly illustrate that chronic exposure to moderate hypoxia significantly reduces the magnitude of specific antibody titer following vaccination. Furthermore, the antibody titer in normoxic fish peaked at 14^{th} dpv while moderate hypoxic fish peaked at 21^{st} or 28^{th} dpv, indicating not only suppressed, but also delayed, antibody response under moderate hypoxia. Grabowaski *et al.* (2004) [25] evaluated antibody response in *O. niloticus* following immunization with formalin killed *Flavobacterium columnare* and the kinetics of the antibody response agreed with the antibody response of normoxic vaccinated fish in our study. The relatively low antibody titer in sea bass, *Dicentrarchus labrax* exposed to hypoxia compared with fish exposed to mild hyperoxia was noted in a previous study [26]. Moreover, a significant decrease of antibody titer in channel catfish following exposure to acute hypoxia and challenged with a low dose of *Edwardsiella ictaluri* was also reported [13]. Hypoxia-mediated immune suppression in fish and increased susceptibility to

infectious organisms has been reported in other studies [19, 20]. These authors indicate that the observed immune suppression results from stress caused by hypoxic treatments. It is widely accepted fact that stress could alter the energy metabolism of fish in order to maintain homeostasis and adapt to stressful conditions [27,28, 29]. Overall, such a clear mobilization or alteration of patterns in energy reserves, such as glucose, triglycerides, non-esterified fatty acids, was not identified in moderate hypoxic fish in our study, even though high individual variability was observed in nearly all the detected parameters. Therefore, it is unlikely that specific antibody titer was reduced as a response to chronic stress caused by moderate hypoxia, as we failed to detect any stress-mediated alteration in plasma chemistry. On the other hand, since lymphocyte proliferation, differentiation and specific antibody production are energy-demanding, it is reasonable to assume that suppressed and/or delayed antibody response might be due to suppressed cellular activities, which serve as counterbalanced system to conserve energy, in moderate hypoxic fish. This assumption was supported by our results of absolute blood lymphocyte count, as they were significantly higher in normoxic fish compared to moderate hypoxic fish at 14^{th} dpv, although 21^{st} dpv those differences were non-significant. This result is in accordance with the kinetics of the antibody response we observed in normoxic vs. moderate hypoxic groups. This suggests that moderate hypoxia drives other unknown factors that might directly affect the rate of lymphocyte proliferation and differentiation hence it likely the reason for reduced and delayed antibody response under moderate hypoxia. A positive correlation between blood lymphocyte count and serum antibody titer was reported in Nile tilapia immunized with sheep red blood cells [30].

The vaccination experiment was conducted as two independent experiments with similar experimental plans except for the differences in fish transfer date, to determine the critical time-frame following vaccination where hypoxia might have profound impacts on antibody titer. In

experiment one, moderate hypoxia fish were transferred to normoxic conditions immediately after vaccination. Here, antibody titer was markedly enhanced up to the level of normoxic fish antibody titer, 14th dpv onward where no differences could be detected between transferred fish and normoxic fish. In contrast, fish kept in normoxic conditions and transferred to moderate hypoxia had a significant decrease in antibody titer compared to fish in normoxic conditions since 7th dpv. Interestingly, in experiment two, where transfers from moderate hypoxia to normoxia or normoxia to moderate hypoxia were done at 7th dpv, the antibody titer of moderate hypoxia to normoxia transferred fish was significantly restored at 21st and 28thdpv, although antibody titer somewhat below than normoxic antibody titer. In contrast, following transfer of vaccinated normoxic fish to moderate hypoxia condition, antibody titer was significantly reduced from that of the normoxic group. Those results merely reflect that even though antibody titer can be restored by increasing DO following vaccination, optimum DO at early post-vaccination appears to play an important role in antibody production.

To address the potential role of moderate hypoxia on immune-related gene expression spleens sampled at 0h, 3h, 3rd and 7thdpv were analyzed. This time protocol was chosen because most of the detected genes expression was reported at early post-vaccination time points in previous studies [31]. In the present study, expression of IL-1β, MHC class II β, TCR β, IgM, BCAF were detected. IL-1β is a pro-inflammatory cytokine [32, 33]. The expression of IL-1β was significantly up-regulated in the normoxic group at 3h pv (post vaccination) and 3 dpv compared to the moderate hypoxic group, with a pronounced increase (~66 fold) at 3h pv. Such a negative impact of acute hypoxia on IL-1β induction in Nile tilapia has been reported previously [19]. Significantly higher expression of IL-1β was reported in rainbow trout, *O. mykiss* immunized with *Yersinia ruckeri* [34]. Our finding is also further supported by Kavamme *et al*. (2013) [20], where chronic hypoxia

impaired IL-1β expression in the head kidney of Atlantic salmon, *S. salar,* following intra-peritoneal injection of *V. anguillarum* vaccine. Following vaccination, significant up-regulation of IL-1β in other fish species has been previously reported [35, 36, 37], and authors suggest that this is likely a systematic innate immune response to the bacteria immunization.

At 24h pv and 3rd dpv, a significant up-regulation of MHC class IIβ in the normoxic group was observed. MHC class II β, known to form cell surface receptors in antigen-presenting cells such as macrophages and B cells [38, 39, 40,41].MHC class IIβ expression in rainbow trout is significantly up-regulated following vaccination with *Y. ruckeri*. Furthermore, Atlantic salmon vaccinated against *Aeromonas salmonicida, V. anguillarum* and *Vibrio salmonicida* also showed significant expression of spleen MHC class II compared to the non-vaccinated group [42]. Moreover, Japanese flounder, *Paralichthys olivaceus* vaccinated with *Edwardsiella tarda* antigen combined with Freund's incomplete adjuvant significantly up-regulated MHC IIα expression in spleen at 24h post vaccination [43].

T cell receptor β is known to function in ligand binding [44,45]. In our study, TCR β expression in spleen of normoxic fish following 3rd and 7th dpv was significantly up-regulated. A similar result was obtained by Raida and Buchmann, (2007) [34] following vaccination of rainbow trout against *Y. ruckeri* at 72h post vaccination. However further research is necessary to examine to what extent these gene expressions are involved in fish antibody production processes following vaccination with inactivated bacteria.

B cell activating factor (BCAF) expression was not significantly different between our normoxic and moderate hypoxic groups. It is noteworthy that expression of BCAF had greater individual variability. In contrast to the BCAF expression profile, IgM transcript was significantly up-

regulated in the normoxic group following 3rd dpv compared to the moderate hypoxic group. A clear time-dependent elicit of IgM transcription level in orange- spotted grouper, *Epinephelus coioides,* has been reported following vaccination with formalin killed *Vibrio alginolyticus* [46]. Furthermore, Atlantic salmon receiving oil adjuvant commercial vaccine were able to elicit spleen IgM transcription [47]. In contrast, Raida and Buchmann (2007) [34] failed to find significant up-regulation of IgM transcript level in rainbow trout following vaccination. These discrepancies between different studies may be due to the differences in primers designed for surface-bound and secreted forms of antibodies, different experimental conditions, and differences in experimental fish species used. If we examine the gene expression pattern in moderate hypoxic vs. normoxic fish during the first week of vaccination, it is clear that moderate hypoxia causes overall suppression of transcription level of genes involved in regulating antibody response at early post-vaccination. These results further strengthen the idea that optimum oxygen at early post-vaccination, where important immune regulatory pathways are active, is an important factor in achieving maximum antibody production following vaccination.

Specific antibody production against target pathogens used as surrogate marker to detect the vaccine efficacy and it has shown that antibody production is positively correlated with protection of Nile tilapia against common pathogenic bacteria species [48, 49]. Therefore, to evaluate the possible anti-*V. anguillarum* activities of serum in vaccinated moderate hypoxic and normoxic fish, serum collected from vaccinated and PBS injected fish was subjected to antimicrobial assay. The bactericidal activity of vaccinated normoxic fish was significantly higher at 7th and 14th day following vaccination compared to the moderate hypoxic fish. Those differences in antimicrobial activities between experimental groups disappeared following adsorption and precipitation of antibody, suggesting that serum anti-*V. anguillarum* activities are precisely based on the specific

antibody present in the serum. This view is further supported by the experiment done on complement mediated serum killing, where anti-*V. anguillarum* activities were markedly inhibited in serum treated with EGTA and $MgCl_2$. As in mammals, two main complement pathways are responsible for antibacterial activity in fish against gram-negative bacteria [50]. The classical complement pathway is known to activate through antigen antibody complex in the presence of Ca^{2+}, while the alternative pathway can activate in the absence of such an interaction between antigen and antibody [51]. In our study, we selectively inhibited the classical complement pathway by chelating Ca^{2+} in the serum by adding EGTA + $MgCl_2$. Furthermore, both classical and alternative pathways were abolished by chelating Mg^{2+} in the serum using EDTA. When the classical complement pathway is inhibited but the alternative pathway is active, serum anti-*V. anguillarum* activities were markedly reduced (~40% reduction) in vaccinated fish. This reflects that the higher serum killing observed in vaccinated fish is via the classical complement pathway, which activates by specific antibody presence in the serum. In agreement with our study, Boesen*et al*. (1999) [50] and Ourth and Bachinski, (1987) [52] found that serum anti *V. anguillarum* activities in rainbow trout and channel catfish were initiated and amplified via an antibody - dependent classical complement pathway. Moreover, the differences we observed in serum killing between the EGTA+ $MgCl_2$ treated groups and the EDTA treated group were non-significant. Therefore, we can assume that the potential contribution of the alternative pathway in serum killing was weaker. Following vaccination, serum lysozyme activity failed to show any significant differences between vaccinated groups at all the detected time-points. The observed differences in anti-*V. anguillarum* activities between normoxic and moderate hypoxic serum did not therefore seem linked to lysozyme activity. Accordingly, the observed low anti *V. anguillarum* activities in moderate hypoxic fish following vaccination are apparently due to low antibody production. Such

a positive correlation between antibody production and serum antimicrobial activity was also found in Atlantic halibut, *Hippoglossus L*. following vaccination against *V. anguillarum* [53].

Following booster dose, antibody production as well as serum antimicrobial activity found to be not different between moderate hypoxic and normoxic groups suggesting that booster dose may be the possible counter measure to improve the vaccine efficacy in Nile tilapia kept at moderate hypoxic environment. Furthermore, booster dose significantly increased the antibody production in Nile tilapia and this might be reflecting the clonal expansion of B cells following vaccination as seen in mammals. However, detail study is necessary to confirm this idea. High dose vaccination also increased the antibody production in moderate hypoxic fish but magnitude of antibody production was far more below than booster vaccination. Even though these modifications in vaccination program appear to be better counter measures to mitigate moderate hypoxia mediated suppression on antibody production and antibody mediated serum killing, it should noteworthy that there are several antibodies mediated immune mechanisms, those that involved in host defense may not recover as serum killing. Therefore, it is too early to make solid conclusion as booster or high dose as valid approach or better counter measures. Further studies are necessary to evaluate the efficacy of booster or high dose vaccine to improve other immune mechanisms those that we did not studied.

Findings in this Chapter reinforce the notion that persistence of moderate hypoxia in fish farms may be the reason fish are not getting the degree of protection expected following vaccination. Overall, it is noteworthy that moderate hypoxia can persist unnoticed in fish aquaculture systems, since severe behavioral, morphological and biochemical alterations are not observed. However, it is clear that there are profound negative impacts on antibody production in vaccinated Nile tilapia, hence directly affecting vaccine efficacy.

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CHAPTER 3

Clearance mechanisms of *S. agalactiae* in vaccinated *Oreochromis niloticus* (Nile tilapia) are modulated by moderate hypoxia, thereby increased the susceptibility following experimental challenge.

1

Abstract

Streptococcus agalactiae is emerging bacterial pathogen in tilapia aquaculture industry around the world and strain specific vaccines are known to provide protection against this pathogen. The protection offer via vaccination can modulates by several factors including persistence of lower dissolved oxygen (DO) in the rearing water. Lacks of detailed knowledge on potential immune mechanisms those involve in clearing of this pathogen in fish following vaccination and influence of lower oxygen level or hypoxia on those mechanisms have made it difficult to optimise the vaccination programs to gain the maximum protection. In view of those gaps this study aim to reveal the immunological basis of protection offered via vaccination and influence of moderate hypoxia (55 ±5% DO) on those mechanisms in Nile tilapia.

Serum antibody titer found to be significantly higher in normoxic vaccinated fish compared to the moderate hypoxic vaccinated fish at 15^{th} and 30^{th} dpv. *S. agalactiae* appear to be resistance for serum killing even when presence of the specific antibodies. The cumulative motality following challenge was significantly lowered in vaccinated normoxic fish compared to the moderate hypoxic vaccinated fish and control fish reflecting that pre challenge antibody titer is correspondence with the survival. Lowest mortality among control groups was found in normoxic control fish suggesting that DO dependency of the pathogen clearance mechanisms. The blood and tissue pathogen burden detection studies revealed that normoxic vaccinated fish are free from *S. agalactiae* in their blood at all the detected time points. In contrast, moderate hypoxic vaccinated fish took more than 5 days for total clearance of pathogen in their blood. Pathogen burden in tissues and blood appear to be correlated with mortality and rapid clearance of bacteria in blood appear to lower the cumulative mortality in fish. Highest bacteria burden in blood, head kidney and brain of moderate hypoxic control group compared to the normoxic

control group might have linked to the compromised innate immune clearance mechanisms under moderate hypoxic condition.

In vitro studies revealed that phagocytosis and intracellular reactive oxygen species (ROS) production were significantly lowered by moderate hypoxia. Furthermore, presence of specific antibodies and higher antibody amount in the opsonising serum increased the phagocytosis, ROS production and lowered intracellular survival of *S. agalactiae* in HKLs. These data demonstrated that higher antibody level as well as optimum DO positively correlate with pathogen clearance ability of phagocytes. Therefore, higher cumulative mortality in moderate hypoxic fish even after vaccination not only linked to the lower antibody production but also to lower phagocytic functions. Overall this study highlighted that mechanism of vaccine based protection in Nile tilapia against *S. agalactiae* is appear to be antibody dependent phagocytosis mechanisms and efficacy of this mechanism is DO dependent hence emphasized the needs of optimum oxygen to gain the maximum protection offered via vaccination.

3.1 Introduction

Streptococcus agalactiae is gram positive encapsulated bacterium known as Group B *Streptococcus* (GBS) causes meningoencephalitis in Nile tilapia [1]. This ubiquitous bacterium has evolved several strategies to avoid host immunity hence control or prevention is biggest challenge in the aquaculture industry [2, 3, 4]. Recently, studies proved that vaccination is attractive and effective prophylactic measure against *S. agalactiae* infection [5, 6, 7]. The capsule specific antibodies presence the serum following vaccination improved opsonophagocytic killing of this pathogen in mouse and revealed that killing was clearly accompanied by oxidative burst of macrophages [8]. In addition, specific antibodies in serum against this pathogen found to be positively correlated with higher survival in Nile tilapia following challenge [5].

Previous study (Chapter 2) clearly demonstrated that specific antibody titer as well as serum bactericidal activities in vaccinated Nile tilapia against *Vibrio anguillarum* is significantly lowered following exposure to moderate hypoxia. In addition, Welker *et al*. (2007) [9] found that channel cat fish exposed to sub lethal hypoxia had lower antibody titer at subsequent challenge with *Edwardsiella ictaluri*. Those findings are highlighting that hypoxia is negatively affecting on antibody production of fish. On the other hand, according to the *in vitro* studies, hypoxia exposure significantly reduced reactive oxygen species production (ROS) in anterior kidney leukocytes of *Fundulus heteroclitus* [10]. Another study pointed out that, phagocytic capability and phagocytic index in Nile tilapia are decreased following exposed to hypoxia [11]. According to those findings, hypoxia not only affects on antibody production, but also on phagocytes function. Furthermore, Bunch and Benjo, (1997) [12] found that hypoxia exposure increases susceptibility of Nile tilapia to *Streptococcus* sp. Similarly, short time sub lethal hypoxia (1mg/L) exposure induced stress response in Nile tilapia which

increased mortality following experimental challenge with *S. agalactiae* [13]. Several recent studies have been reported that Streptococcosis in fish directly or indirectly driven by environmental factors, such as dissolved oxygen, pH, salinity and temperature [14, 15, 16]. Collectively, these scientific evidences led us to hypothesize that hypoxia exposure may reduce *S. agalactiae* clearance abilities in vaccinated Nile tilapia thereby increase susceptibility to Streptococcosis even after vaccination.

Up to date, most *S. agalactiae* vaccination studies detected percent survival, mortality or antibody production as measures of vaccine efficacy. Apart from those parameters, mode of protection offered via these vaccines were less concerned hence it is not clear that blood and tissue clearing mechanisms in vaccinated fish following exposure to *S. agalactiae.* On the other hand, influence of environmental changes such as hypoxia on those potential clearance mechanisms also less studied. In view of these gaps, this study conducted to reveal the impact of moderate hypoxia on *S. agalactiae* clearance ability in vaccinated and non-vaccinated Nile tilapia. Based on our previous work, in this study, moderate hypoxia defined as a low oxygen condition where no morphological, behavioural or blood biochemical alteration could observe in Nile tilapia except packed cell volume and lymphocyte count. Apart from detection of cumulative mortality and antibody titer, I examined number of culturable bacteria in blood, head kidney and brain following challenge with *S. agalactiae* to reveal that pathogen clearance ability and its correlation with vaccination and ongoing exposure to moderate hypoxia. Furthermore, several *in vitro* studies carried out with head kidney leukocytes and serum in order to reveal the impact of moderate hypoxia and anti- *S. agalactiae* antibody on leukocytes killing abilities and serum bactericidal abilities. Larger body of experimental evidences expected to gather in this study and their inter - connection will broaden our knowledge on vaccine based protective mechanisms in Nile tilapia against *S. agalactiae* and impact of moderate hypoxia on these protective mechanisms.

3.2. Materials and Methods

3.2.1 Fish and rearing conditions

Nile tilapia cultured in Tokyo university of marine science and Technology weighing 55±12.3 g with no history of *Streptococcus agalactiae* were acclimatised 3 weeks for moderate hypoxic (55±5% DO) or normoxic (85±5% DO) conditions adjusted by manipulating aeration and directly injecting N_2 or O_2 gas to the tanks through aerators connected with flow meter (Kofloc, Kyoto, Japan). Dissolved oxygen and temperature in each tank was measured using oxygen meter (Mettler Toledo, Switzerland) 3 times per day with 6 h intervals and all other water qualities were recorded daily. The fish were randomly assigned to twelve, 60 L glass tanks of 20 fish per each connected with recirculation system. Water temperature, pH, ammonia, nitrate and nitrite were 25±0.5°C, 7.2±0.2, less than 2 mg/L, less than 10 mg/L and less than 0.5 mg/L respectively. The fish were fed with commercial dry feed (Nippai, Kanagawa, Japan) twice per day until satiation and feeding was ceased 24 h before the sampling and restored following sampling. Photoperiod held constant at 12 h dark and 12 h light per day. Water flow rates in each tank was adjusted as 1 L /min in order to avoid any other changes in water qualities except dissolved oxygen and water in the storage tank and settling tank of the recirculation system was replaced 3 times per week except during challenge period where water have been replaced twice per week. To confirm the absence of *Streptococcus agalactiae* in experimental fish population, randomly selected 12 fish were euthanized in aerated water bath contained 2-Phenoxyethanol (300 µl/L) (Wako, Japan), and brain, kidney, spleen samples were cultured on Columbia agar (Difco Laboratories) supplemented with 5% ovine blood (CBA) and incubated for 48 h at 25°C. The blood samples were collected from caudal vein puncture with 2.5 ml syringe (Pre coated with heparin) fitted with 24 G needle. Those blood samples were centrifuged at 3500 rpm, 15 min, 4°C; serum separated and stored in 4°C or -20°C. Separated

serum used for the slide agglutination with formalin killed *S. agalactiae*. All the tested fish were confirmed to be free from *S. agalactiae* with bacteriological examination and none of the examined serum samples shown agglutination with *S. agalactiae*. Once confirmed the absence of *S. agalactiae*, fish were used for the following experiments. The fish handling and treatment were done according to the ethical principles proposed in administrative guideline offered by Head of Science and Minister of Education in Japan.

3.2.2 *In vivo* experiments.

3.2.2.1. Vaccine preparation and vaccination.

Beta haemolytic, catalase negative, *S. agalactiae* (serotype III JP17) isolated from brain of diseased Nile tilapia showing clinical signs of meningoencephalitis and eye opacity was used in this experiment. The bacterium was confirmed as *S. agalactiae* using species specific primers [32]. *S. agalactiae* grown on Columbia agar supplemented with 5% ovine blood (CBA) at 25^0C for 48h. Resulted colonies were suspended in Todd Hewitt Broth (THB) and grown for 24 h at 25 ^{0}C in 100 rpm shaking water bath. Following incubation, bacteria were pelleted, washed twice with 1×PBS and re-suspended in the 1×PBS for challenge test (1.3×10^8 CFU/ml). The bacterium was grown in similar manner for 48 h, treated with 3% formaldehyde, kept in 4^0C over night for inactivation, washed 3 times with sterile 1×PBS and re - suspended in the 1×PBS for vaccination (1×10^{10} CFU/ml) or agglutination test (OD = 0.5 at 600 nm). To confirm the inactivation, 500µl of inactivated bacteria suspension was spreaded over TSA plates and incubated at 25°C for 24 – 48 h. Fish in to be vaccinated groups were injected intra peritoneal route with 100µl of prepared formalin killed pellet. Before vaccinate or challenge, fish were anaesthetised using 2-phenoxyethanol to reduce the stress and immediately after injection, fish were kept in a well aerated water tank until they recover. Following recovery, fish were

transferred to the initial treatment tanks. For the control groups, 100µl sterile PBS was similarly injected. Two control groups were maintained in moderate hypoxic and normoxic conditions.

3.2.2.2. Post vaccination sampling

Fish were sampled at o day (before vaccination) 7^{th} 15^{th} and 30^{th} days following vaccination and blood samples were collected, serum was separated as explained before. Blood samples were collected from 3 fish in each group at each sampling point. The serum samples were used to determine agglutination antibody titer, serum lysozyme activities and serum killing activities against *S. agalactiae*.

3. 2.2.3 Agglutination antibody titer

Specific antibody production in serum was evaluated by agglutination assay following a previously described method [chapter 2, 2.2.2.3]. Briefly, 50µl of serum subjected for two fold diluted across 96 well plate. Fifty microliter of formalin inactivated *S. agalactiae* pellets (0.5 OD at 600 nm) were added to each well, mixed and incubated at 25°C for 18 h and observed under the light microscope with lower magnification. Highest serum dilution showing granular dispersed mat with fuzzy edges at the bottom of the well taken as agglutination titer of the sample. Circular compact button at the bottom was considered as negative titer.

3.2.2.4 Serum killing of *S. agalactiae*

Serum killing activities were measured as explained in chapter 2 (2.2.2.4). Briefly, immune and non- immune serum obtained from fish reared under normoxic or moderate hypoxic conditions were added to the 96 well round bottom plate as 50µl serum per each well. The mid log phase *S. agalactiae* suspension was prepared, washed and diluted in 1×PBS to get approximately 1 $\times 10^6$ CFU/ml and 50µl of prepared suspension was added to each well mixed and incubated for 2 ½ h at 25°C with mild shaking (100 rpm). Bacteria suspension incubated with 1 x PBS served as positive control. At this point, 15 µl of MTT (5 mg/mL) was added to each well, mix by pipetting and incubated for 15min at room temperature. Resulted formazan was dissolved in 50 µl of DMSO, OD in each well was read at 570 nm. Serum killing activity was determined by following formula and results were given as % inhibition of *S. agalactiae* relative to the positive control.

$$\% \text{ inhibition of } S.\ agalactiae = \frac{(\text{OD of positive control} - \text{OD of the sample})}{\text{OD of positive control}} \times 100$$

3.2.2.5 Serum lysozyme activities

Immune and non- immune serum obtained from Nile tilapia in each oxygen conditions were used for the lysozyme assay. Serum lysozyme activities were measured according to previously described turbidometric method [chapter 2, 2.2.2.6]. Lysozyme activity in each sample was measured as reduction of absorbance units at 450 nm and expressed as lysozyme concentration (µg/mL).

3.2.2.6 Challenge experiment

For the challenge experiment, intra-peritoneal injection method was used. Bacteria culture for the challenge experiment was prepared as explained before. Series of preliminary experiments were conducted to find the suitable challenge dose. For that purpose, fish were injected with different doses of live *S. agalactiae* and infection pattern was studied for 2 weeks. Twelve fish were tested for each challenge dose and the dose which is able to disseminate pathogen to the brain and kidney within 24 h and causes 50 ±7% mortality during 2-week post injection was selected as challenge dose for the experiment to observe tissue distribution pattern of this pathogen. The dose for the challenge experiment was selected as 1.3×10^8 CFU/ ml. At the 30th day following vaccination, fish were placed in an aerated bucket contains 2-Phenoxyethanol at a strength sufficient to induce light anaesthesia within 2-4 min of introduction and injected with 0.1ml of prepared bacteria suspension via intra-peritoneal route. Following injection, fish were returned to their original treatment tanks and observed and confirmed their recovery. Fish were fasted day before the challenge and feeding was resumed 24 h post challenge.

Throughout the experimental period, fish in each oxygen condition was observed for behavioural morphological abnormalities. Dead fish were removed daily and head kidney and brain swabs of freshly dead fish were spreaded on CBA to recover *S. agalactiae* and isolates were confirmed as S. *agalactiae* as explained before.

3. 2.2.7. Clearance of *S. agalactiae*

Fish were sampled at 1st, 3rd, 5th, 7th and 15th days post challenge. Three fish from each experimental group were randomly sampled at each sampling date to collect blood, brain and head kidney samples. Blood was collected to a heparinized Eppendorf tube and kept on ice.

Fish were decapitated; head kidney and brain were collected aseptically and transferred to the L- 15 medium ((Leibowitz, Sigma, no antibiotic supplemented) kept on ice until use for the bacteria re-isolation.

Head kidney and brain samples collected at 1st, 3rd, 5th, 7th and 15th days post challenge were weighed, homogenised separately as 100 mg in 5 ml sterile PBS. Resulted suspensions were centrifuged, supernatant were collected and serially diluted in TSB. One millilitre of each dilution was plated on TSA and CBA, incubated at 25°C for 48 h prior to CFU determination. CFU was expressed as mean CFU (n = 3 from each group at each sampling date) and bacteria count was given as CFU/g of tissue. In the case of blood samples, 100 µl of heparinized blood was added into the 4.9 ml of distilled water (pH = 11) to disrupt the cells and release any bacteria surviving in the cells. Resulted suspension was serially diluted in TSB, plated, incubated and CFU were calculated as explained before.

The CFU /ml of blood = No of CFU counted on plate × dilution factor × 10.

The CFU /g of tissue = No of CFU counted on plate × dilution factor × 10

3.2.3*In vitro* experiments

3.2.3.1 Head kidney leukocytes isolation

To isolate head kidney leukocytes (HKLs), healthy non vaccinated Nile tilapia (n =12) reared in normoxic condition were anaesthetised with 2-Phenoxyethanol, decapitated, head kidney tissue was removed aseptically and transferred in to a 15 ml tube with L-15 medium supplemented with 1% fatal bovine serum (FBS), 100 U/ml streptomycin/penicillin. The head kidney tissue was gently crushed and passed through 100 µm cell strainer (BD falcon). Resulted cell suspension was used for the leukocyte isolation as shown in figure 3.1. Briefly cell

suspension layered over 34% and 51% percoll (GE health care) density gradients, centrifuged at 800 g for 30 min at 4^0C. The leukocytes were collected at the 34-51% percoll interface and washed 3 times with L-15 medium containing 1% FBS. Resulted cell suspension was tested for viability using 0.2% trypan blue (Sigma St Louis, MO, USA), viable cell population was calculated using C-chip haemocytometer (Improved Neubauer type haemocytometer, Digital Bio, Japan) and adjusted to 1 x 10^6 cells/ml.

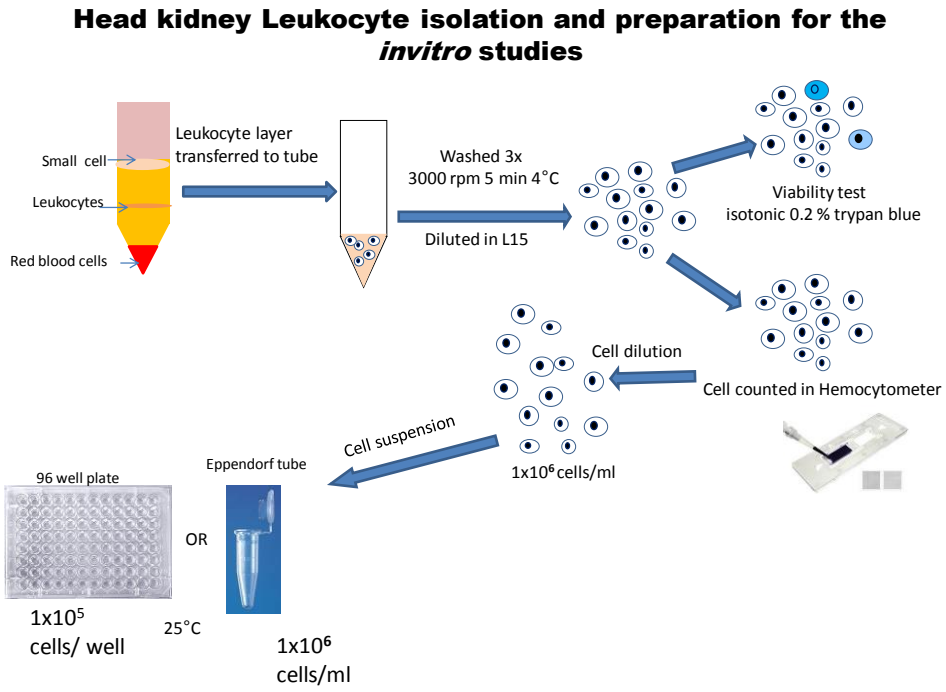

Figure 3.1: Diagrammatic view of experimental procedure used for the isolation of head kidney leukocytes.

3.2.3.2. Blood collection and serum separation

Blood samples were collected and serum was separated as explained before. Serum samples stored at 4°C used for the assays within 24 h and samples stored at -20°C were used whenever necessary.

3.2.3.3. Fluorescence bead preparation and opsonisation for phagocytic assay

Green fluorescence beads (1µm**,** Polyscience Ink, Warrington, PA) were used for the oxygen dependent phagocytosis experiments. Before each experiment, fluorescence beads were suspended in 1xPBS, pelleted at 13 000g, 60 min at 4^0C in high speed cooling centrifuge. The pelleted beads were re-suspended in the PBS and opsonised with homologous serum (1:20 serum to bead suspension ratio) for 1 h at 4^0C. Following opsonisation, bead suspension was washed with 1 x PBS, volume adjusted to give 5 x 10^7 beads /ml and incubated with cells at the ratio of 1:50 (cell: bead).

3.2.3.4 DO dependent phagocytosis activity (*invitro*)

HKLs were isolated and prepared for the experiment as described before. Isolated HKLs suspensions were allowed to equilibrate in the 12 well cell culture plates (BD Falcon) at 25^0C for 24 h prior to the experiment. To obtained the moderate hypoxic condition or hypoxic condition (25% DO saturation), L-15 medium was gassed for 3-4 h before begin the experiment by bubbling the nitrogen and oxygen gas in to the medium via special glass apertures fitted with flow meters which connected to the nitrogen and oxygen outlets. Oxygen meter (Metler Toledo) fitted to the glass apertures used to measure the oxygen tension in the medium. For normoxic condition the medium gassed with oxygen. Preliminary experiments were conducted to observe oxygen retention time in gassed medium + cells in each oxygen conditions and confirmed that

within experimental period, DO in each oxygen treatment varies within the range of ± 5% from original DO. Cells were incubated in 12 well plate as 1ml of cell suspension per each well and prior to running the assay, plate was centrifuged, supernatant was removed and medium was replaced with 900µl L-15 medium gassed to get moderate hypoxia or normoxia. Cells were allowed to equilibrate at each oxygen condition for 2h and at the beginning and at the end of the experimental period, oxygen level in each well was measured using oxygen sensor to confirm that cells were exposed to hypoxia, moderate hypoxia or normoxia throughout the experimental period. Preliminary experiments were conducted to assay the impact of gassing and hypoxia on cell viability and confirmed that cells in hypoxic conditions are equally viable as cells in normoxic condition. Opsonised fluorescence beads (Polyscience Ink, Warrington, PA) (1:50 cells: bead ratio, pre washed and opsonised with homologous serum obtained from Nile tilapia) were used for the phagocytic assay and beads were suspended in L-15 medium to get the indicated cell to bead ratio and resulted suspensions were also gassed to moderated hypoxic or normoxic conditions. At the end of the 2 h incubation period, plates were spun for 5 min at 250 g, 25°C, supernatant removed, 900 µl of gassed bead suspensions were added and incubated for 2 h. At this point plates were spun for 10 min at 250 g 25°C, supernatant removed, ice cold PBS was added to stop the cellular activities, cell smears prepared on glass slides (12 smears per each group), fixedwith methanol, allow to dry and 500 cells from each slide were randomly counted under fluorescence microscope to calculate phagocytic capacity under each oxygen tension. Phagocytic capacity was calculated according to following equation.

$$\text{Phagocytic capacity} = \frac{\text{Number of leukocytes with engulfed fluorescence bead}}{\text{Total leukocytes counted on glass slide (500 Cells)}} \times 100$$

3. 2.3.5 Opsonin-dependent phagocytosis activity (*invitro*)

In this experiment, head kidney leukocytes were isolated separated and prepared for the phagocytic assay as explained before. Blood samples were collected from vaccinated Nile tilapia reared under normoxic and moderate hypoxic conditions (at 30^{th} day post vaccination) and used to get the immunized serum and blood from PBS injected fish was used to collect non immunized serum. The mid log phase *S. agalactiae* suspension was diluted in PBS to get the approximately 2×10^7 CFU/ml (1:20 cell: bacteria ratio) and divided in to three groups before the opsonisation. One group was opsonised with immunised serum collected from normoxic vaccinated fish (Titer = Log_{10} 2.20). Next group was opsonised with immunised serum collected from moderate hypoxic vaccinated fish (Titer = Log_{10} 1.51) and remaining group was opsonised with non-immunized serum collected from PBS injected fish. Opsonisation was done as 1:10, serum: bacteria suspension ratio for 30 min at 25°C in rotary shaker. Following the opsonisation cells were washed with 1×PBS to remove unbound serum component and re-suspend in L-15 medium (free from antibiotics and FBS) to gain 2×10^7 CFU/ml. Twelve well plate contained HKLs (1ml cell suspension in each well which contained 1x 10^6 cells /ml) was spun, supernatant removed, opsonised bacteria suspensions were added and incubated for 1 h at 25°C in orbital shaker. Phagocytosis was stopped by adding ice cold PBS as explained before and cell smears were made, stained with May-Grünwald-Giemsa stain solution (Wako chemical, Japan), bacteria engulfed cell number counted and phagocytic capacity was determined as explained before. In each experiment, the group of leukocytes were used for the phagocytic experiment exactly as explained but incubated at 4°C. The membrane attachment of bacteria at 4°C used as control to bacteria adhesion.

3.2.3.6 DO dependent production of reactive oxygen species

Intracellular superoxide production was measured using nitro blue tetrazolium assay (NBT assay) as explained by Secombes, (1990) [17], with few modifications. Briefly, head kidney leukocytes were isolated and prepared as explained before and prepared cell suspension was allowed to equilibrate at hypoxic, moderate hypoxic condition, or normoxic conditions as explained in DO dependent phagocytosis assay. At the end of the 2h incubation period at each oxygen conditions, cell plate was spun at 1800 g 25°C for 5 min and supernatant was replaced with phenol red free L-15 medium (Gassed to obtain each oxygen conditions). Immediately, 62.5 µl of NBT from stock solution 6 mg/ml made in 1x PBS and 50 µl of heat inactivated Nile tilapia serum were added to each well. Superoxide production was initialized by adding 20 µl of stimulant, zymosan to each well to get final ratio of 1: 20 cell: zymosan and incubated for 1 h at 25°C. At this point plate was centrifuged at1800 g at 25°C for 5 min, supernatant replaced with 70% methanol and centrifuged again at 1800 g, 25°C for 5 min. The cell button was air dried for 15-20 min and 450 µl of 2 M KOH added and sonicated for 15 min. Five hundred microliters of DMSO (Sigma, Mo, USA) was added into sonicated suspension, mixed and incubated for another 5 min. Resulted suspension was transferred to 96 well plate as 100 µl per well and optical density was measured at 620 nm in microplate reader. The amount of NBT reduction caused by cells alone was also measured and used as a base line. ROS production was given as absorbance units at 620 nm.

3.2.3.7 Opsonin - dependent production of reactive oxygen species

Opsonin - dependent reactive oxygen species production was measured using nitro blue tetrazolium assay (NBT assay) as explained before. In this experiment, all cells are in normoxic conditions. Following equilibrate at 25°C 24 h, cell plate was spun at 1800 g 25°C for 5 min and supernatant was replaced with phenol red free L-15 medium. Immediately, 62.5 µl of NBT from stock solution 6 mg/ml made in 1x PBS was added to each well. Superoxide production was initialized by adding 100 µl of stimulant, *S. agalactiae* opsonized with immunised serum collected from normoxic vaccinated fish or moderate hypoxic vaccinated fish or non-immunized serum collected from PBS injected fish as explained in opsonin-dependent phagocytosis assay. Cell plate was incubated for 1 h at 25°C. At this point plate was centrifuged at1800 g at 25°C for 5 min, supernatant replaced with 70% methanol and ROS production was measured as explained before (3.2.3.6). The amount of NBT reduction caused by cells alone were measured and used as a base line. ROS production was given as absorbance units at 620nm.

3.2.3.8 Intracellular survival of *S. agalactiae* in head kidney leukocytes (HKLs)

HKLs were prepared for the experiment as explained in opsonin based phagocytic assay (3.2.3.5). Live *S. agalactiae* was opsonised with immune and non- immune serum as explained above. HKLs were incubated with *S. agalactiae* in a ratio of 1:20 cell: bacteria at 25°C for 1 h with shaking. At the end of the 1h incubation period, combination of penicillin and streptomycin (100 U/ ml) was added to kill the extracellular bacteria and incubated for 1h at 25°C. This antibiotics combination and concentration were confirmed to effective in complete killing of *S. agalactiae* without having any effect on survival or function of HKLs. Then cell plate was spun at 1800 g 25°C for 5 min, supernatant removed and cells were washed two times with antibiotics free L-15 medium and re-suspended fresh antibiotics free L-15 medium. At this point 100µl of

each cell suspensions was added to the 1.9 ml sterile DW (pH=11), incubated 5 min at room temperature, vortexes and disrupted cell suspension was serially diluted in TSB. Diluted suspensions were plated on TSA and incubated at 25°C for 24-48 h. Resulted bacteria colonies were counted in each group and intracellular bacteria count at 2 h time point considered as initial count and percentage intracellular survival at 4 h and 8 h time points were given as ratio to the initial count in each group.

3.2.4. Statistics

In vitro phagocytosis and ROS productions experiments were repeated three times as independent test series. All the data from *in vitro* and *in vivo* experiments were analysed using one-way ANOVA or unpaired two tailed t-test with a 95% confidence interval. Following ANOVA, Multiple comparisons between groups were performed using Turkish post hoc test. The $p< 0.05$ was considered as significant difference. XLSTAT software was used to perform all statistical analysis in this study.

3.3. Results

3.3.1. *In vivo* studies

3.3.1.1. Serum antibody titer

Serum antibody titer was determined with agglutination assay and a significantly higher ($p< 0.05$) antibody titer was detected in normoxic vaccinated fish at 15th and 30thdpv (Mean Log_{10}±SD, 1.81±0.25 and 2.20±0.14 respectively) compared to moderate hypoxic vaccinated fish at 15th (Mean Log_{10}±SD = 1.47±0.21) and 30th (Mean Log_{10}±SD= 1.51±0.33) days post vaccination (Fig. 3.2). Antibody titer in normoxic vaccinated group showed a gradual increase from 15th to 30th day post vaccination while antibody titer in moderate hypoxic group appear to maintain at the same level within this period.

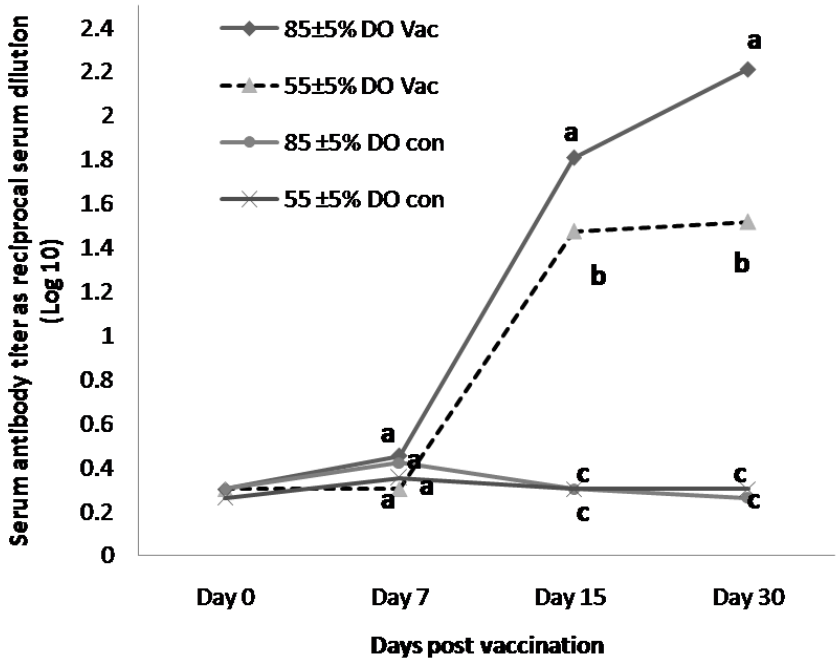

Figure 3.2. Serum agglutination antibody titer against *S. agalactiae* in Nile tilapia at 0,7th 15th and 30th days post vaccination. Agglutination antibody titer was given as log_{10} of the reciprocal serum dilution which shows visible clumping or agglutination. Each value represents mean of triplicates measurement of 3 fish and error bars omitted for clarity. Different letters at the same time point indicates significantly difference ($P < 0.05$) results.

Prominent antibody titer against *S. agalactiae* in vaccinated groups was observed at 15th and 30th day post vaccination. Both vaccinated groups were demonstrated significantly higher ($p < 0.05$) antibody titer compared to their respective controls those that received dose of sterile PBS.

3.3.1.2. Serum bactericidal activities (SBA) against *S. agalactiae*

SBA against *S. agalactiae* shows non-significant results between experimental groups regardless of oxygen condition. It should be noteworthy that SBA was lower in all groups (3-7% inhibition) might reflecting the inability of serum to kill this pathogen regardless of the presence or absence of specific antibodies against *S. agalactiae*.

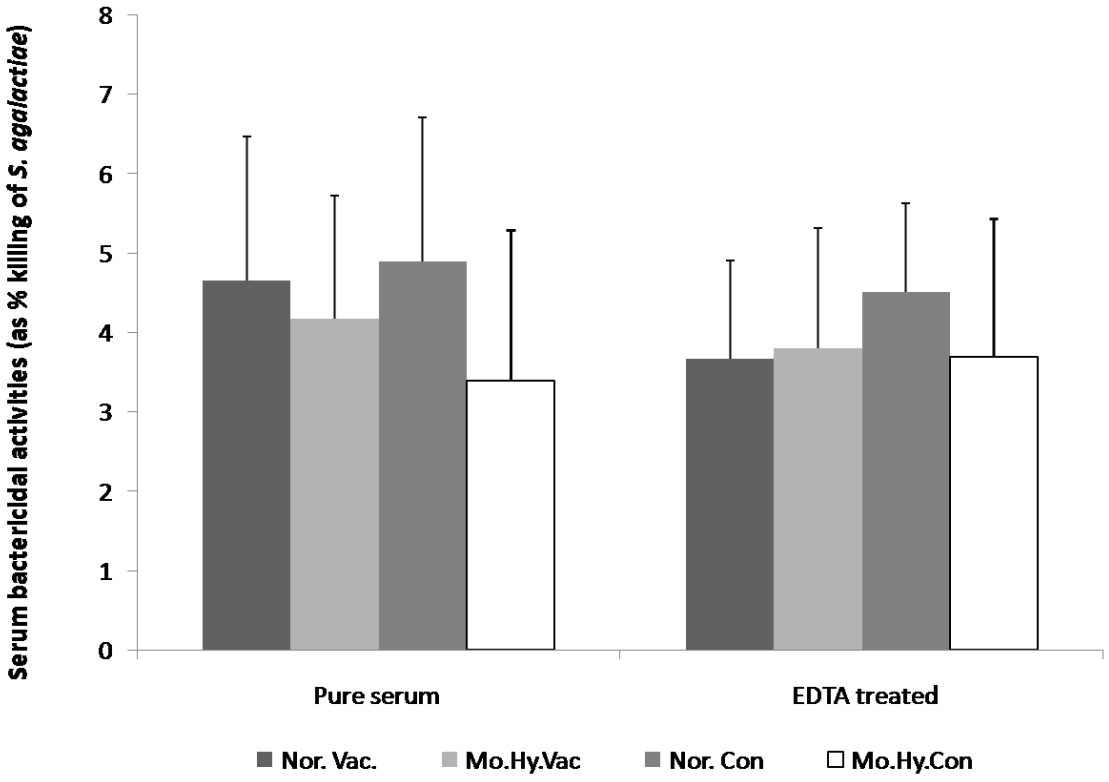

Figure 3.3. Serum Bactericidal Activity (SBA) as % inhibition of *S. agalactiae* in Nile tilapia (n=16) kept under moderate hypoxic or normoxic conditions. Fish were vaccinated with *S. agalactiae*or injected with sterile PBS (Control groups) and SBA was measured at 30th day post vaccination. The values are mean of 4 fish ± SD (n=16). The serum killing of *S. agalcatiae* did not different between groups before and after EDTA treatment. Normoxic vaccinated (Nor.Vac), moderate hypoxic vaccinated (Mo.Hy. Vac), normoxic control (No. Con), moderate hypoxic control (Mo.Hy. Con).

3.3.1.3. Serum lysozyme activities

Serum lysozyme activities were shown non-significant results between groups regardless of the vaccination and DO level (Fig.3.4).

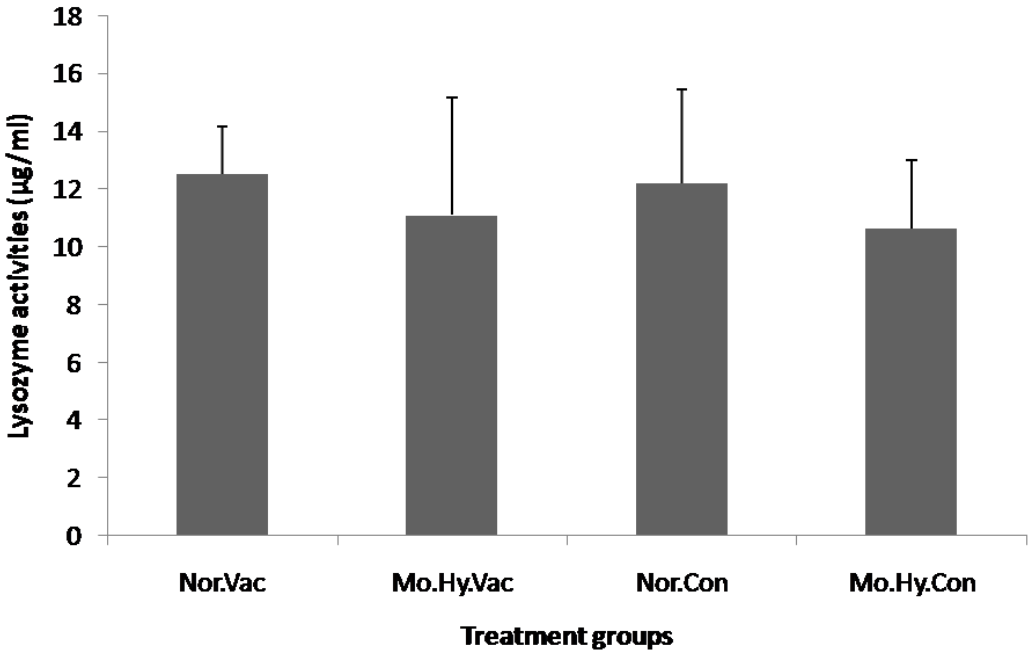

Figure.3.4. Serum lysozyme activity in Nile tilapia (n=16, four fish per each group). Immune and non-immune serum collected from fish at 30th dpv was incubated with *Micrococcus lysodeikticus* for 15 min and reduction of absorbance at 450 nm was measured. Absorbance values converted to the concentration using standard curve created with known concentrations of hen egg white lysozyme and results expressed as lysozyme concentration (µg/mL). Each value is mean±SD

3.3.1.4. Cumulative mortality following challenge (as a percentage)

Behavioural and morphological abnormalities those are known to cause by *S. agalactiae* were observed in diseased fish. Clinical signs of eye opaqueness, irregular swimming, loss of appetite, lethargy, haemorrhages at the base of the pectoral fin were prominently observed in most of the fish in control groups and some of the fish in moderate hypoxic vaccinated group throughout the study period. In contrast, fish in normoxic vaccinated group was not shown

severe clinical signs except loss of appetite which was only exhibited at first two days following challenge. The vaccinated Nile tilapia had a significantly lower cumulative mortality ($P < 0.05$) compared to the non-vaccinated controls. At normoxic condition, cumulative mortality of vaccinated Nile tilapia (5.5%) was significantly lower ($P < 0.05$) than the vaccinated fish kept at moderate hypoxic condition (20%) (Fig 3.5). The PBS injected control fish reared at moderate hypoxic condition seemed more susceptible (74.5% Cumulative mortality) to *S. agalactiae*, compared to the control fish at normoxic condition (45.4% Cumulative mortality) (Fig 3.5).

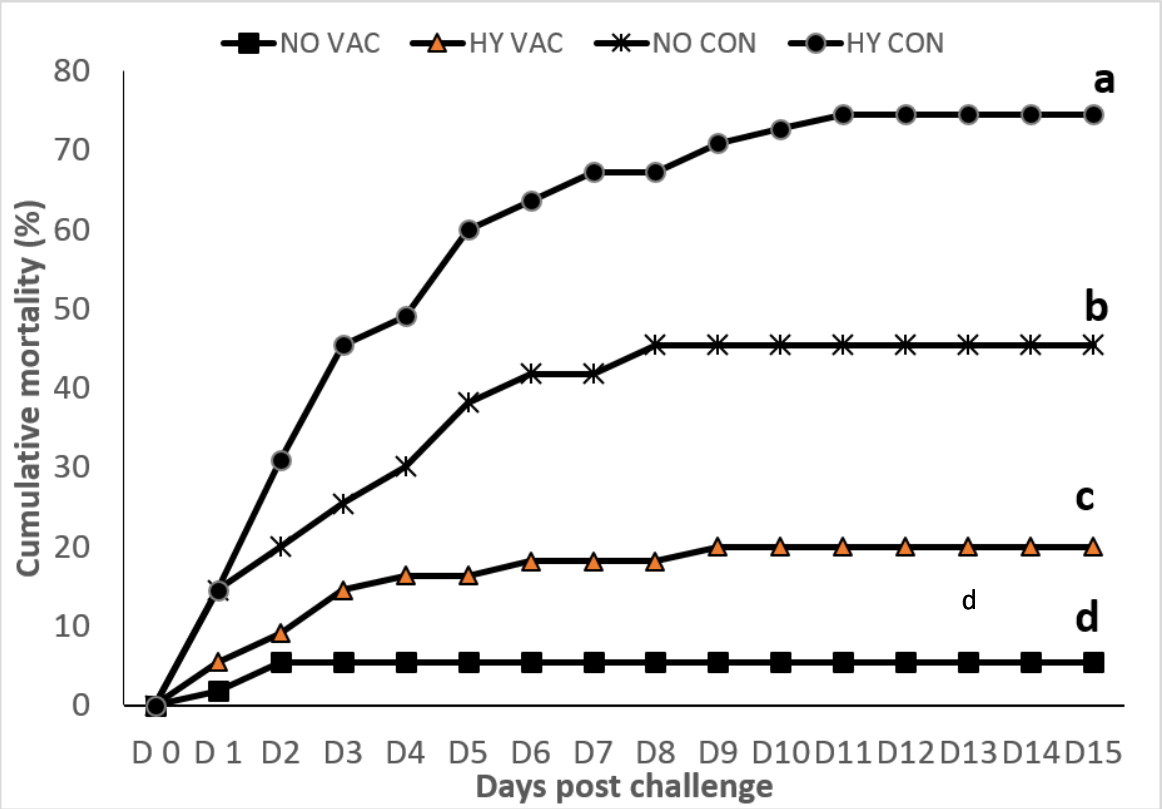

Figure 3.5: Cumulative mortality (%) of vaccinated and non-vaccinated Nile tilapia kept in moderate hypoxic or normoxic conditions following I.P challenge with *S. agalactiae* (1.3×10^7 CFU / fish). Mortality was observed up to 15 days' post challenge. Two hundred fifty fish were used in challenge experiment as 62 fish per each group. Different letters at the end of each line indicates significant difference ($p < 0.05$). Normoxic vaccinated (NO VAC), moderate hypoxic vaccinated (HY VAC), normoxic control (NO CON), moderate hypoxic control (HY CON).

Protection in vaccinated fish following challenge was appeared to be correspondent with pre-challenge antibody titer and DO level in the treatment groups. Mortality was prominent during 1-7 days following challenge in all treatment groups. Fish in all groups started dying after 1day post challenge (dpc) and in normoxic vaccinated group mortality was stabilised within first two dpc while moderate hypoxic vaccinated group took 5 dpc (Fig.3.5). The mortalities in normoxic control appear to be stabilised at 8 dpc while moderate hypoxic control group at 10-12 dpc. *S. agalactiae* was re-isolated from the head kidney and brain of fish died in challenge experiment. Isolates were confirmed as *S. agalactiae* as explained in materials and methods.

3.3.1.5 Distribution of *S. agalactiae* in brain, blood and head kidney of Nile tilapia

The distribution of *S. agalactiae* in head kidney, brain and blood at 1st, 3rd, 5th, 7th and 15th days post challenge was detected in vaccinated and PBS injected control fish kept in moderate hypoxic or normoxic conditions and results summarized in Table 3.1. Fish were sampled randomly (3 fish from each group at each date) from each group at each sampling date and tissue and blood samples were cultured on TSA and CBA to detect the viable bacteria count. During experimental period, overall bacteria burden was significantly higher ($P < 0.05$) in control groups compared to the vaccinated groups (Table 3.1). Among vaccinated groups, normoxic vaccinated fish shown lowest bacteria burden in brain and head kidney at all the detected time points and their blood was found to be free from pathogen at all the detected time points indicates rapid clearance of bacteria from the peripheral blood in this group (Table 3.1). In contrast, moderate hypoxic vaccinated group took more than 5 days to clear the pathogen from the blood stream. The pathogen burden in brain was also had the similar trend where pathogen has totally cleared in brain tissue after 1-day post challenge in normoxic vaccinated

group while vaccinated moderate hypoxic fish took more than 5 days for total clearance (Table 3.1) reflecting lowered clearance rate under moderate hypoxic conditions. In contrast to the peripheral blood and brain, head kidney tissue had highest bacteria count in all the treatment groups at all the detected time points might be due to ability of this pathogen to survive in residence immune cells in this tissue. However, normoxic vaccinated group had significantly lower ($P < 0.05$) bacteria count in head kidney compared to the other groups throughout the study period reflecting that importance of vaccination, higher antibody production and optimum oxygen on effective clearance of this pathogen. It is noticeable that both normoxic and hypoxic control groups had significantly higher ($P < 0.05$) pathogen burden in blood and tissues compared to the vaccinated fish and these data clearly correlates with higher mortalities observed in control groups. Non-vaccinated Nile tilapia kept in normoxic condition exhibited significantly low ($P < 0.05$) bacteria burden compared to the moderate hypoxic control fish in tissues and blood at all the detected time points suggesting that importance of DO on innate immune mechanisms that involve in clearance of this pathogen. Comparison of tissue and blood susceptibility for *S. agalactiae* clearly showed that lower tissue and blood infection is directly correlates with higher antibody level in blood and optimum oxygen level in the water (Fig. 3.5 and Table 3.1). Although higher bacteria number was detected in head kidney tissue, early clearance of bacteria from blood and brain seems to be important for the survival of Nile tilapia following challenge.

Table 3.1. Burden of *S. agalactiae* in brain, head kidney and blood of Nile tilapia at 1st, 3rd, 5th, 7th and 15th days (D) post challenge. Values were given as CFU / g of tissue or CFU/ ml of blood determine by counting bacteria on the TSA plate. Different superscript letters at the same time point indicates significantly different results ($p < 0.05$). At each sampling date 3 fish were randomly sampled from each group.

Tissue	Experimental groups	Days post challenge				
		1 D	3 D	5D	7D	15 D
Brain (CFU/ g)	85 ± 5% DO Vac	$1.33\times 10^{2\,a}$	$0^{\,a}$	$0^{\,a}$	$0^{\,a}$	$0^{\,a}$
	55 ± 5% DO Vac	$5.24 \times 10^{3\,b}$	$4.66\times 10^{3\,b}$	$1.01 \times 10^{2\,b}$	$0^{\,a}$	$0^{\,a}$
	85 ± 5% DO Con	$3.88 \times 10^{4\,c}$	$8.55 \times 10^{3\,c}$	$3.74 \times 10^{4\,c}$	$4.86 \times 10^{2\,b}$	$0^{\,a}$
	55 ± 5% DO Con	$3.37 \times 10^{7\,d}$	$1.87 \times 10^{5\,d}$	$1.46 \times 10^{5\,d}$	$8.73 \times 10^{3\,c}$	$1.37\times 10^{3\,b}$
Blood (CFU/ ml)	85 ± 5% DO Vac	$0^{\,a}$	$0^{\,a}$	$0^{\,a}$	$0^{\,a}$	$0^{\,a}$
	55 ± 5% DO Vac	$5.64 \times 10^{3\,b}$	$1.97 \times 10^{3\,b}$	$3.56 \times 10^{2\,b}$	$0^{\,a}$	$0^{\,a}$
	85 ± 5% DO Con	$8.83 \times 10^{5\,c}$	$1.72 \times 10^{4\,c}$	$0^{\,a}$	$3.83 \times 10^{2\,b}$	$0^{\,a}$
	55 ± 5% DO Con	$2.29\times 10^{8\,d}$	$3.02 \times 10^{5\,d}$	$1.87 \times 10^{4\,c}$	$1.94 \times 10^{4\,c}$	$0^{\,a}$
Head kidney (CFU/ g)	85 ± 5% DO Vac	$5.82\times 10^{5\,a}$	$1.39 \times 10^{4\,a}$	$7.43 \times 10^{2\,a}$	$3.38 \times 10^{3\,a}$	$4.99 \times 10^{2\,a}$
	55 ± 5% DO Vac	$1.92 \times 10^{6\,b}$	$2.83 \times 10^{4\,b}$	$2.603 \times 10^{3\,b}$	$1.12 \times 10^{4\,b}$	$8.07\times 10^{3\,b}$
	85 ± 5% DO con	$7.20 \times 10^{6\,c}$	$3.17 \times 10^{5\,c}$	$6.29 \times 10^{4\,c}$	$3.05 \times 10^{4\,c}$	$6.13 \times 10^{3\,b}$
	55 ± 5% DO Con	$2.39 \times 10^{8\,d}$	$9.09 \times 10^{6\,d}$	$2.91 \times 10^{5\,d}$	$9.44 \times 10^{4\,d}$	$2.64 \times 10^{4\,d}$

3.3.2. *In vitro* studies

3.3.2.1. DO dependent phagocytic activities and reactive oxygen species production in HKLs

Phagocytic capacity of HKLs were significantly lowered ($P < 0.05$) at hypoxic (49.9%)and moderate hypoxic (44.5%) conditions compared to the cells kept in normoxic condition (62.9%) (Fig. 3.6 A). HKLs phagocytic activity was shown non-significant variation between cells kept in moderate hypoxic condition and hypoxic conditions.

Intracellular reactive oxygen species generation or respiratory burst was known as oxygen dependent immune mechanisms therefore lower ($P < 0.05$) ROS production in hypoxic ($OD_{620} = 0.77$) and moderate hypoxic ($OD_{620} = 1.13$) HKLs might be driven by lower dissolved oxygen in the cell culture medium during experimental period (Fig. 3.6 B). In contrast normoxic cells were shown comparatively higher ($OD_{620}= 1.38$) intracellular ROS production. However, the significant different ($P < 0.05$) was only seen between normoxic and hypoxic cells. Intracellular ROS generation was assessed by NBT reduction assay.

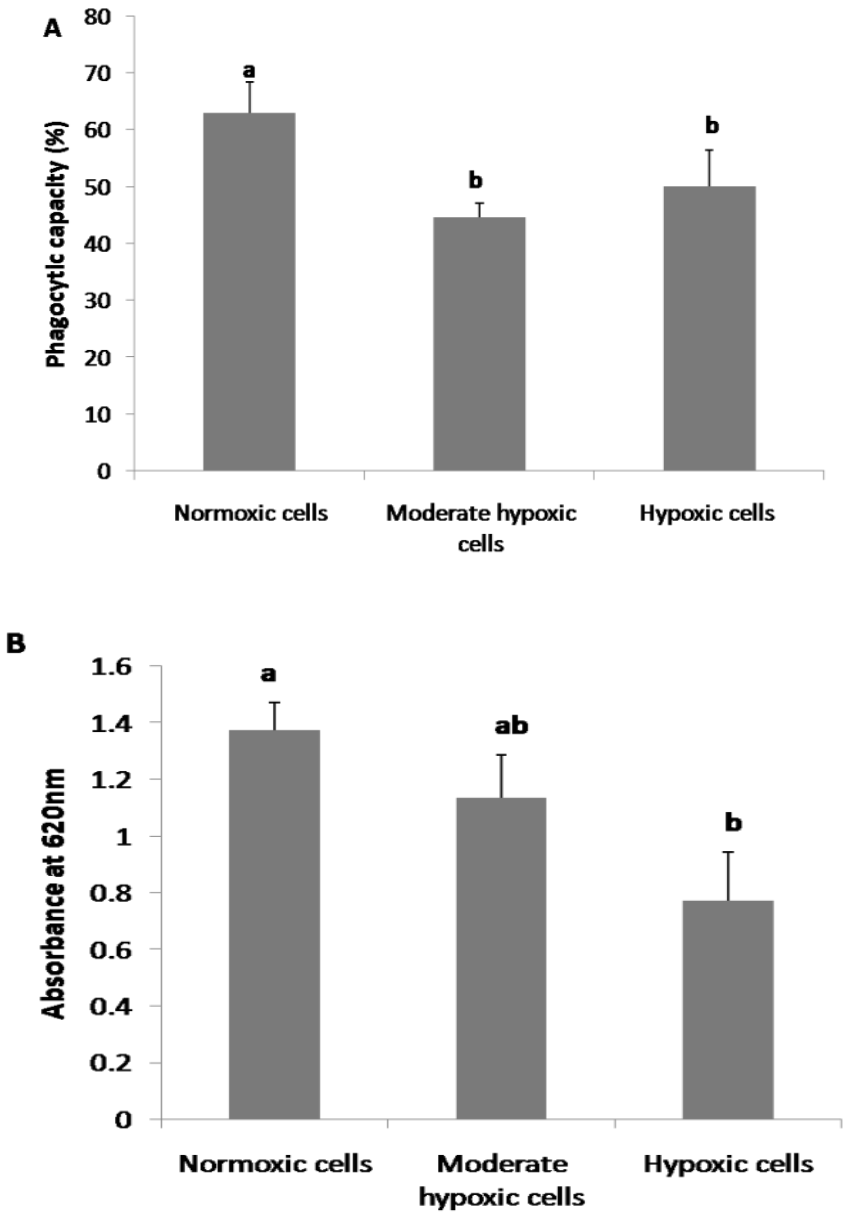

Figure3.6. Comparison of DO dependent phagocytic capacity (A) and reactive oxygen species production (B) in HKLs held under different oxygen conditions (hypoxia, moderate hypoxia, normoxic). Phagocytic capacity was determined by counting the number of cells with engulfed fluorescence beads. Intracellular production of ROS was stimulated with zymosan and NBT reduction was measured at 620nm. Results were given as mean ± SD of 3 independent experiment (n=12). Different letters above the bar indicate significantly different results ($P < 0.05$) between groups.

3.3.2.2. Opsonin dependent phagocytic activities and reactive oxygen species production in HKLs

Effect of opsonin on phagocytic capacity and ROS production of HKLs isolated from Nile tilapia (n = 12) were shown in figure 3.7. Serum samples were obtained from vaccinated normoxic, vaccinated moderate hypoxic or non-vaccinated Nile tilapia. *S. agalactiae* opsonised with serum obtained from vaccinated fish or control fish and incubated with leukocytes. Phagocytic capacity was determined by counting the number of cells with internalised bacteria and it was significantly higher ($P < 0.05$) when cells incubated with normoxic immune serum opsonised bacteria (49.6%) than moderate hypoxic immune serum opsonised bacteria (30%).

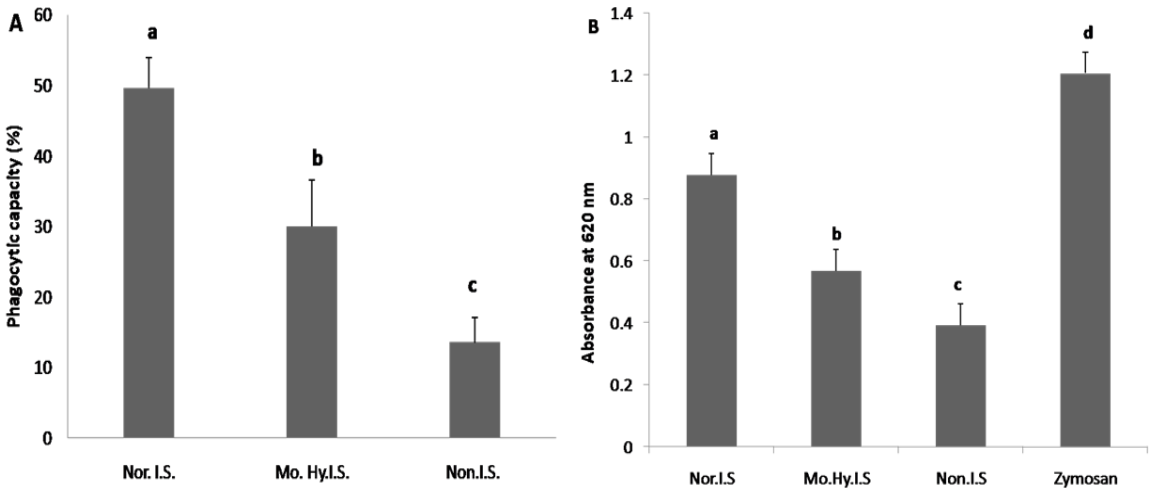

Figure 3.7. Comparison of opsonin dependent phagocytic capacity (A) and reactive oxygen species production (B) in HKLs incubated with *S. agalactiae* opsonise with normoxic immune serum (Nor.I. S) or moderate hypoxic immune serum (Mo. Hy. I. S) or non-immune serum (Non. I. S). Phagocytic capacity was determined by counting the number of cells with engulfed bacteria. Intracellular production of ROS was stimulated by bacteria coated with different serum or zymosan particles and NBT reduction was measured at 620 nm. Results were given as mean±SD of 3 independent experiment (n = 12). Different letters above the bar indicate significantly different results ($P < 0.05$) between groups.

Internalisation of immune serum opsonised bacteria was significantly ($P < 0.05$) higher than the phagocytosis of non- immune serum opsonised bacteria (13.5%) reflecting the efficient uptake of *S. agalactiae* is depends on the presence of specific antibodies in the opsonin serum.

As shown in Fig. 3.7 B, when HKLs were incubated with normoxic immune serum opsonised bacteria, their ROS production was considerably ($P < 0.05$) increased (OD_{620}=0.878) than cells incubated with moderate hypoxic immune serum opsonised bacteria (OD_{620}=0.568). ROS production in HKLs was significantly ($P < 0.05$) lowered when incubated with non - immune serum opsonised bacteria (OD_{620} = 0.393) demonstrating that inefficient ROS generation when internalised via antibody independent pathways. It should be noted that *S. agalactiae* induced comparatively lower ROS production in HKLs compared to the zymosan (OD_{620} = 1.206) HKLs.

3.3.2.3. Intracellular survival of *S. agalactiae* in HKLs

Head kidney leukocytes (HKLs) were incubated with *S. agalactiae* pre-opsonised with sera obtained from vaccinated normoxic fish, moderate hypoxic fish or PBS injected control fish at 30th days post vaccination. HKLs were incubated with bacteria as 1:50, cell: bacteria ratio for 1 h at which point HKLs were treated with antibiotics to kill the extracellular bacteria. Cells were lysed, surviving bacteria were released at 2 h, 4 h and 8 h time points. Surviving bacteria enumerated by counting CFU on TSA plates as described in materials and methods. Number of bacteria internalised at 2 h time point taken as initial viable bacteria count. Higher internalisation was seen when bacteria opsonised with immune serum. At 4 h and 8 h time points, a significant ($P < 0.05$) reduction of viable bacteria count was detected when cells treated with normoxic immune serum coated bacteria (4 h: 49.25%, 8 h: 29.73%) compared to the cells treated with non-immune serum opsonised *S. agalactiae* (4 h: 89.87%, 8 h: 75.63%) where

bacteria appear to be resistance for intracellular killing. This might be reflecting specific antibody mediated internalisation is important in order to trigger the efficient intracellular killing activities against *S. agalactiae* (Fig3.8).

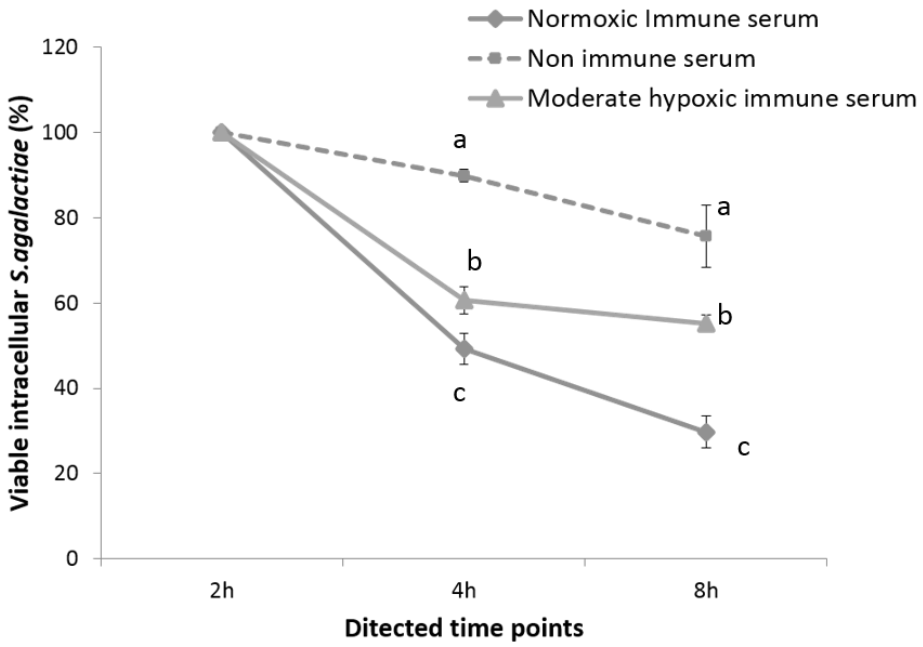

Figure3.8. Effect of opsonisation of *S. agalactiae* with immune serum or non-immune serum on their survival in head kidney leukocytes (HKLs). The viable bacteria count at 4 h and 8 h time points were given as a percentage from initial viable bacteria count which was detected at 2 h time point. Data represent mean ± SD of 3 independent experiments (n = 9). Different letters at the same time point represent significantly different ($P < 0.05$) results.

When bacteria opsonised with moderate hypoxic serum, intracellular survival was markedly decreased from 100% to 60.62% during 2 - 4 h incubation period. However, after 4 h, intracellular killing of remaining bacteria seems to be slow and ~55% bacteria survived at 8 h time point. Initial internalisation was higher when bacteria coated with normoxic immune serum compared to the moderate hypoxic immune serum and control serum (approximately 10-12 CFU, 4-8 CFU and 3-5 CFU per cell respectively). Higher intracellular survival of *S.*

agalactiae in cells treated with moderate hypoxic immune serum coated bacteria might be due to low ROS generation due to lower internalisation of bacteria in this group. However, this should be subjected for further studies.

3.4. Discussion

The aim of this research was to understand the *S. agalactiae* clearance ability and protective mechanisms in vaccinated Nile tilapia under normoxic and moderate hypoxic conditions. Vaccines offer long lasting protection to the fish against infectious organisms by producing specific antibodies and keeping immunological memory against target pathogen [5, 18, 19]. Specific antibodies known to have broad spectrum of functions such as binding to the pathogen, causing pathogen aggregation, immobilizing the pathogen, interfering the pathogen attachment to the receptors, activation of complement cascade to lyses the pathogen, increase opsonophagocytosis and intracellular killing etc. [20, 21, 22]. Therefore, apart from specific antibody production, I examined some of those immune mechanisms and their antibody dependency and DO dependency to reveal the potential antibody dependent pathogen clearing effector mechanisms those that responsible for the protection in vaccinated fish against *S. agalactiae*. Furthermore, co- correlations of specific antibody production and those effector mechanisms with bacteria burden and cumulative mortality in vaccinated Nile tilapia were also studied following experimental challenge.

Following vaccination, significantly higher specific antibody titer was detected at 15th and 30thdpv and specific antibody titer in normoxic vaccinated fish was significantly higher than moderate hypoxic vaccinated fish. Furthermore, exposure to moderate hypoxia increased susceptibility of vaccinated Nile tilapia to *S. agalactiae* which resulted in higher cumulative mortality (20%) compared with normoxic vaccinated fish (5.5% cumulative mortality) following experimental challenge. These results clearly demonstrated pre-challenge specific antibody titer in vaccinated fish corresponded with protection where higher anti *S. agalactiae* antibody level lowered cumulative mortality in Nile tilapia following challenge. In consistence with our study, Pasnik *et al*. (2005) [5] found a positive correlation between protection and anti-

S. agalactiae antibody titer in the Nile tilapia following experimental challenge and explained specific antibodies play an important role in immunity of Nile tilapia against *S. agalactiae*. Several other vaccination studies have been shown that pre-challenge specific antibody level against target pathogen is directly correspondence with level of protection in fish following experimental challenge [18, 19, 23]. Therefore, lower antibody response resulted by exposure to moderate hypoxia in this study appear to be the potential reason why Nile tilapia had comparatively higher susceptibility to *S. agalactiae* even after vaccination.

Interestingly, normoxic vaccinated fish demonstrated effective clearance of bacteria in tissue and blood following experimental challenge compared with moderate hypoxic vaccinated fish. This is apparently due to join forces of higher circulating antibodies and antibody dependent immune mechanism those that have played considerable role in preventing survival and dissemination of pathogen to target organs such as brain and head kidney. Absence of specific antibodies directed against *S. agalactiae* in control groups might have caused lower clearance hence higher bacteria burden was found in their blood and tissues compared with vaccinated fish. It should be noted that even though control fish had higher bacteria burden, normoxic control fish appear to clear pathogen effectively in blood and brain compared to the moderate hypoxic control fish. This might be due to DO dependency of pathogen clearing effector mechanisms.

Lower cumulative mortality and lower blood and tissue bacteria burden seem to be correlated with higher anti-*S. agalactiae* antibody level in Nile tilapia. Therefore, next I examined potential antibody mediated immune mechanisms those that might have played important role in protection of Nile tilapia against *S. agalactiae*.

The complement cascade is important defensive measure consist off with three activating pathways those ultimately merged to one mechanism which responsible for formation of

membrane attack complex [24]. This membrane attack complex is responsible for killing of pathogen by forming pores on their membrane [25,26]. The classical complement pathway is known to initiate by antibodies [27,28]. In this study, serum obtained from vaccinated fish exhibited very low bactericidal activity regardless of the oxygen level and it was not different from bactericidal activity of serum of PBS injected fish. These findings confirm the inefficiency of complement pathways or other serum antimicrobial components on serum killing of *S. agalactiae*. Apparently low bactericidal activity observed in the serum might be due to direct lysing via lysozyme activities or growth inhibition of bacteria caused by binding of other antimicrobial proteins during experimental period. The serum lysozyme activities were also found to be not different between experimental groups. It has been shown that due to some surface components such as BibA [29], group B streptococcus can prevent activation of complement hence impede the generation of membrane attack complex via classical complement pathway [30]. Other studies also explained that terminal sialic acid on the capsule of group B streptococcus facilitates to prevent direct activation of complement via alternative pathway [31,32,33]. Shelby *et al*. (2002) [34] observed significantly higher survival in Nile tilapia following injection of heat inactivated anti- *S. iniae* serum and it was not significantly different from the survival of non-heat inactivated anti- *S. iniae* serum injected fish suggesting that other than specific antibodies, complement and other serum antimicrobial proteins have less importance in protection of fish against *S. iniae*. Based on the results of serum killing activities in this study together with scientific studies those that discovered virulence strategies of *S. agalactiae* those are responsible to avoid complement mediated killing, I suggest that complement based immune mechanisms or other serum antimicrobial components alone ineffective in killing or clearing of *S. agalactiae* in Nile tilapia.

Among the different functions of pathogen specific antibodies, specific antibody based opsonophagocytosis is known as one of the most important function against Streptococcosis in

mammals [35,36,37,38]. Furthermore, studies have highlighted that failures in initial phagocytosis mediated killing mechanisms might allow persistence and establishment of Streptococcosis in animal [8, 38). Phagocytosis is the process where several cells in innate immune system such as neutrophil, macrophages can recognize, internalize and kill the pathogen via receptor mediated mechanisms [39,40]. The phagocytes pathogen recognition process can be non- opsonic via direct recognition of pathogens surface molecules by receptors such as scavenger receptors, lectin receptors or opsonic based recognition via Fc receptor or complement receptors [39]. Opsonins are usually antibodies; complement components, mannan binding lectin, protein A, etc. [41]. Opsonized pathogen recognition, internalization and killing proved to be more efficient than non-opsonic phagocytosis [38,41,42]. Therefore, *in vitro* studies, I evaluated impact of opsonisation of *S. agalactiae* with immune and non-immune serum on phagocytosis process in HKLs. Significantly higher phagocytic capacity was found when opsonized with immune serum than non-immune serum. This result simply pointed out that phagocytosis of *S. agalactiae* is mainly via antibody mediated pathways. In consistence with our findings, specific antibody mediated opsonophagocytosis of *S. iniae* was reported in peritoneal macrophages of Japanese flounder (*Paralichthys olivaceus*) [43]. Sakai *et al.* (1989) [44] also found higher phagocytic activity in kidney leukocytes of rainbow trout when immunized with *S. iniae* formalin killed cells than control fish. Furthermore, I examined the efficacy of opsonophagocytosis when opsonized with immune serum with lower antibody level (Titer $\log_{10}$= 1.8), and higher antibody level (Titer $\log_{10}$ =2.408). Results exhibited that phagocytic efficiency is depend upon the amount of specific antibodies in serum. Previous study has described opsonophagocytosis of *S. iniae* by trout macrophages was decreased with the dilution of the immune serum and suggested that antibody level might be important for the efficacy of this process [38]. Group B streptococcus such as *S. agalactiae* known to bear sialylated capsular polysaccharide hence able to avoid complement deposition on their surface

and complement activation hence can avoid complement based opsonophagocytosis [31, 45]. In contrast to the complement, presence of strain specific antibodies directed against group B streptococcus known to act as most important opsonin which can induce effective phagocytosis [8, 38,45]. These studies suggested that Group B streptococcus uptake is mainly via specific antibody dependent pathways and findings of this study further confirmed this idea. Moreover, I evaluated reactive oxygen species production (ROS) in head kidney leukocytes following exposure to *S. agalactiae*. ROS production was significantly higher when cells incubated with immune serum opsonised *S. agalactiae* than non- immune serum opsonised *S. agalactiae*. Furthermore, a significantly higher ROS production was observed when cell treated with normoxic immune serum (having higher specific antibody level) coated bacteria than moderate hypoxic immune serum coated bacteria indicating that efficacy of ROS production is driven by level of specific antibodies in the serum. Efficient engulfment of normoxic immune serum coated bacteria might have caused higher ROS generation in the HKLs. Internalization via opsonin dependent pathways have been shown to stimulate higher reactive oxygen species production(ROS) in phagocytes than internalize via non opsonin pathways [8, 43]. Shutou e*t al.* (2007) [46] observed strong ROS production in peritoneal macrophages of Japanese flounder when *S. iniae* opsonised with immune serum than normal serum. Furthermore, GBS internalised by mouse bone marrow macrophages in the absence of specific antibodies did not induce ROS production (38). In this study, I provide evidence that opsonisation with immune serum and amount of specific antibodies are not only important for opsonophagocytosis, but also for intracellular ROS production. ROS production or respiratory burst is important innate immune mechanisms where activated phagocytes increased consumption of oxygen and reduce it in to water in a step-wise process catalysed mainly via membrane-bound NADH oxidase [47]. In this process, intermediate products such as hydrogen peroxide, superoxide anion, hydroxyl radicals will form [48]. These products are highly antimicrobial hence inefficient ROS

generation will create the weak hostile environment in phagocytic cells. Such a condition in phagocytic cells will aid intracellular survival of pathogen hence they can avoid other important immune mechanisms of the immune system [49]. Zlotkin *et al.* (2003) [38] explained that failures in phagocytosis mediated killing of *Streptococcus* will aid disease establishment via intracellular survival and dissemination of pathogen. Considering those possibilities, to find out the fate of internalized *S. agalactiae*, I conducted the intracellular survival assay at 2h, 4h, 8h post exposure. Interestingly, when bacteria opsonised with non- immune serum, bacteria appear to be more resistance to phagocytic killing compared to the bacteria opsonised with immune serum where efficient killing was noticed after 4 h and 8h post exposure. Furthermore, I found that higher percentage of bacteria surviving in the HKLs when opsonized with moderate hypoxic immune serum contained relatively low antibody level than normoxic immune serum (contained relatively higher specific antibody level) coated bacteria. However, it should be noted that there is no evidence of growth of bacteria in the leukocytes even when opsonised with normal serum during the experimental period of this study. These findings highlight the importance of specific antibodies and amount of antibodies in serum for opsonophagocytosis based killing of *S. agalactiae*. Previous studies also explained that if GBS engulfed by phagocytes in the absence of antibodies, they are much resistance to phagocytes bactericidal mechanisms [8,50]. Locke *et a*l. (2006) [51] explained that, in the absence of specific antibodies, capsule of *S. iniae* reduces phagocytosis and it is the main reason for higher mortality in rainbow trout following challenge compared to the capsule deficient mutant which is phagocytosed efficiently hence no mortality was observed. Long-time residing in leukocytes will enable pathogen to invade other tissues and cause systemic infection by avoiding other immune mechanisms. Inefficient phagocytosis, inadequate ROS production and longer survival in phagocytic cells appear to be the potential reasons for longer and higher survival of *S.*

agalactiae in the blood and tissues of vaccinated moderate hypoxic fish in this study compared to the vaccinated normoxic fish.

Regardless of antibody mediated opsonophagocytosis, direct effect of moderate hypoxia on phagocytic function was examined *in vitro* and found that phagocytic capacity and ROS production in head kidney leukocytes were considerably suppressed following exposure to moderate hypoxia. When fish expose to ongoing moderate hypoxia, similar thing may happen in which phagocytic cells activity will markedly reduce and it will aid the infection status. McGovern *et al* (2010) [52] suggested that global reduction of respiratory burst in neutrophils impairs their killing capacity against *S. aureus*. Boleza *et al.* (2001) [10] found that hypercapanic hypoxia markedly suppresses the ROS production in anterior kidney cells of *Fundudulus heteroclitus.* Furthermore, Choi *et al.* (2003) [11] demonstrated significantly lowered phagocytic function under hypoxia in peripheral blood leukocytes of Nile tilapia. The reactive oxygen species production of other vertebrates such as rat, human and mouse also known to sensitive for the hypoxia and several studies found that respiratory burst of their leukocytes were suppressed following exposure to hypoxia [47,49,53].

According to the results of antibody dependent phagocytic functions and intracellular survival assay in this study, it is reasonable to suggest that specific antibody mediated phagocytic process is potential effector mechanism which important for the clearance of *S. agalactiae* in vaccinated fish following experimental challenge. Furthermore, efficacy of this effector mechanism is significantly rely on optimum dissolved oxygen as well as on higher amount of anti-*S. agalactiae* antibodies in Nile tilapia

In summary, this study clearly demonstrated specific antibody production in Nile tilapia against *S. agalactiae* is suppressed by moderate hypoxia. Furthermore, lower tissue and blood bacteria burden in vaccinated fish following experimental challenge appear to be correspondent

with higher antibody titer. Moreover, lower bacteria burden and higher antibody titer exhibited positive correlation with lower cumulative mortality in vaccinated fish after experimental challenge with *S. agalactiae*. Complement cascade or other antimicrobial components in serum appear to be inefficient for killing or clearing of *S. agalactiae* while opsonophagocytosis appear to be potential antibody mediated effector mechanism which is responsible for clearing *S. agalactiae* in vaccinated fish following experimental challenge. The efficacy of opsonophagocytosis exhibited clear correlation with amount of specific antibody in the opsonising serum. On the other hand, regardless of specific antibodies, phagocytosis as well as ROS production was found to be oxygen dependent. All these results suggesting that *S. agalactiae* clearance mechanism in vaccinated fish is might be phagocytic process and this process is antibody dependent and DO dependent hence higher cumulative mortality exhibited in vaccinated moderate hypoxic fish following experimental challenge is corresponded with lower antibody titer as well as lowered phagocytic function. However, role of other cellular immune mechanisms those that did not examine in this study in clearing S. *agalactiae* in vaccinate fish also should not be underestimated hence this should subject to further studies

In conclusion, large body of experimental evidence in current study demonstrated that moderate hypoxia lowered antibody production as well as antibody mediated opsonophagocytosis in Nile tilapia which ultimately increased susceptibility of fish to the Streptococcosis even after vaccination hence emphasised the importance of optimum oxygen to gain maximum protection against *S. agalactiae* following vaccination via antibody and DO dependent immune mechanisms.

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CHAPTER 4

General discussion

4. 0 General discussion

This study was conducted to understand the impact of moderate hypoxia on vaccine efficacy in Nile tilapia. One of the most compelling finding is moderate hypoxia supressed and delayed antibody production in vaccinated fish. Furthermore, optimum oxygen at first week following vaccination appear to be important to gain maximum antibody production. Altered antibody production positively correlated with inefficient serum bactericidal activities, phagocytic activities and increased pathogen survival in blood and tissues of vaccinated fish. Those alterations ultimately increased mortality in vaccinated fish following challenge. The dynamic of the altered immune response in moderate hypoxic fish following vaccination together with higher cumulative mortality clearly indicating that moderate hypoxia is immune modulator in Nile tilapia which can lower the protection offered via vaccine thereby vaccine efficacy.

Why optimum oxygen is important to gain maximum protection in vaccinated Nile tilapia

The main purpose of this study was to investigate the influence of moderate hypoxia on vaccine efficacy in Nile tilapia. In my study I used two different bacteria species (*V. anguillarum* and *S. agalactiae*) having different biochemical properties as well as different cellular organisations as gram positive and gram negative. Those differences may contribute for the different virulence mechanisms hence mechanism of vaccine based protection might also be different. Considering all those factors together with moderate hypoxia, we examined several immune mechanisms, particularly antibody production under normoxic and moderate hypoxic conditions and their co correlation with vaccine efficacy. The design of overall study

expected to give comprehensive research outcome regarding influence of moderate hypoxia on vaccine efficacy in Nile tilapia.

Even though I conducted this research as two separate set of experiments with two different bacteria species, simply by analysing two data sets, I found that regardless of the bacteria species, moderate hypoxia modulates vaccine efficacy in negative manner by supressing and delaying antibody response in vaccinated Nile tilapia. In addition, I examined antibody mediated effector mechanisms in immune system such as serum bactericidal activities and opsonin mediated phagocytic activities. The results revealed that these mechanisms are altered along with antibody level in the serum. Those alterations will predispose fish to an infectious disease such as Vibriosis. Furthermore, in second half of this study, observed percent survival and tissue bacteria burden following challenge with *S. agalactiae* and results highlighted that higher percent survival and lower tissue and blood bacteria burden are positively correlated with higher antibody amount in serum. Therefore, I suggest that lower antibody production in moderate hypoxic fish following vaccination is the basis for an increased susceptibility of Nile tilapia to the Streptococcosis. Overall, current study stress that moderate hypoxia is poor water quality condition which modulates several immune mechanisms along with antibody production in negative manner thus consider as key role player on vaccine efficacy in Nile tilapia.

Although Nile tilapia is known to tolerate many environmental extremities, abiotic factor like moderate hypoxia altered their immunity thereby vaccine efficacy. Therefore, it is reasonable to assume that fish species having higher sensitivity to the dissolved oxygen will equally or severely affect than observed here. Furthermore, hypoxic conditions reported in tilapia farms are far more intense and known to show diurnal fluctuations hence will exert a substantial stress on fish. Moreover, in aquaculture systems, hypoxia is integrated with

ammonia and nitrate toxicities, hypercapnia, higher temperature hence additive effect of all these negative forces on fish immunity will be far higher? than we observed with hypoxia alone hence under these conditions, vaccines may offer limited protection to the fish. Therefore, we suggest that in aquaculture industry, optimisation of culture conditions specially dissolved oxygen is equally important as vaccination to obtain better protection following vaccination.

How moderate hypoxia modulates immune and non-immune mechanism in Nile tilapia

Many studies have explained that sub lethal or lethal hypoxia induces stress response in fish [1, 2, 3]. As an immediate response, fish shows several behavioural alterations such as loss of appetite, reduced activities, gulping air on water surfaces, increased gill ventilation rate etc [1]. These behavioural alterations help to increase oxygen uptake, delivery as well as to lessen potential impact of hypoxic stress on general physiology [1, 4]. The moderate hypoxic condition I used in this study failed to induce such a hypoxic stress mediated behavioural changes hence suggest that moderate hypoxia is not a stressful condition which can induce behavioural stress response in Nile tilapia.

In general term, as a next strategy to overcome stress caused by hypoxia, chain of physiological and biochemical changes occurs in fish where endocrinal system induces to release several pituitary and chromaffin tissue originated hormones [4, 5, 6]. Those hormones further induce release of several stress hormones such as cortisol and catecholamine's, known to have ability to regulate fish osmoregulation and metabolism to reduce energy turn over (1, 4, 7). As a result, several blood biochemical variations could be observed in fish such as hyperglycaemia, higher triglycerides levels due to mobilisation of energy reserves to cope with energy crisis caused by existing stress [5, 6, 7]. In addition, changes in plasma chemistry

analytes such as Na^+, K^+, Cl^- could also be observed as a result of altered osmoregulation (5). Surprisingly in this study none of those blood biochemical parameters were shown significant difference between moderate hypoxic and normoxic fish thus reflecting moderate hypoxia is not strong enough to induce biochemical stress response in Nile tilapia. In contrary, several studies pointed out hypoxia around or lower than 1mg/L is able to induce biochemical stress response such as increased cortisol or blood glucose level in Nile tilapia [2,8]. The oxygen level in moderate hypoxic fish in this study is far more high (3.9 - 4.5 mg/L) than those studies and even close to the oxygen level they considered as normoxic treatment hence comparison of our research out come with those studies is appear to be unreasonable. However, it should noteworthy that in this study, I did not examine stress hormone level in Nile tilapia following exposure to moderate hypoxia.

The existence of molecular sensors in cellular environment for oxygen sensing is known for long and several studies have explained that hypoxia induces ubiquitous heterodimeric DNA-binding protein or transcription factor called hypoxia-inducible factor (HIF) [9, 10] which can activate the transcription of many hypoxia-inducible genes those that promote survival under hypoxia such as erythropoietin [9] which can increase red blood cell production. Gracey *et al.* (2001) [11] explained that mudsucker (*Gillichthys mirabili*), tolerate hypoxia by down-regulating genes responsible for cell growth and protein synthesis as an energy saving counter mechanism facilitates survival under hypoxia. Even though we failed to recognise stress induced behavioural or blood biochemical alterations, significantly higher packed cell volume, significantly lower lymphocyte number, marked reduction of antibody production as well as reduced phagocytic activities and ROS production (*in virto*) were observed in Nile tilapia kept under moderate hypoxic condition. As suggested by other studies [12], higher red blood cell volume might be adaptation to increase oxygen uptake and

transport while other alterations appear to be cellular adjustments to reduce energy consumption under moderate hypoxia. However, as I did not observe stress mediated responses following exposure to moderate hypoxia, it is difficult to admit to the fact that alterations in lymphocyte count and leukocytes functions are driven by stress response in Nile tilapia. Therefore, this should be subjected for further studies to evaluate whether these alterations driven by stress hormones or HIF mediated transcription regulations in Nile tilapia.

In addition to altered packed cell volume, lymphocyte number and antibody production, I noticed altered transcription level of immune related genes in moderate hypoxic fish following vaccination. Few studies done on hypoxia mediated transcription regulation in fish revealed that transcription levels of genes encoding for protein synthesis, locomotion and cell proliferation were affected in negative manner [13,14]. As explained before, hypoxia induces HIF which has ability to inhibit or supress transcription level of genes encoding energetically costly anabolic processes. Several studies pointed out that possible involvement of HIF on immune related gene expressions [1,13] therefore, we can assume that overall suppression of transcription level of immune related genes in moderate hypoxic vaccinated fish might be driven by HIF mediated transcription regulation to lower energy expenditure under moderate hypoxia. However, I did not examine HIF transcription level in moderate hypoxic fish in this study.

Taking together, reduced lymphocyte count, increased packed cell volume, lower antibody production and supressed immune related gene expression in vaccinated moderate hypoxic fish apparently an allostatic reaction or adaptive responses to save energy in moderate hypoxic fish rather than stress response. Whether these alterations are allostatic reaction or stress response, they have caused profound negative impact on fish immunity thereby disease resistance. However, the amount of data gather in this study is not enough to

draw a complete picture on exact mechanisms underlying these alterations hence detail study on stress hormone secretion and HIF mediated transcription regulation under hypoxic conditions is necessary.

What is the possible relationship between expression of immune related genes and vaccine efficacy?

As a part of this study I examined the expression patterns of few immune related genes such as IL-1ß, TCR ß, MHC class II ß, B cell activating factor and IgM. As mammals, fish also known to possess antigen recognition receptors. Group of those receptors such as pattern recognition receptors (PRRS) recognise pathogen associated molecular pattern and recognition is not pathogen specific [15]. In contrary, another group of receptors such as immunoglobulin and T cell receptors (TCR) having ability to recognise pathogen or processed pathogenic molecules and this recognition apparently pathogen specific [16, 17]). In either way, pathogen primed immune cells such as macrophages can initiate production and release of proinflammatory cytokines such as Interleukin-1β (IL-1β) which is having crucial role in host defence as inflammatory mediator and promoter of immune cell proliferation and differentiation [18]. Following induction by pathogen and cytokines, immune cells can engulf, kill, process and present antigenic molecules to induce other cell types such as T helper cells. Presentation of processed antigen carried out by molecules called MHC [2]. The antigen presenting process is essential to induce T cells and following activation, T cell induces B cell maturation in order to produce specific antibodies and immunological memory. Fish are lacking some of the component in adaptive immune system compared to the mammals, yet able to mount effective immune response against pathogen. Therefore, following vaccine induction, I analysed the transcription level of genes those that appear to have role in some of the immune mechanisms I mentioned here and found that they were significantly upregulated

in response to the bacterial vaccine in normoxic fish. Higher induction of IL-1β was seen and might be indicating effective and immediate inflammatory response to the bacterial vaccine which could have mobilised immune cell to the site of injection. Cytokines are usually having multiple sources, also multiple functions, and multiple targets [19,20] hence exact mechanism of IL-1β upregulation remain unclear. On the other hand, gap between benefit of this pro-inflammatory cytokine in inflammatory process and over expression related cellular cytotoxicity is narrow [1] hence without knowing exact transcription level of this cytokine where benefits turn in to tissue or cell damage, it is difficult to draw a conclusion as higher up regulation is always beneficial to the fish. Therefore, here I can only assume that higher induction of IL-1β might be beneficial for the vaccinated fish to induce other immune cells.

As explained before MHC II beta is important for the presenting of processed antigen to the T cells by professional phagocytic cells such as macrophages. In mammals, activated and antigen loaded professional antigen presenting cells such as dendritic cells known to migrate to the lymph node or spleen where antigen presentation will take place. In fish, due to they are lacking of lymph node, scientist suggest that this process could occur in the melanomacrophage centers in spleen and head kidney [2, 21]. In our study we found significantly higher transcription level of MHC II in the spleen of normoxic vaccinated fish might be indicating antigen presenting process occur in spleen following vaccination. MHC II molecule not only found in macrophages, but also in B cells, granulocytes, T cells and even in the epithelial cells [19]. Other than in professional antigen presenting cells, MHC II expression in other cell types assume to be mediated by cytokines such as interferon γ [2]. Therefore, significant upregulation of MHC II we have seen in spleen following vaccination might be due to increased antigen processing and presentation or as a response to the cytokine induction. Antigen presentation is necessary for T cell activating hence MHC II upregulation

can assume to be important in helper T cell activation. Even though inactivated vaccines known to induce humoral arm of adaptive immunity, surprisingly we observed significant up regulation of T cell receptor (TCR ß) expression in vaccinated fish. This might be a response to the antigen presentation or induced cytokine activities [22]. Significant up regulation of TCR ß have seen in Nile tilapia [23] as a response to the bacteria induction. However, exact function of this gene in fish following pathogen induction or vaccination is yet to be understand therefore this should subject for further studies. Furthermore, significantly higher expression of IgM was found in spleen following vaccination and this might be indicating increased IgM expression in B cells to produce antibodies following vaccine induction.

In summary, we examined significant up regulation of few immune related gene expressions in vaccinated Nile tilapia under normoxic condition. The transcription level of those genes can be modulated by several factors as well as their exact function in vaccine immunology in fish is largely unknown. On the other hand, by analysing transcription of few genes, it is difficult to draw a conclusion on molecular basis of vaccine induction in which enormous amount of genes and their interactions might have involved in single process. Therefore, by analysing gene expression induction pattern in vaccinated Nile tilapia in this study I can only suggest that these genes might have direct or indirect role in vaccine based immunity hence detail study is necessary to confirm their role in vaccine immunology which is out of the scope of current study.

In this study, for the first time, I revealed that moderate hypoxia negatively affects on vaccine efficacy in Nile tilapia hence research outcome of this study will broaden our knowledge on manipulation of vaccine immunology under moderate hypoxic condition. Furthermore, data presented here will be important to optimise future vaccination programs in

aquaculture industry in order to gain maximum benefits from vaccination and to become a viable and sustainable industry by limiting opportunities for infectious diseases.

Finally, I summarize some remained problems in this research and propose future works to progress our knowledge and apply them to solve disease issues in aquaculture industry.

1) Moderate hypoxia did not induce behavioural or physiological stress responses yet seems to be affecting on cell proliferation and protein synthesis. The exact mechanism underlying those alterations is largely unknown hence future work is necessary to understand this phenomenon.

2) Booster vaccination and high dose vaccination appear to reduce the negative impact caused by moderate hypoxia on antibody production yet detail studies are required with experimental infection with several pathogenic bacteria species in order to accept those counter measures as better solution to improve vaccine efficacy in Nile tilapia under moderate hypoxia.

3) Future research should examine wider range of gene expression patterns in vaccinated Nile tilapia under moderate hypoxia with comprehensive technologies such as microarray to understand the molecular basis of moderate hypoxia on fish immunity.

Proposed mechanism of moderate hypoxia mediated immune modulation in Nile tilapia and its co- correlation with vaccine efficacy

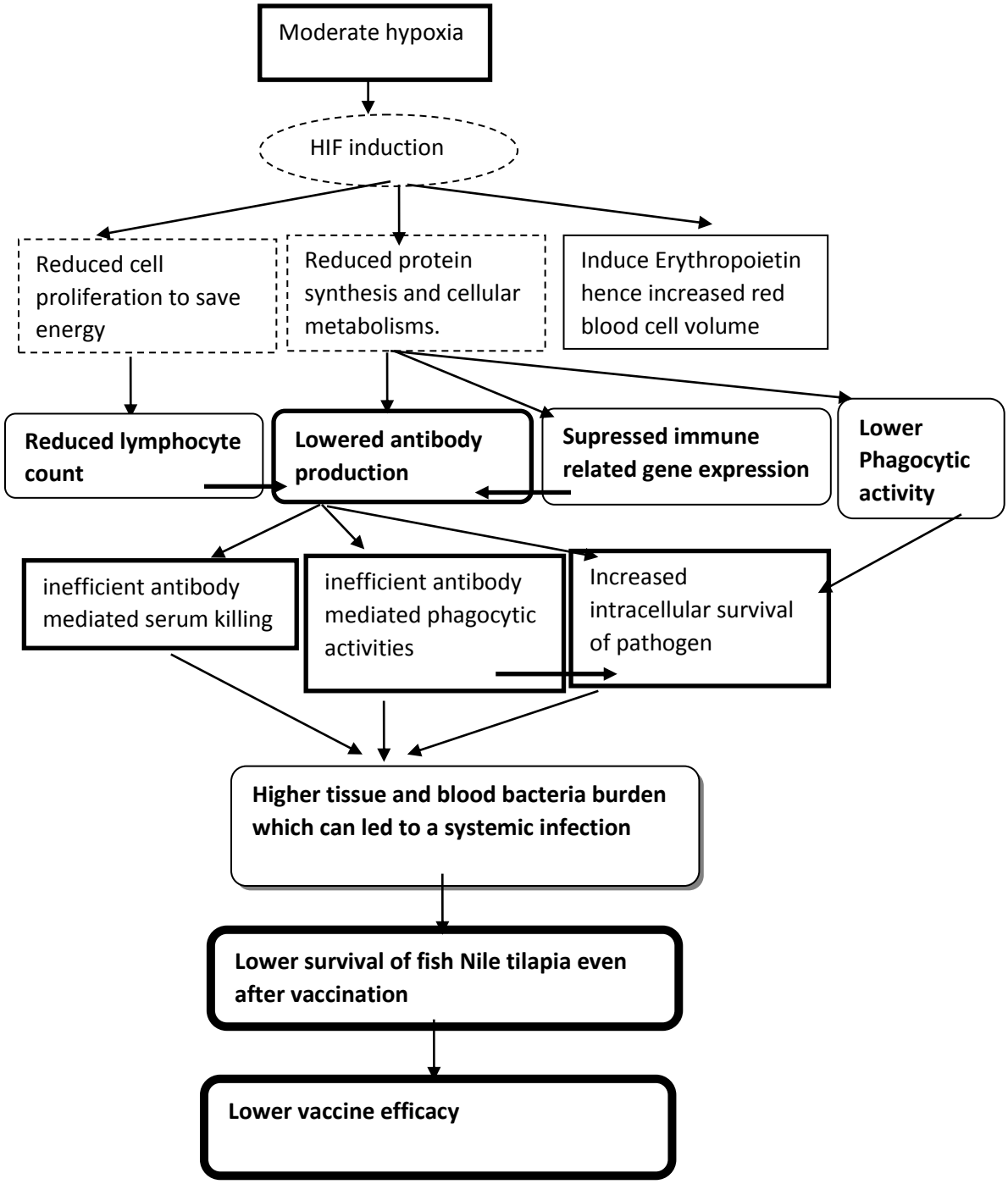

Figure 4.1 Diagrammatic view of moderate hypoxia mediated immune modulation in Nile tilapia and its co correlation with vaccine efficacy. Solid line boxes indicted the findings of this study while dash line boxes indicated proposed pathways.

4.1 **References**

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