Final report

Project Title: Francisella-infected tilapia and their susceptibility to *Streptococcus agalactiae* infection

BY

Researcher: Asst.Prof.Dr. Wanna Sirimanapong

Mentor: Prof. Boonsirm Withyachmnarnkul

July/2018

Contact No. MRG5980064

Final Report

Project Title: Francisella-infected tilapia and their susceptibility to *Streptococcus agalactiae* infection

Asst.Prof.Dr. Wanna Sirimanapong

Prof. Boonsirm Withyachmnarnkul

Faculty of Veterinary Science

Mahidol University

Project Granted by the Thailand Research Fund

Abstract

Project Code : MRG5980064

Project Title : Francisella-infected tilapia and their susceptibility to *Streptococcus agalactiae* infection

Investigator : Asst.Prof.Dr. Wanna Sirimanapong

Prof. Boonsirm Withyachmnarnkul

E-mail Address : wanna.sri@mahidol.edu

boonsirm.wit@mahidol.ac.th

Project Period : 2 May 2016 – 2 May 2018

Abstract : Hybrid red tilapia (*Oreochromis niloticus × Oreochromis mossambicus*) were obtained from a commercial farm that had experienced a disease outbreak caused by *Francisella noatunensis* subsp. *orientalis* (*Fno*). This bacterium infects the head-kidney of the fish, an organ involved in haematopoiesis in fish, including the generation of white blood cells that play a defensive role against pathogens. The haemato-immunological functions of the *Fno*-infected red tilapia were therefore tested, including a number of haematological parameters [haematocrit (Hct), total red blood cell (RBC) count, mean corpuscular volume (MCV), total white blood cell (WBC) count, and differential white blood cell count and total plasma protein content], and innate immune parameters such as lysozyme and peroxidase activity and respiratory burst and phagocytic activities of head-kidney macrophages. Most of the parameters examined in the *Fno*-infected fish were comparable to those of normal fish; the percentage of the peripheral lymphocytes was the only parameter found to be significantly lower ($p<0.05$) than that of the normal fish. Both *Fno*-infected and normal fish were then experimentally challenged with the bacterium *Streptococcus agalactiae*, and the haemato-immunological parameters determined at 3, 6, 12, 24 and 48 h post-infection (hpi). The haematocrit and total RBC count of the *Fno*-infected fish were significantly lower

than those of the normal fish, while the plasma lysozyme, peroxidase and respiratory burst activities of the *Fno*-infected fish was significantly higher than that of the normal fish at many time-points post-challenge. Other parameters were more or less comparable among the groups. Despite normal, or even higher, levels of innate immune activity in the *Fno*-infected fish, they had significantly higher and more rapid onset of mortality than the normal fish following the *S. agalactiae* challenge, while the non-challenged, normal and *Fno*-only-infected fish had 100% survival during the 2-week trial period. The reason for the rapid and high mortality observed in the *Fno*-infected fish when infected with *S. agalactiae* is unknown, but one possibility is that erythropoiesis taking place in the head-kidney is affected by the presence of the *Fno*.

Keywords : *Francisella noatunensis* subsp. *orientalis* (*Fno*), *Streptococcus agalactiae*, red tilapia, immune system

Introduction

Members of the genus *Francisella*, family Francisellaceae, are Gram negative, non-motile, non-sporulating, aerobic, pleomorphic coccobacilli prokaryotic cells, with an intracellular nature and have a wide geographical and ecological distribution. *Francisella* spp. are facultative intracellular pathogens of macrophages and other various cell types of humans, rabbits, rodents, non-human primates, amoebas, arthropods and fish. *Francisella noatunensis* is one of the most recently recognized species within the genus, and is the causative agent of the emerging disease "aquatic francisellosis" recently reported worldwide. The disease affects several marine and fresh water aquatic organisms, including tilapia, cod and Atlantic salmon. In Thailand francisellosis caused by *F. noatunensis* subsp. *orientalis* appears to be an emerging problem in tilapia aquaculture (Nuyen et al., 2015; Ruane et al., 2015).

Nile tilapia (*Oreochromis niloticus*) and hybrid red tilapia (*Oreochromis* sp.) is an economic fish species in aquaculture industry. The global production of tilapia and other cichlids species was 715,128 tons in 2006 and 784,276 tons in 2011, but was down to 710,535 tons in 2012 (FAO, 2014). Asia and the Pacific countries provide the main of world aquaculture supply of tilapia (Romana-Eguia et al., 2004). Thailand has ranked 5th or 6th the highest tilapia production country. One of the major problems of tilapia aquaculture is its high mortality during swim-up (newly hatched) and fingerling periods and sporadic disease outbreaks during juvenile and adult period. The most common pathogens associated with mortality in tilapia are *Streptococcus agalactiae*, *S. iniae*, *Aeromonas hydrophila*, *A. sobria*, *Flavobacterium columnare* and *Francisella* spp. (Ke et al., 2010; Iregui et al., 2011; Li and Cai 2011; Yardimci and Aydin, 2011; Figueiredo et al.,

2012; Suebsing et al., 2014). Clinical manifestations include swimming errors, skin lesion and exophthalmos. There seemed to be no pathognomonic signs of infection by any particular pathogens, thus lab identification is essential to indicate the causative agent. In fact, even the pathogen is identified in association of the fish clinical manifestation; it is still inconclusive whether the pathogens are the causative agent or opportunistic one, or even contaminations from water environment. Koch's postulate should be strictly required to identify any pathogen as primary causative agent (Evans, 1976). Thus far, few of them have been documented. *Streptococcus agalactiae*, *S. iniae* and *Francisella asiatica* are among those that are well-documented (Bowser et al., 1998; Lau et al. 2003; Hernandez et al., 2009; Jeffery et al., 2010; Ke et al., 2010; Iregui et al., 2011; Figueiredo et al., 2012; Jantrakajorn et al. 2014).

Literature review

Francisellosis *Francisella* spp. are pleomorphic cocco-bacilli of variable size from 0.5 to 1.7 µm, and with somewhat fastidious nature. They are facultative intracellular bacteria; therefore they can survive and replicate inside the fish's cells as well as in the environment. They have a common dependency on the amino-acid cysteine for growth (Ottem et al., 2009; Colquhoun and Duodu, 2011). *Francisella* spp. are divided into certain subspecies according to their DNA sequences. *F. noatunensis* subsp. *orientalis* (*Fno*; synonym, *F. asiatica*) is present in tropical and temporate areas, while *F. noatunensis* subsp. *noatunensis* (*Fnn*) seems to be found more in the temperate environments (Colquhoun and Duodu, 2011).

The infected fish are usually observed on the water's surface, or near aeration, and gasping. They may have skin lesions, frayed fins, petechial hemorrhage on and around the pectoral fins, exophthalmos, fungal patches and pale gills (Jeffery et al., 2010). Their visceral organs show abnormalities such as empty intestines, large gall bladders, chronic granulomatous lesions in the gills, spleen, anterior kidney, liver and heart (Ottem et al., 2009; Jeffery et al., 2010; Colquhoun and Duodu, 2011).

As detected by molecular biology methods, several disease outbreaks in variety of aquatic species are found to be infections by both *Fno* and *Fnn* (Hsieh et al., 2006; Ottem et al., 2009; Soto et al., 2009, 2013; Jeffery et al., 2010; Sridhar et al., 2012). Moreover, the important factors in the cultivability and persistence of *Fno* in both marine and freshwater microcosms are temperature and salinity. The bacteria *Fno* survive better in seawater, and its persistence is inversely related to water temperature. This is in consistent with the field finding that *Fno* outbreaks occur more in low temperature condition, in contrast to *S.*

agalactiae outbreaks that occur more often in warm temperature condition. The pathogenic properties of the bacteria suspended in water microcosms appear to decrease after only 24 h and become non-infective after 2 days in the absence of the fish host (Colquhoun and Duodu, 2011; Soto and Revan, 2012). The update report of francisellosis distribution in the world, from 2001 in Taiwan to 2013 in Thailand, is shown in figure 1 (Nguyen et al., 2015). The disease affects both seawater and freshwater fish, as well as mollusks (Table 1) (Birkbeck et al., 2011). Tilapia are highly susceptible to *Fno*, while cods are to *Fnn*, which results in high mortality rate (Colquhoun & Duodu 2011).

Figure 1 The geographical distribution of francisellosis in fish (black dots: *Francisella noatunensis* subsp. *orientalis* and red dots: *F. noatunensis* subsp. *noatunensis* (from Nguyen *et al*., 2015).

Tilapia Immunology; As mentioned, francisellosis causes damage to spleen and anterior kidney among other visceral organs, and it is well known that both organs are important for fish immunological functions, therefore it is possible that fish infected with *Francisella* sp. may have impaired immunological functions. Fish immunology is an evolution from that of invertebrate to vertebrate (Corbel 1975; Plouffe et al., 2005; Saurabh

and Sahoo, 2008). Fish are the first animals in the Phylum Chordata that possess both innate and adaptive immune systems (Magnadottir, 2010). The innate response, being non-specific, is the first line of defense against invading pathogens, while the adaptive immune response is for specific recognition and destruction of pathogens. Knowledge of the function of the immune system is essential for the successful development of disease prevention strategies for fish, especially for the development of vaccines and selective breeding for disease-resistance traits (Watts et al., 2001).

Information regarding the immune system of fish infected with *Francisella* sp. is scarce, and non-existent in tilapia. Vojtech et al. (2009) reported that when the zebrafish was intraperitoneally injected with *Francisella* sp., the bacteria replicated rapidly in the liver, kidney and spleen beginning at 12 h post-injection, and its titers rose steadily, leveled off, and decreased by 7 days post-injection. The fish responded to the infection by the up-regulations of interleukin (IL)-1β, γ- interferon (IFN-γ), and tumor necrosis factor mRNA, beginning at 6 h post-injection and persisting for up to 7 days post-injection. In addition, exposure of the zebrafish to heat-killed *Francisella* sp. revealed a significant induction of IL-1β highly specific to the live bacteria. When the Atlantic cod was intraperitoneally injected by live *F. noatunensis*, significant up-regulations of IFN-γ and IL-1β expressions were observed in the spleen and anterior kidney from 15 to 60 days post-injection (Ellingsen et al., 2011). In the fish intestine, IFN-γ was significantly up-regulated after 30 days post-injection. In addition, IL-10 was up-regulated in the intestine of cohabitant fish from day 30 to day 60. Soto et al. (2010) found the ability of *Fno* to invade tilapia anterior kidney-derived macrophages and replicate vigorously within them, causing apoptosis and cytotoxicity in the macrophages at 24 and 36 h post-infection. Intracellular localization and innate immune responses following *Fnn* infection of Atlantic cod (*Gadus morhua*) macrophages demonstrated a weak activation of the inflammatory response genes as measured by

increased expressions of the IL-1β and IL-8 and down-regulation of IL-10 (Bakkemo et al., 2011).

Table 1. Susceptible hosts of *Francisella* spp. (from Birkbeck et al., 2011)

Species	**Country**	***Francisella* spp.**	**Life stage**	**Freshwater /marine**	**References**
Atlantic cod (*Gadus morhua*)	Norway	*Francisella noatunensis* subsp. *noatunensis*	Adult	Marine	Zerihun et.al., 2011
Atlantic salmon (*Salm salar* L.)	Chile	*Francisella* spp.	Parr	Freshwater	Birkbeck et.al., 2007
Tilapia	Taiwan	*Francisella* spp.	Fry - Adult	Freshwater / marine/ brackish	Hsiech et.al., 2006
	Central S. America		Fry-adult (1–>1000 g)	Freshwater	Mauel et. al., 2007
	Hawaii, USA		Fry-adult (?)	Freshwater	Mauel et. al., 2003
	Costa Rica		Adult	Freshwater	Soto et.al., 2009
	UK		Fry-adult	Freshwater	Jeffery et. al., 2010
	Thailand	*F. noatunensis* subsp. *orientalis*	Fry-adult (?)	Freshwater	Nguyen et.al., 2015
Three-line grunt (*Parapristipoma trilineatum*)	Japan	*Francisella philomiragia*	Fry-adult (?)	Marine	Kamaishi et. al., 2005
Ornamental cichlid	Taiwan	*Francisella* spp.	Juvenile to adult	Freshwater	Hsich et.al., 2007
Hybrid striped bass (*Morone chrysops* × *M. saxatilis*)	South California, USA	*Francisella* spp.	Juvenile - adult (10–350 g)	Freshwater	Ostland et. al., 2006
Giant abalone [*Haliotis* (*Nordotis*) *gigantean*]	Japan	*Francisella* spp.	Adult	Marine	Kamaishi et. al., 2010

In the *in vitro* assay, Soto et al. (2010) reported the interaction of wild-type *Fno* and the intracellular growth locus C (ΔiglC) mutant *Fno* and showed that both *Fno* strains were resistant to killing by normal sera. While the wild-type *Fno* is able to invade and cause apoptosis and cytotoxicity of the macrophages in the tilapia anterior kidney, the ΔiglC *Fno* cannot unless the gene is restored. Using ΔiglC *Fno* as live attenuated vaccine, Soto, et al. (2011) was able to protect tilapia against wild-typed *Fno*. Brudal, et al. (2015) was also successful in using outer membrane vesicles of *Fnn* as vaccine to protect zebrafish against *Fnn*. At present, an attenuated vaccine against *Francisella* sp. has been patented (Hawke and Soto, 2012).

Streptococcosis; For most tilapia farmers, infection by *Francisella* spp. has not been readily recognized. The reason may be that when disease outbreaks occur, farmers and researchers at farms usually identify the cause of the problem from the presence of culturable bacteria spp., but not pathogens that require sophisticated method to identify, such as *Francisella* spp. and iridovirus. The bacteria most commonly reported in associated with tilapia disease outbreaks are *S. agalctiae*, *S. iniae*, *Flavobacterium columanare*, *Aeromonas hydrophila*, *Edwardseilla tarda* and others.

One of the bacterial species that are most frequently revealed in the outbreaks is *Streptococcus* spp. and the disease is commonly known as streptococcosis. *Streptococcus* spp. are Gram-positive, fermentative, catalase-negative, oxidase-positive, non-motile and form spherical colonies at 1mm size in diameter (Austin and Austin, 2007). Up to the present, six species of them are found pathogenic to the fish; they are *S. iniae*, *S. agalactiae*, *S. dysgalactiae*, *S. phocae*, *S. parauberis* and *S. ictaluri* (Domeénech et al., 1996; Colorn et al., 2002; Gibello et al., 2005; Pasnik et al., 2009; Yang, 2009; Geng et al., 2012;). The bacteria cause disease outbreaks in farmed fish worldwide with increasing economic losses. For instances, *S. iniae* caused mortality of hybrid tilapia farms in Texas (Perera et al., 1994),

of Nile tilapia farms in South America (Figueiredo et al., 2012), and red tilapia and Asian sea bass (*Lates calcarifer*) in southern Thailand (Suanyuk et al., 2010). *Streptococcus agalactiae* was identified in fish samples from several disease outbreaks in tilapia farms in Malaysia (Zamri-Saad et al., 2010) and Thailand (Suanyuk et al., 2008).

Clinical signs of streptococcosis include darkening skin, erratic or disorientated swimming behavior, exophthalmos or opaqueness of the eyes and inflammation or ulceration of the abdominal skin. Gross pathology of the visceral organs includes accumulation of ascetic fluid, brain and liver hemorrhage, enlarged spleen and kidneys and turgid gallbladder (Zhang et al., 2008). It is commonly known that outbreaks of *S. agalactiae* infection in tilapia are associated with high temperature in rearing water, such as in summer.

Since the streptococcosis is a common disease outbreaks in farmed tilapia, whether as primary or opportunistic infections, researches on vaccination against *S. agalactiae* and *S. iniae* and the fish immune responses to the pathogens are relatively numerous. Numbers of reports on *S. iniae* vaccination are more than that on *S. agalactiae* (Table 2), probably because of higher prevalence of *S. iniae* than S. agalactiae infections in the Western world. The vaccines are either formalin-killed bacteria, heat-killed bacteria, bacterial proteins, recombinant proteins, or live attenuated vaccines (Evan et al., 2004; Pasnik et al., 2005; Nur-Nazifah et al., 2014). Like other kinds of vaccines, the live vaccines have been found to be more effective than of the dead ones (LaFentz et al., 2008; Pridgeon et al., 2013). It is also worthwhile to note that tilapia swim-ups and fingerlings that survive streptococcosis outbreak are likely to survive better in natural environment than the naïve fish. It seems that those that survive have live *Streptococcus* sp. vaccine naturally.

Table 2. *Streptococcus* vaccines in fish

Type of vaccine	Fish species	References
Streptococcus iniae		
Formalin-killed	rainbow trout (*Onchorynkus mykiss*)	Eldar et al., 1997
	Nile tilapia	Klesius et al., 2000
	Asian seabass (*Lates calcarifer*)	Aviles et al., 2013
	Grouper (*Epinephelus coioides*)	Huang et al., 2014
Extra-cellular proteins	Olive flounder (*Paralichthys olivaceus*)	Shin et al., 2007
Live	Hybrid striped bass	Locke et al., 2010
Streptococcus agalactiae		
Modified bacterin	Nile tilapia	Evan et al., 2004
Whole-cell + β-haemolysin	Nile tilapia	Hardi et al., 2013
Streptococcus faecalis		
Formalin-killed	Aferican catfish (*Clarias gariepinus*)	Badran et al., 2010

From the above information, it is not clear if several disease outbreaks of tilapia that cause mass mortality is primarily caused by *S. agalactiae* (or other types of bacteria), or the discovered bacteria are opportunistic pathogens of the fish that have already been infected by *Francisella* spp. (or other types of non-lethal pathogens, e.g, iridovirus) that cause sub-optimum immunological functions of the fish. Upon being stressed by environmental factors (high temperature, high stocking density, etc.), these "sub-optimum immunological functioned" fish thus become easily susceptible to the opportunistic pathogens. With this hypothesis, this study therefore aims at proving that tilapia with underlying *F. noatunensis* infection is more susceptible to *S. agalactiae* (and other types of bacteria) than those without. And to substantiate the hypothesis, immunological responses of tilapia are compared between the fish that have underlying *F. noatunensis* infection and those without.

Objectives

1) To detect francisellosis in farmed tilapia in Thailand and identify *Francisella* spp. by molecular biology tools
2) To compare immunological profiles of *Francisella* spp.-infected and *Francisella* spp.-free tilapia of the same population
3) To compare susceptibility of *Francisella* spp.-infected and *Francisella* spp.-free tilapia to *S. agalactiae* challenge. The challenge serves as a model for other types of bacterial infections.

Research methodology

Experimental animals

Three hundred hybrid red tilapia (*Oreochromis niloticus × Oreochromis mossambicus*), 200-300 g in weight, which had survived a natural *Fno* infection were collected from a commercial farm site in Nakhornsawan Province, Thailand. Ten of these fish were randomly collected and their head-kidneys screened for the presence of residual *Fno* by polymerase chain reaction (PCR) using the method described by Soto *et al*., (2009), all of which were found to be positive for the bacterium. This group of red tilapia was therefore considered to be sub-clinically infected with *Fno* without any clinical manifestation of the disease. Three hundred control hybrid red tilapia of the same size were collected from another farm in the same Province that had no previous history of any disease outbreak, including infection with *Fno.* Ten of these fish were also randomly sampled and their kidneys screened for presence of *Fno* by PCR, all of which were found to be *Fno*-negative. The fish were brought to the wet lab at Mahidol University, Bangkok, and stocked in 10 re-circulatory 350-L fiberglass tanks, with 30 individuals per tank. The normal and *Fno*-infected fish were kept separated to avoid possible cross contamination between groups.

Each of the two groups was further divided into a group to be experimentally infected with *S. agalactiae* and another that would not be infected with the pathogen, resulting in 4 groups altogether: G1, normal fish without *S. agalactiae* challenge; G2, *Fno*-infected fish without *S. agalactiae* challenge; G3, normal fish with *S. agalactiae* challenge; and G4, *Fno*-infected fish with *S. agalactiae* challenge. Five tanks were used for each group with 30 fish per tank (150 fish per group). Water quality was monitored daily to ensure optimal water

conditions for the fish, with the following water quality values maintained: dissolved oxygen, >4 ppm; total ammonia nitrogen, <0.5 ppm; total nitrite, <0.5 ppm; pH, 7.0-7.5 and water temperature, 28 ± 1 ^{o}C. A water exchange of 20-30% was performed daily.

Haematological and immunological parameters

One fish was randomly sampled from each tank (5 fish per group) to assess their haemato-immunological parameters at 0 days post-infection (dpi), before the *S. agalactiae* challenge was performed; thus, the fish tested were 10 normal control fish (from G1 and G3 combined) and 10 *Fno*-infected fish (from G2 and G4 combined). The parameters measured included haematological parameters [haematocrit (Hct), total red blood cell (RBC) count, mean corpuscular volume (MCV), total white blood cell (WBC) and differential WBC count], plasma-related related parameters (lysozyme activity, peroxidase activity and total protein levels) and head-kidney macrophage activity [respiratory burst (RBA) and phagocytic activities].

Haematology

Blood samples were collected from the caudal vein of the fish and all the parameters described determined using the protocols outlined by Sirimanapong *et al*., (2014). Briefly, 1 mL of blood was withdrawn from the tail vein of individual fish and immediately mixed with heparin (200 U/mL). The withdrawn blood, 100 µL, was immediately used to determine Hct, total RBC count, total and differential WBC counts and MCV. The rest of the blood was centrifuged at 3,000× g for 5 min, plasma collected, and kept at -70 ^{o}C for further analysis.

The Hct values were determined by placing well-mixed blood into haematocrit capillary tubes and centrifuging at 3,000 ×g for 5 min, and calculated as a percentage of packed RBC volume against the whole blood volume. For total RBC and WBC counts, blood

(20 µL) was added to 4 mL Natt-Herricks's solution and mixed thoroughly before counting on a Neubauer chamber. From the values of Hct and total RBC count, MCV was determined according to the formula: MCV (fL) = (Hct × 10)/total RBC (×10^6 cells/µL). WBC differential count was carried out by preparing a smear of whole blood on microscopic slides, stained by Wright–Giemsa and examined under light microscopy and the % of neutrophils, lymphocytes, monocytes, eosinophils, basophils and thrombocytes determined. At least 200 cells were counted for each differential count.

Plasma-related parameters

The plasma was thawed and lysozyme activity, plasma peroxidase activity and total protein content determined. Lysozyme activity was determined according to the method previously described by Morgan *et al*., (2008). Briefly, a suspension of 0.2% (w/v) *Mycobacterium lysodeikticus* (Sigma, UK) was prepared in 0.04 M phosphate buffer saline (PBS) and 190 µL of the bacterial mixture was placed into microtitre plate wells, and control wells contained only the buffer. Plasma samples (10 µL) were added into the wells to make a final volume of 200 µL $well^{-1}$. Each plasma sample tested consisted of 4 replicates. The wells were incubated at room temperature (32 ± 1^oC) and the absorbency at 540 nm read at 1 and 5 min after the incubation. One unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance at 0.001/min.

Plasma peroxidase was determined using a modification of the method outlined by Quade and Roth (1997) and Sitjà-Bobadilla *et al*. (2005). The plasma sample (15 µL) and Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (135µL) were added to the flat bottomed well (96-well plate) followed by the addition of 50 µL of 20 mM 3,3',5,5'-tetramethylbenzidine in substrate buffer (40 mM acetic acid) containing 0.1% H_2O_2. The reaction was stopped with 50 µL of 2 M H_2SO_4 and read with an ELISA reader at 450 nm.

Total plasma protein was analysed using a Bradford assay (Bradford, 1976). A protein standard series (from 0 to 2 mg/mL) were prepared by diluting 6 mg bovine serum albumin in PBS. Twenty µL of sample or standard was added to the wells of a 96 well plate, to which is then added 200 µL of Bradford solution (Sigma, UK) and mixed thoroughly on a plate shaker for 30 sec. After 5 min the plates were read at an absorbance of 595 nm on a microplate reader. A standard curve of protein concentration against absorbance at 595 nm was plotted and the level of total protein in the samples determined.

Head-kidney macrophage activity

Samples of head-kidney were collected to examine head-kidney macrophage activity i.e. RBA with and without stimulation by phorbol myristate acetate (PMA), reactive oxygen species (ROS) stimulation index and phagocytosis, all of which were performed as previously described by Sirimanapong *et al*., (2015). To determine the RBA, head-kidney phagocytes were isolated and the cell suspension was placed into a 96 well tissue culture plate (100 µL/well), 10 wells per fish, and incubated at 28-30 ^{o}C for 2 h. After incubating, the non-adherent cells were removed before adding 100 µL of *p*-nitroblue tetrazolum (NBT) solution to four replicate wells and 100 µL of NBT solution containing PMA to another four replicate wells, and 100 µL lysis buffer to two remaining wells to determine the number of attached cells. The plates were incubated for 1 h at 28-30 ^{o}C. Meanwhile the number of adherent cells was determined by counting the number of released nuclei with a haemocytometer. The RBA was stopped after 1 h by fixing the cells with 100 % (v/v) methanol. The plates were washed three times with 70 % (v/v) methanol and the wells were air-dried. The insoluble formazan in the wells was dissolved by adding 120 µL 2 M KOH (BDH, UK) and 140 µL DMSO (Sigma, UK). The content of each well was mixed carefully and air bubbles removed with a needle. The absorbance of the wells was measured at 610 nm using a micro-plate reader

(Synergy HT; Bio Tek Instruments, Winooski, VT, USA) and the results expressed as an absorbance at 610 nm per 10^6 cells. The ROS stimulation index was calculated from the equation PMA-stimulated ROS/mean unstimulated ROS.

Phagocytic activity by head-kidney macrophages was determined by placing 100 µL of the head-kidney cell suspension onto microscope slides, which were then placed in a moist incubation chamber for 1 h at room temperature (32 ± 1^{o}C) to allow adherence of cells to the slide. Yeast, *Saccharomyces cerevisiae*, 10^7 yeast cells/mL in L-15 medium (Sigma, UK), was used as the phagocytic particles and 100 µL of the yeast suspension was placed into one circle of attached cells and 100 µL of the medium, as control, into the second circle. The slides were incubated for 1 h at room temperature (32 ± 1^{o}C) to allow phagocytosis to take place and then washed with L-15 medium. The cells were fixed with 100% methanol (100 µL) for 5 min and stained with Giemsa stain, and the number of phagocytes containing yeast was counted for a total 200 phagocytes. The phagocytic index (PI), phagocytic activity (PA), and phagocytic capacity (PC) were determined as described previously (Findlay and Munday, 2000).

<u>*Streptococcus agalactiae* challenge</u>

A virulent strain of *S. agalactiae,* isolated from a field outbreak, was cultured in tryptone soy agar (TSA, Oxoid England) for 24 h. Three to five colonies of the bacteria were randomly selected from the plate and placed into 100 mL of sterile tryptic soy broth (TSB). The bacteria were cultured to late logarithmic growth in a shaking incubator (150 rpm, 28 ^{o}C) (Incu-shakerTM 10L, Benchmark, NJ) for 24 h and the concentration of the bacterial suspension determined using spectrophotometer at OD_{600} nm and the concentration confirmed with a plate counting method as colony forming units (Suanyuk *et.al*., 2008).

The fish in G1 and G3 were individually injected with 0.1 mL of TSB, while those in G2 and G4 were individually injected intraperitoneally with 0.1 mL TSB containing 2.4 $\times 10^7$ cfu/mL *S. agalactiae.* At 3, 6, 12, 24 and 48 h post-injection, one fish per tank (thus, five fish per group at each time-point) was randomly sampled and determined for haemato-immunological parameters as described. Mortality was monitored twice daily for 2 weeks in seperate tanks using 24 fish per tank in five replicate tanks per group. Moribund fish were clinically assessed and tissue swabs were cultured to determine the presence of *S. agalactiae* in TSA and confirmed by PCR assay.

Statistical analysis

All data were analysed using one-way analysis of variance (ANOVA), general linear model, and pairwise comparison (Turkey) of means. All statistical tests were performed using SPSS software (Version 19) under license to the Mahidol University. The differences were considered statistically significant when $p<0.05$. Principal Components Analyses (PCAs) were conducted within Statistica 10 (StatSoft, Inc. 1984-2011, Tulsa, Oklahoma, USA) on the raw data obtained for 24 measured immunological parameters. An ANOVA with a post-hoc Tukey HSD test with both Bonferroni and Holm's correction (Holm, 1979) applied to account for multiple comparisons were subsequently ran on the factor scores for the first principal component derived from each analysis. Statistically significant differences were accepted at $p<0.05$.

Result

Before performing the *S. agalactiae* challenge, the haemato-immunological parameters of the normal and *Fno*-infected fish were compared and most of the parameters tested were not statistically different between the two groups (Table 3). The only parameter that was statistically different was the percentage of lymphocytes, which was significantly lower ($p<0.05$) in the *Fno*-infected fish compared to the normal fish (i.e. approx. 85% of those compared to the normal fish).

Following the *S. agalactiae* challenge, many changes were observed in the parameters measured. The Hct, total RBC count and MCV of all groups of fish, measured at 3, 6, 12, 24 and 48 hours post-infection (hpi), are shown in Figure 2. Although a comparison was made between the 4 groups at each time-point, the difference between G3 (normal fish infected with *S. agalactiae*) vs G4 (Fno-infected fish infected with *S. agalactiae*) was of particular interest. Following the *S. agalactiae* infection, the Hct of the G3 and G4 fish were generally lower than those of their respective controls, G1 and G2, with statistically significance differences obtained at several time-points: G1 vs G3, at 3, 12 and 48 hpi; G2 vs G4, at 12 and 48 hpi. Comparing between G3 vs G4, the Hct of G4 fish was significantly lower than that of the G3 fish at 12 and 48 hpi, suggesting that the *Fno*-infected fish had become more anaemic than the normal fish following infection with *S. agalactiae*.

The RBC value of the G3 fish was significantly higher than that of G1 fish at 24 hpi, while the RBC values of the G4 fish was significantly lower than that of G2 fish at 12 hpi. Comparing between G3 vs G4 fish, the RBC count of G4 was significantly lower than that of G3 at 12 and 24 hpi. Therefore, *S. agalactiae* infection in normal fish had either “no-effect” or resulted in a higher RBC count, while the infection in the *Fno*-infected fish had either “no-effect” or resulted in a lowering of the RBC count.

The values of the MCV obtained for the G3 fish was significantly lower than that of G1 fish at 12 and 24 hpi, while corresponding values in the G4 fish did not differ to those of the of G2 fish at any time-point. No significant difference was detected between the G3 fish and the G4 fish at any time-point. Therefore, infection with *S. agalactiae* caused a decrease in the MCV value in the normal fish, but not the *Fno*-infected fish.

The total WBC count, and the differential count for lymphocytes and thrombocytes for the 4 groups of fish are presented in Figure 3. No difference was found in the percentage of neutrophils, eosinophils and monocytes between the groups of fish, and therefore the data for these have not been presented in Figure 3. The total WBC count of G3 fish was significantly higher than that of G1 fish at 6, 12, 24 and 48 hpi, whereas the WBC count of the G4 fish was only significantly higher than that of G2 fish at 48 hpi. Between G3 and G4 fish, the latter had significantly lower levels of WBC than the fish in G3 at 12 hpi. The results, therefore, suggested that there is an increase in the total WBC count in both normal and *Fno*-infected fish in responded to the *S. agalactiae* infection, but this response was statistically higher in normal fish at certain points in the infection.

When comparing the percentage of lymphocytes present in G1 and G3 fish, these values were significantly higher at 12 hpi and significantly lower at 48 hpi in G3 fish compared to G1 fish, while no significant difference was seen between G2 vs G4 fish. When the percentage of lymphocytes present in the blood of the normal (G3) and *Fno*-infected fish (G4), following *S. agalactiae* challenge was compared, the percentage was significantly lower in the G4 fish at 0 hpi, but significantly higher at 24 hpi, and overall the percentage of lymphocytes seemed very variable between these two groups over the infection period.

The percentage of thrombocytes did not differ in the four groups of fish, except at 12 hpi when the value of *Fno*-infected fish (G4) was significantly higher than that of other groups.

The levels of lysozyme and peroxidase activity and total protein levels in plasma of fish over the duration of the study are shown in Figure 4. High levels of fluctuation could be seen in lysozyme activity and no significant differences could be seen between the groups at any time-point, except at 3 hpi, when the activity in G4 fish was significantly higher than that of the G3 fish. When peroxidase activity was examined in the different groups, significantly higher levels were seen in G3 fish than that of G1 fish at 48 hpi, and significantly higher levels were seen in G4 fish compared to G2 fish at 12, 24 and 48 hpi. Between G3 vs G4 fish, the peroxidase activity was significantly higher in G4 fish compared to that of G3 fish at 12 and 24 hpi, but significantly lower at 48 hpi. The results, therefore, suggested a general increase in the level of plasma peroxidase activity after *S. agalactiae* challenge, both in the normal and *Fno*-infected fish, and the response in the *Fno*-infected fish was more than that of the normal fish during 12-24 hpi, but had lower response after that.

For plasma protein levels, significantly higher levels of protein were seen in normal fish infected with *S. agalactiae* (G3) than that of normal fish (G1) at 48 hpi, while significantly higher were seen in *Fno*-infected fish infected with *S. agalactiae* (G4) compared to *Fno*-infected fish (G2) at 12 hpi. Comparing between the G3 vs G4 fish, the protein level *Fno*-infected fish infected with *S. agalactiae* (G4) was significantly higher than that of the normal fish infected with *S. agalactiae* (G3) at 12 hpi. The protein level was, therefore, increased by the *S. agalactiae* infection and the response in the *Fno*-infected fish was earlier (12 hpi) than that of the normal fish (24 hpi). And, again, the response of the *Fno*-infected fish seemed to decline after 24 hpi, while that of the normal fish continued to rise at 48 hpi.

Head-kidney macrophages function was also examined in the 4 groups of fish (RBA and ROS values shown in Figure 5 and phagocytosis activity shown in Table 4). For RBA activities and ROS stimulation index without PMA stimulation, these values were significantly higher in G3 fish than those measured in G1 fish at 12 and 48 hpi, while values

in G4 fish was significantly higher than those of G2 fish at 12, 24 and 48 hpi. When values were compared between the G3 vs G4 fish i.e. normal fish infected with *S. agalactiae* compared to *Fno*-infected fish infected with *S. agalactiae*, the levels produced by G4 macrophages was significantly higher than macrophages from G3 fish at 24 and 48 hpi. The response was similar in the presence of PMA stimulation, except with higher values were obtained. The results therefore suggest that the RBA is increased in both normal and *Fno*-infected fish in responded to *S. agalactiae* infection and this response was significantly higher in the *Fno*-infected fish in the latter part of the trial.

The ROS stimulation index was, however, comparable in all groups at all time points, except at 48 h, in which the level seen with G4 macrophages was significantly lower than those of G2.

Generally, the phagocytic activity of head-kidney phagocytes was not significantly different in any of the groups of fish, except at 24 hpi when the level in the G4 fish was significantly higher than that of the G3 fish (Table 4); the phagocytic index was also similar between groups. When comparing the phagocytic index between G1 and G3 fish, there was a significantly higher activity in G3 fish than the G1 fish at 12 hpi, and between the G2 and G4 fish, the latter had a significantly higher index than G2 fish at 24 hpi. Between G1 vs G2 fish, the phagocytic index of G2 fish was significantly higher than that of G1 at 3 hpi, whole no significant difference was seen between G3 vs G4 fish at any time-point.

No significant difference was detected in the phagocytic capacity when G2 and G4 fish and G3 and G4 fish were compared. There was a significant difference between G1 vs G3 fish however, with G3 fish having a significantly lower PC3 at 12 hpi, but significantly higher PC6 at 24 hpi and significantly higher PC>6 at 3 hpi.

The survival of the fish following the *S. agalactiae* challenge is shown in Figure 5, showing that G4 died rapidly and the survival was less than 10 % at 2 dpi, while the mortality of G3 was less than 10 % at 2 dpi. From days 11 to 14, the survival in G4 (the *Fno*-infected fish infected with *S. agalactiae*) was 3.3 (± 3.5) %, while 46.7 (± 5.4) % of fish survived in G3 (normal fish infected with *S. agalactiae*). No fish died in the two non-challenged groups [G1 (normal fish) and G2 (*Fno*-infected fish)] with 100% survival. The moribund fish had an erratic swimming pattern, exophthalmos and skin lesions. Swabs taken from head-kidney, liver and spleen, streaked on agar produced colonies of bacteria, which were characterised as *S. agalactiae* from their biochemical profile.

Separate rounds of PCA analysis were run for each time point in the immunology-based study (i.e. at t=0, 3, 6, 12, 24 and 48 hours post-infection; see Figure 7). The component loadings for the 24 measured immunological parameters (Hct, MCV, RBC count, WBC count, Differential WBC count (Monocyte, Lymphocyte, Eosinophil and Thrombocyte), Respiratory burst activity (RBA) and RBA with PMA), ROS, Lysozyme activity, Phagocytic activity, Phagocytic index, Phagocytic capacity (PC1, PC2, PC3, PC4, PC5, PC6 and PC6+), Total protein and Plasma peroxidase) used in each PCA analysis are given in Table 5, 6 and shows which had a major effect in separating the fish through each principal axis. From this, in general, RBA, RBA with PMA and HCT were the key parameters separating specimens through the first principal component at 12-48 hours post-infection, while total protein, thrombocytes and lymphocytes, among others were the key variables acting through the second principal component (see Figure 7 and Table 5, 6). Significant differences between the groups tested with a one-way ANOVA were seen at 12 (p=3.042 $\times 10^{-5}$), 24 (p=0.0004) and 48 (p=2.40 $\times 10^{-6}$) hours post-infection. Bonferroni and Holm post-hoc multiple comparison tests between the pairs of test groups are summarised in Table 7.

Table 3. Haemato-immunological parameters of hybrid red tilapia compared between normal and *F. noatunensis*-infected fish

	Normal	*Fno*-infected
Haematology		
Haematocrit (%)	29.0 ± 3.1	27.2 ± 3.6
Total RBC count (x 10^6 cells/µL)	1.68 ± 0.48	1.58 ± 0.31
Mean corpuscular volume (fL)	189.8 ± 71.0	178.3 ± 41.2
Total WBC count (x 10^4 cells/µL)	1.41 ± 0.40	1.43 ± 0.19
WBC differential count (%)		
Neutrophil	21.5 ± 4.5	24.3 ± 6.0
Eosinophil	3.1 ± 1.9	3.5 ± 2.8
Monocyte	2.3 ± 0.8	3.0 ± 1.9
Lymphocyte	42.7 ± 3.3	36.4 ± 7.5*
Thrombocyte	30.4 ± 6.9	32.8 ± 7.1
Innate immune response		
Lysozyme activity (unit/mL)	257.9 ± 110.8	266.2 ± 165.3
Peroxidase activity (450-nm absorbance)	3.7 ± 0.9	3.6 ± 0.7
Total protein (mg/mL)	322 ± 88	320 ± 30
Respiratory burst activity		
Reactive oxygen species index	1.88 ± 0.35	1.97 ± 0.39
Phagocytic activity		
Phagocytic activity (%)	90.3 ± 1.7	89.8 ± 4.1
Phagocytic index (yeast cells/phagocyte)	3.5 ± 0.2	3.5 ± 0.3
Phagocytic capacity (%)		
1-yeast cell/phagocyte	14.4 ± 2.9	16.2 ± 4.0
2-yeast cell/phagocyte	20.3 ± 5.3	15.9 ± 3.7
3-yeast cell/phagocyte	13.8 ± 4.1	12.6 ± 2.7
4-yeast cell/phagocyte	12.1 ± 4.2	13.5 ± 2.7
5-yeast cell/phagocyte	6.0 ± 1.6	6.2 ± 0.8
6-yeast cell/phagocyte	4.5 ± 1.2	5.1 ± 0.9
>6-yeast cell/phagocyte	5.5 ± 1.6	5.5 ± 1.4

Fno, *F. noatunensis*; RBC, red blood cell; WBC, white blood cell. *p<0.05; ** (610-nm absorbance for 10^6 cells); Values represent the mean value of 10 fish ±SD

Table 4. Phagocytosis of cells isolated from head-kidney of normal and *Fno*-infected red tilapia

	No *S. agalactiae* challenge		*S. agalactiae* challenge	
	G1 (Normal)	**G2 (*Fno*-infected)**	**G3 (Normal)**	**G4 (*Fno*-infected)**
Phagocytic activity (%)				
3 hpi	88.0 ± 2.9	92.4 ± 5.9	89.8 ± 3.6	90.0 ± 7.3
6 hpi	88.8 ± 1.3	89.0 ± 2.8	88.4 ± 2.7	91.8 ± 2.6
12 hpi	91.0 ± 2.7	91.0 ± 3.3	93.4 ± 4.0	93.8 ± 3.2
24 hpi	91.2 ± 1.5^{ab}	88.8 ± 5.5^{a}	88.8 ± 3.7^{a}	94.0 ± 1.6^{b}
48 hpi	86.0 ± 5.9	84.8 ± 1.9	85.4 ± 2.2	83.6 ± 2.3
Phagocytic index				
3 hpi	3.1 ± 0.5^{a}	3.8 ± 0.3^{b}	3.5 ± 0.3^{ab}	3.5 ± 0.6^{ab}
6 hpi	3.3 ± 0.1	3.4 ± 0.4	3.3 ± 0.3	3.5 ± 0.3
12 hpi	3.5 ± 0.2^{a}	3.6 ± 0.3^{ab}	4.0 ± 0.4^{b}	3.8 ± 0.1^{ab}
24 hpi	3.8 ± 0.2^{ab}	3.5 ± 0.3^{a}	3.6 ± 0.1^{ab}	3.9 ± 0.1^{b}
48 hpi	3.0 ± 0.3	3.0 ± 0.2	3.0 ± 0.1	2.9 ± 0.2
Phagocytic capacity 1 (PC1)				
3 hpi	19.9 ± 6.2^{ab}	14.1 ± 1.7^{a}	18.6 ± 4.4^{a}	21.6 ± 4.8^{b}
6 hpi	20.1 ± 2.1	17.8 ± 5.8	20.2 ± 2.8	18.1 ± 3.5
12 hpi	19.1 ± 2.8^{a}	16.8 ± 3.2^{ab}	13.8 ± 3.8^{b}	14.9 ± 3.0^{ab}
24 hpi	15.4 ± 3.5	15.9 ± 4.0	17.3 ± 1.9	14.0 ± 3.3
48 hpi	22.5 ± 3.9	23.4 ± 1.6	23.7 ± 2.3	24.9 ± 2.9
Phagocytic capacity 3 (PC3)				
3 hpi	13.9 ± 3.2	12.3 ± 2.7	13.4 ± 3.1	11.5 ± 4.4
6 hpi	13.1 ± 3.1	14.1 ± 1.8	14.7 ± 1.2	14.4 ± 2.1
12 hpi	13.9 ± 1.5^{a}	13.0 ± 0.5^{ab}	12.0 ± 1.3^{b}	13.0 ± 1.6^{ab}
24 hpi	12.7 ± 1.6	15.6 ± 2.2	12.4 ± 3.2	13.2 ± 2.0
48 hpi	16.0 ± 4.2	16.1 ± 2.0	12.6 ± 4.1	16.3 ± 2.4
Phagocytic capacity 5 (PC5)				
3 hpi	5.7 ± 1.3	6.3 ± 1.3	5.2 ± 1.4	5.7 ± 1.6
6 hpi	5.0 ± 1.1	5.7 ± 0.9	6.1 ± 0.9	5.9 ± 2.1
12 hpi	4.8 ± 1.5	5.3 ± 1.8	6.4 ± 1.5	6.3 ± 0.7
24 hpi	5.3 ± 1.0^{a}	6.0 ± 0.7^{ab}	6.6 ± 0.5^{b}	6.7 ± 0.8^{b}
48 hpi	4.1 ± 1.3	4.4 ± 1.3	3.1 ± 1.3	3.4 ± 0.7
Phagocytic capacity 6+ (PC6+)				
3 hpi	3.3 ± 1.8^{a}	6.3 ± 1.4^{b}	6.4 ± 1.3^{b}	6.6 ± 2.7^{b}
6 hpi	5.4 ± 1.2	5.4 ± 1.9	5.0 ± 1.2	5.6 ± 1.1
12 hpi	5.2 ± 0.5	7.0 ± 0.9	7.3 ± 2.5	6.5 ± 1.0
24 hpi	7.5 ± 0.5^{a}	5.9 ± 1.3^{b}	6.5 ± 0.7^{ab}	6.9 ± 0.6^{ab}
48 hpi	6.1 ± 1.6	5.6 ± 1.7	6.2 ± 0.3	6.4 ± 0.9

Different superscripts indicate statistical significance ($p<0.05$); comparison was made between the values of the same row. Phagocytic index = average number of yeast cells in one phagocyte; PC1 (and higher) = percentage of phagocytes containing 1 yeast cell (and higher); hpi, hours post-injection of *S. agalactiae*. Values represent the mean value of 5 fish ±SD

Table 5. The component loadings and the percentage of the variance explained by each variable (n = 24) for each principal components analysis. PCA 1 (*t* = 0 hour post-infection (p.i.); 4 groups; n = 5 replicates per group); PCA 2 (*t* = 3 hpi.); PCA 3 (*t* = 6 hpi.). Each analysis considers four groups of samples: G1 - normal fish not subjected to a *Streptococcus agalactiae* challenge; G2 - *Francisella noatunensis*-infected fish not subjected to a *S. agalactiae* challenge; G3 - normal fish subjected to an *S. agalactiae* challenge and G4 - *F. noatunensis*-infected fish subjected to a *S. agalactiae* challenge. Values above ± 0.70 are shown in a bold font.

Variable	PCA 1 (*t* = 0 hpi.)			PCA 2 (*t* = 3 hpi.)			PCA 3 (*t* = 6 hpi.)		
	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3
HCT	0.484	-0.323	0.520	0.345	0.004	0.480	-0.017	0.488	-0.175
MCV	**0.793**	-0.299	-0.253	0.109	**-0.766**	0.342	-0.442	0.447	0.593
RBC × 10^5 cell/ml	**-0.708**	0.108	0.333	0.022	**0.783**	0.012	0.449	-0.169	**-0.752**
WBC × 10^4 cell/ml	-0.452	-0.281	0.411	0.090	0.157	-0.392	0.232	-0.393	-0.308
Respiratory burst activity (RBA)	-0.646	-0.325	0.483	-0.039	-0.630	-0.581	-0.454	0.418	0.024
Respiratory burst activity (RBA with PMA)	-0.554	-0.349	0.378	0.154	**-0.784**	-0.383	-0.303	0.369	-0.345
Reactive oxygen species (ROS)	0.157	-0.087	-0.188	**0.763**	-0.176	0.259	0.344	-0.234	-0.663
Lysozyme activity	-0.233	0.351	-0.341	-0.233	-0.563	0.158	-0.141	**-0.733**	-0.032
Total protein	-0.591	-0.216	-0.354	-0.377	0.346	0.262	0.313	0.100	0.290
Plasma peroxidase	0.176	-0.477	0.522	-0.664	-0.172	-0.151	-0.278	-0.635	-0.254
Monocyte	-0.193	0.512	0.225	-0.051	0.376	-0.160	0.201	-0.506	0.408
Lymphocytes	0.017	-0.297	0.403	-0.187	-0.155	-0.553	-0.641	-0.515	0.133
Neutrophils	**-0.777**	0.163	-0.072	-0.316	0.139	0.050	-0.272	-0.244	0.215
Eosinophils	-0.222	0.239	-0.297	-0.036	0.678	-0.342	-0.020	-0.233	0.060
Thrombocytes	**0.700**	-0.039	-0.271	0.385	-0.335	0.544	0.528	0.614	-0.288
Phagocytic activity (PA)	-0.175	**0.715**	0.316	-0.682	0.131	0.305	0.495	-0.096	-0.153
Phagocytic index (PI)	0.066	**0.889**	0.264	**-0.939**	-0.050	0.192	**0.896**	0.025	0.234
Phagocytic capacity 1 (PC1)	-0.518	-0.330	-0.318	**0.744**	0.092	-0.571	-0.668	-0.288	-0.407
Phagocytic capacity 2 (PC2)	-0.034	-0.485	-0.478	0.688	0.214	0.306	-0.507	0.516	-0.090
Phagocytic capacity 3 (PC3)	0.352	0.031	0.665	0.177	0.366	0.267	-0.420	0.023	-0.563
Phagocytic capacity 4 (PC4)	0.518	0.095	0.480	-0.161	-0.157	0.367	-0.037	-0.175	0.665
Phagocytic capacity 5 (PC5)	-0.319	0.168	0.214	-0.159	-0.551	-0.045	0.483	-0.400	0.022
Phagocytic capacity 6 (PC6)	-0.595	0.052	-0.207	-0.339	-0.089	0.623	0.501	-0.572	0.273
Phagocytic capacity 6+ (PC6+)	0.309	0.663	-0.115	**-0.800**	0.034	-0.260	0.631	0.629	0.064
% Total variation	0.216	0.145	0.133	0.203	0.168	0.130	0.195	0.176	0.132
Cumulative percentage	21.59	36.08	49.38	20.34	37.16	50.2	19.49	37.09	50.24

Table 6. The component loadings and the percentage of the variance explained by each variable (n = 24) for each principal components analysis. PCA 4 (*t* = 12 hpi.); PCA 5 (*t* = 24 hpi.) and PCA 6 (*t* = 48 hpi.). Each analysis considers four groups of samples: G1 - normal fish not subjected to a *Streptococcus agalactiae* challenge; G2 - *Francisella noatunensis*-infected fish not subjected to a *S. agalactiae* challenge; G3 - normal fish subjected to an *S. agalactiae* challenge and G4 - *F. noatunensis*-infected fish subjected to a *S. agalactiae* challenge. Values above ± 0.70 are shown in a bold font.

Variable	PCA 4 (*t* = 12 hpi.)			PCA 5 (*t* = 24 hpi.)			PCA 6 (*t* = 48 hpi.)		
	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3
HCT	**-0.819**	0.296	-0.147	-0.341	0.373	0.422	**0.768**	-0.249	0.073
MCV	-0.288	-0.441	0.006	-0.513	-0.475	-0.236	-0.073	-0.173	0.546
RBC × 10^5 cell/ml	-0.367	0.563	-0.104	0.204	0.627	0.406	0.471	0.082	-0.427
WBC × 10^4 cell/ml	0.614	0.555	0.053	0.585	-0.286	0.391	-0.607	0.014	0.178
Respiratory burst activity (RBA)	**0.785**	-0.193	-0.431	**0.860**	-0.109	-0.269	**-0.745**	0.080	0.203
Respiratory burst activity (RBA with PMA)	**0.778**	-0.381	-0.323	**0.871**	-0.243	-0.253	**-0.778**	0.003	0.216
Reactive oxygen species (ROS)	-0.168	-0.388	0.364	-0.198	-0.485	-0.163	**0.708**	0.060	0.184
Lysozyme activity	0.086	0.079	-0.240	-0.430	0.026	0.115	0.571	-0.028	0.171
Total protein	0.614	-0.597	-0.032	**0.745**	-0.095	-0.275	-0.606	-0.076	0.135
Plasma peroxidase	**0.812**	-0.437	-0.236	0.458	-0.226	-0.037	-0.676	0.069	0.299
Monocyte	-0.280	-0.031	**-0.735**	0.544	-0.290	0.259	0.332	**0.729**	-0.094
Lymphocytes	0.025	0.687	-0.350	-0.295	-0.557	-0.371	0.489	-0.153	0.330
Neutrophils	-0.444	-0.070	0.596	-0.481	0.607	-0.414	-0.010	0.568	-0.324
Eosinophils	0.026	-0.294	-0.421	0.444	0.571	-0.142	-0.295	0.592	0.181
Thrombocytes	0.547	-0.571	0.493	0.364	-0.244	0.733	-0.291	**-0.828**	-0.194
Phagocytic activity (PA)	0.509	0.418	0.134	0.456	0.321	-0.130	0.309	-0.164	0.416
Phagocytic index (PI)	**0.715**	0.517	0.262	0.370	0.287	-0.145	0.229	-0.484	0.680
Phagocytic capacity 1 (PC1)	-0.671	-0.263	-0.251	-0.015	0.274	0.461	-0.267	0.430	-0.288
Phagocytic capacity 2 (PC2)	-0.238	-0.346	0.242	0.364	-0.259	-0.435	-0.198	0.177	-0.379
Phagocytic capacity 3 (PC3)	-0.174	-0.377	0.108	-0.332	-0.572	0.246	0.445	0.400	0.429
Phagocytic capacity 4 (PC4)	-0.511	-0.162	-0.357	-0.174	0.331	-0.759	-0.066	-0.389	-0.290
Phagocytic capacity 5 (PC5)	0.482	0.161	-0.431	0.352	-0.056	-0.067	0.361	-0.259	-0.486
Phagocytic capacity 6 (PC6)	0.393	0.220	-0.325	-0.220	-0.306	0.224	-0.216	-0.705	-0.159
Phagocytic capacity 6+ (PC6+)	0.461	0.408	0.670	0.288	0.357	0.174	0.030	0.237	**0.893**
% Total variation	0.263	0.155	0.129	0.211	0.139	0.121	0.215	0.143	0.136
Cumulative percentage	26.26	41.79	54.7	21.11	35.04	47.14	21.49	35.82	49.4

Table 7. Bonferroni and Holm post-hoc tests ran on the factor scores for the first principal component derived from each analysis testing for statistical significance ($p<0.05$). Where G1 = normal fish that were not challenged with *Streptococcus agalactiae*; G2 = *Francisella noatunensis*-infected fish that were not challenged with *S. agalactiae*; G3 = normal fish that were challenged with *S. agalactiae*; and, G4 = *F. noatunensis*-infected fish that were challenged with *S. agalactiae*. NS, not significant.

	12 hpi		**24 hpi**		**48 hpi**	
Treatment pair	**Bonferroni**	**Holm**	**Bonferroni**	**Holm**	**Bonferroni**	**Holm**
G1 vs G2	NS	NS	NS	NS	NS	NS
G1 vs G4	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$
G1 vs G3	$p<0.01$	$p<0.01$	NS	NS	$p<0.01$	$p<0.01$
G2 vs G4	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$
G2 vs G3	$p<0.01$	$p<0.01$	NS	$p<0.05$	$p<0.01$	$p<0.01$
G4 vs G3	NS	$p<0.05$	NS	NS	NS	NS

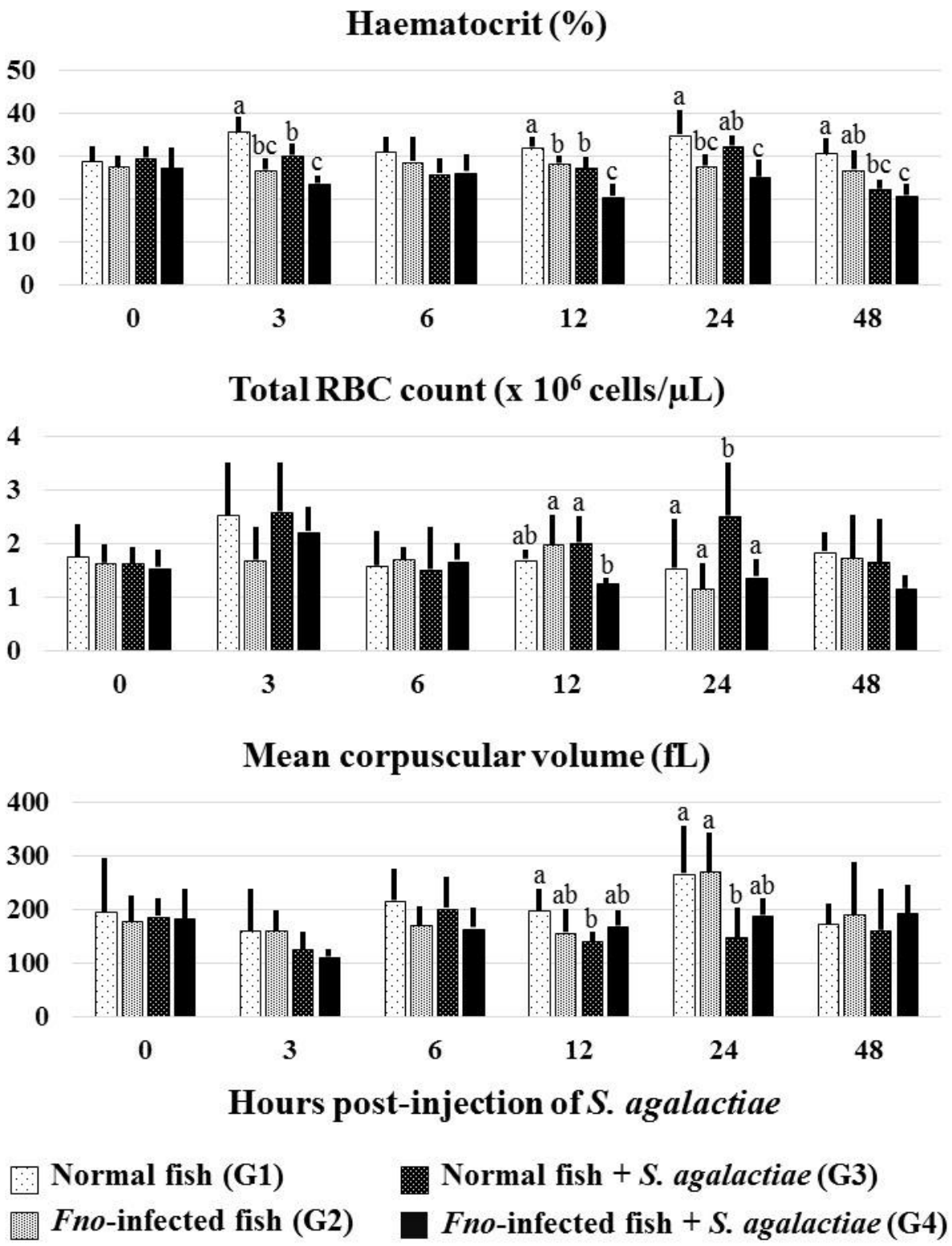


Figure 2. % Haematocrit, total red blood cell count and mean corpuscular volume of normal and *Fno*-infected red tilapia, at time 3, 6, 12, 24 and 48 hours following *Streptococcus agalactiae* challenge. Values are means ± standard deviation with n = 5 per group. Statistical comparison was made among the values of the same time-point; different superscripts indicate statistical significance ($p<0.05$).

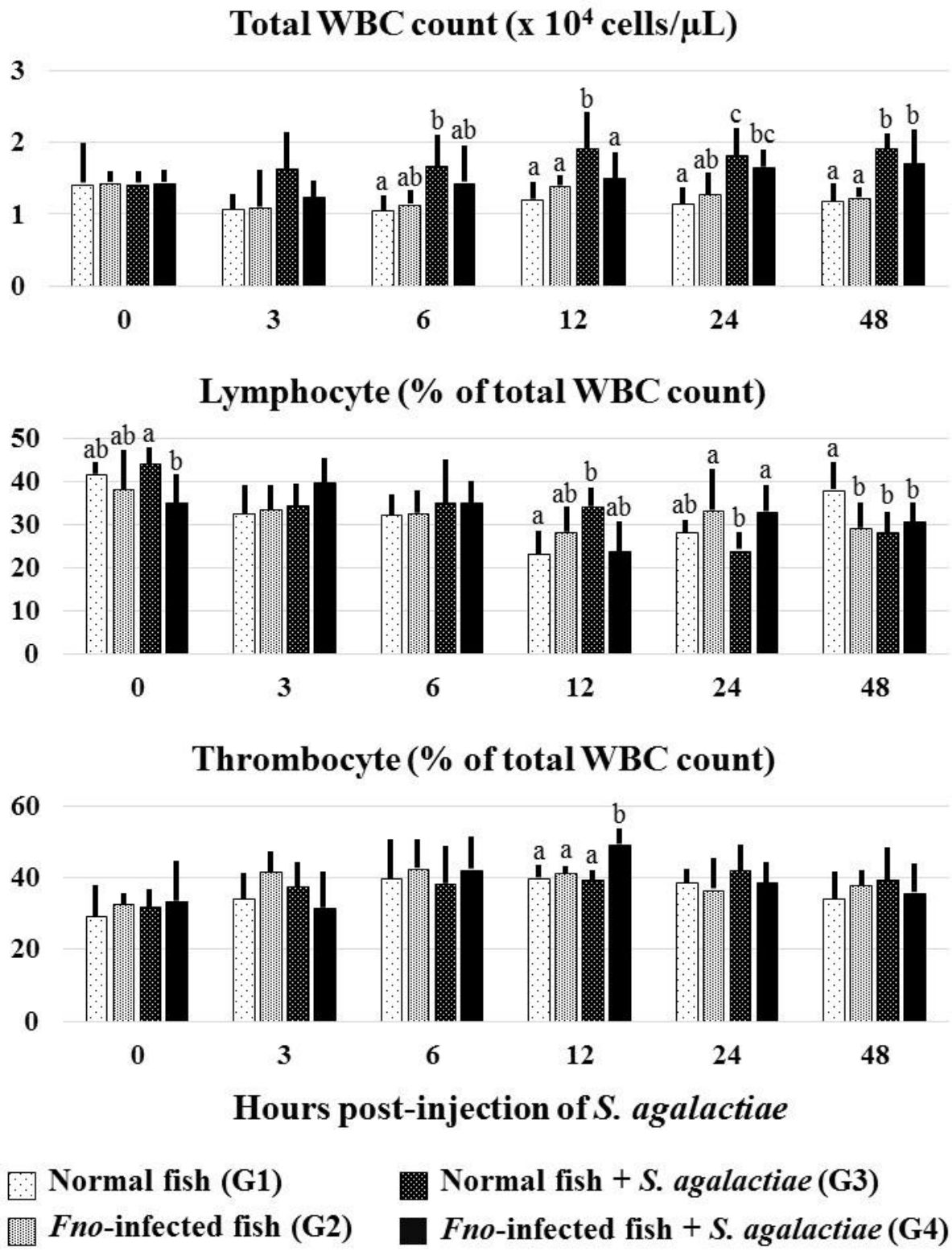


Figure 3. Total white blood cell count and percentage of lymphocytes and thrombocytes of normal and *Fno*-infected red tilapia, at time 3, 6, 12, 24 and 48 hours following *Streptococcus agalactiae* challenge. Values are means ± standard deviation with n = 5 per group. Statistical comparison was made among the values of the same time-point; different superscripts indicate statistical significance ($p<0.05$).

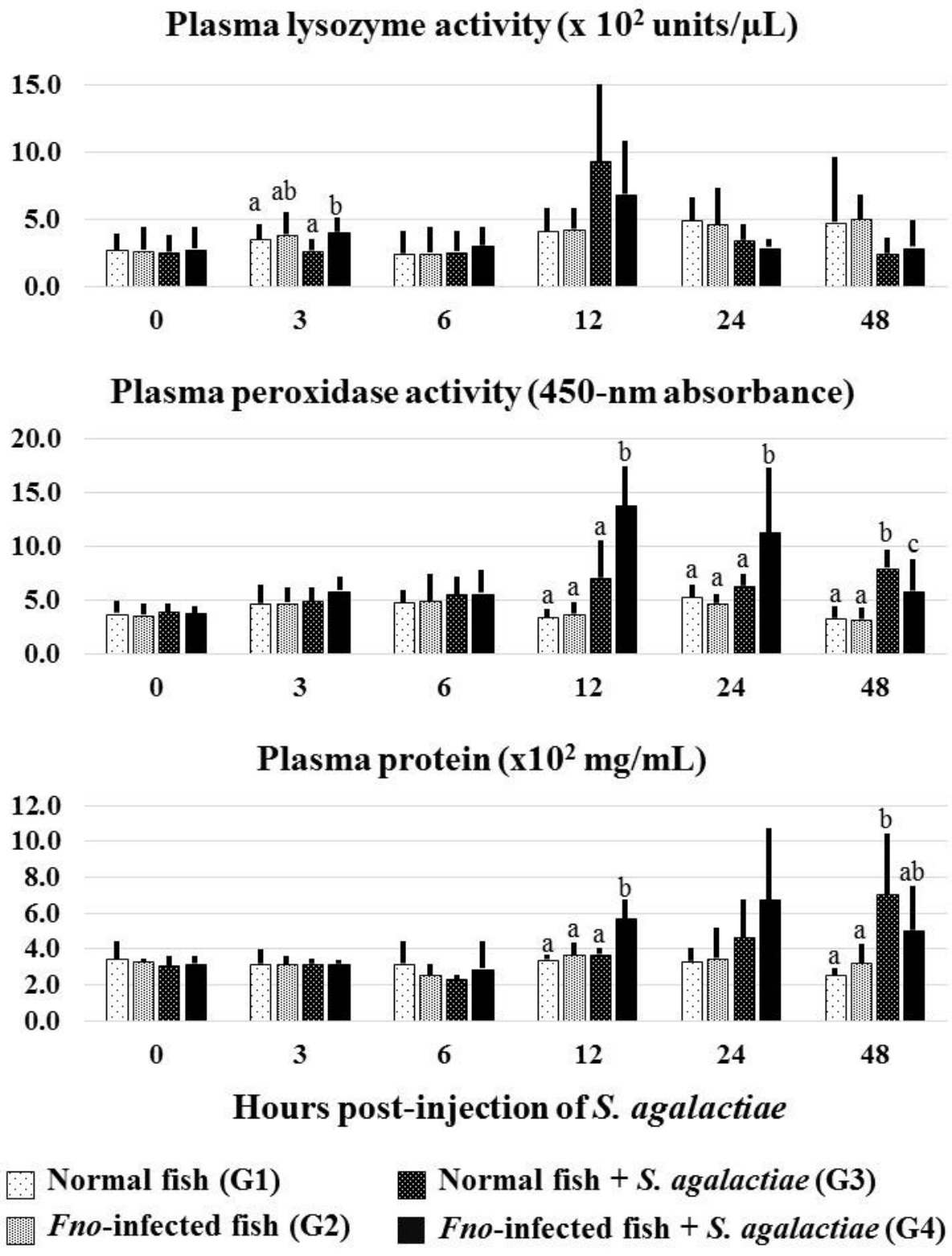


Figure 4. Activities of plasma lysozyme and peroxidase and level of plasma protein of normal and *Fno*-infected red tilapia, at time 3, 6, 12, 24 and 48 hours following *Streptococcus agalactiae* challenge. Values are means ± standard deviation with n = 5 per group. Statistical comparison was made among the values of the same time-point; different superscripts indicate statistical significance ($p<0.05$).

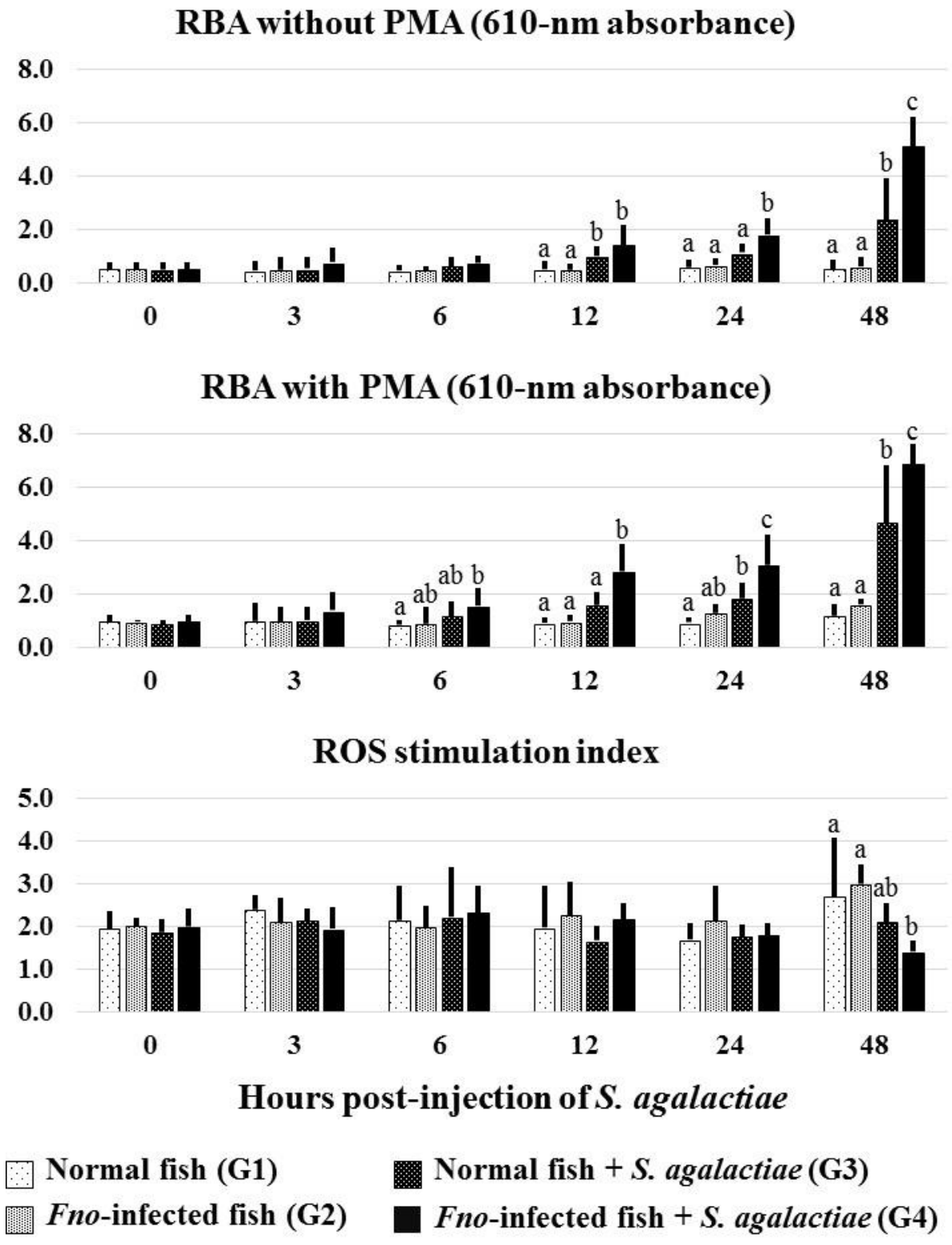


Figure 5. Respiratory burst activities (RBA), with and without phorbol myristate acetate (PMA) stimulation and reactive oxygen species (ROS) stimulation index of normal and *Fno*-infected red tilapia, at time 3, 6, 12, 24 and 48 hours following *Streptococcus agalactiae* challenge. Values are means ± standard deviation with n = 5 per group. Statistical comparison was made among the values of the same time-point; different superscripts indicate statistical significance ($p<0.05$).

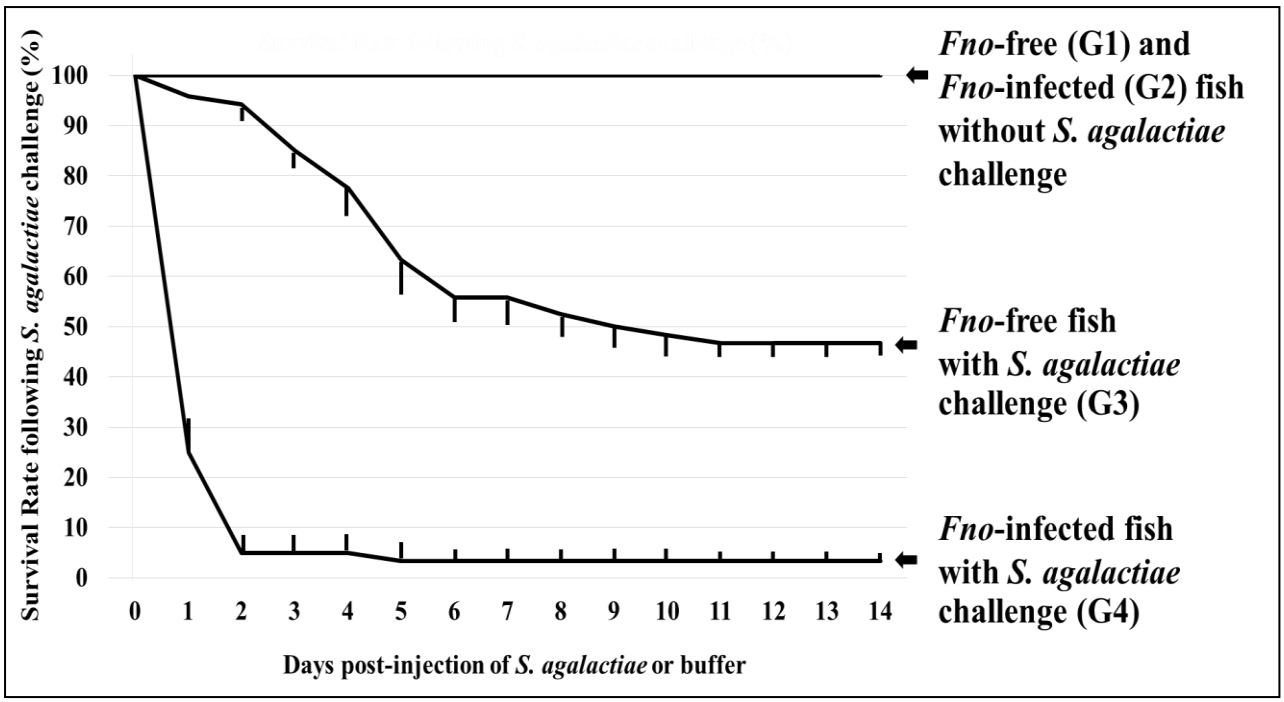


Figure 6. Survival rate of normal and *Fno*-infected red tilapia following *Streptococcus agalactiae* challenge, compared to that of the non-challenge fish. Values are means ± standard deviation with N = 24.

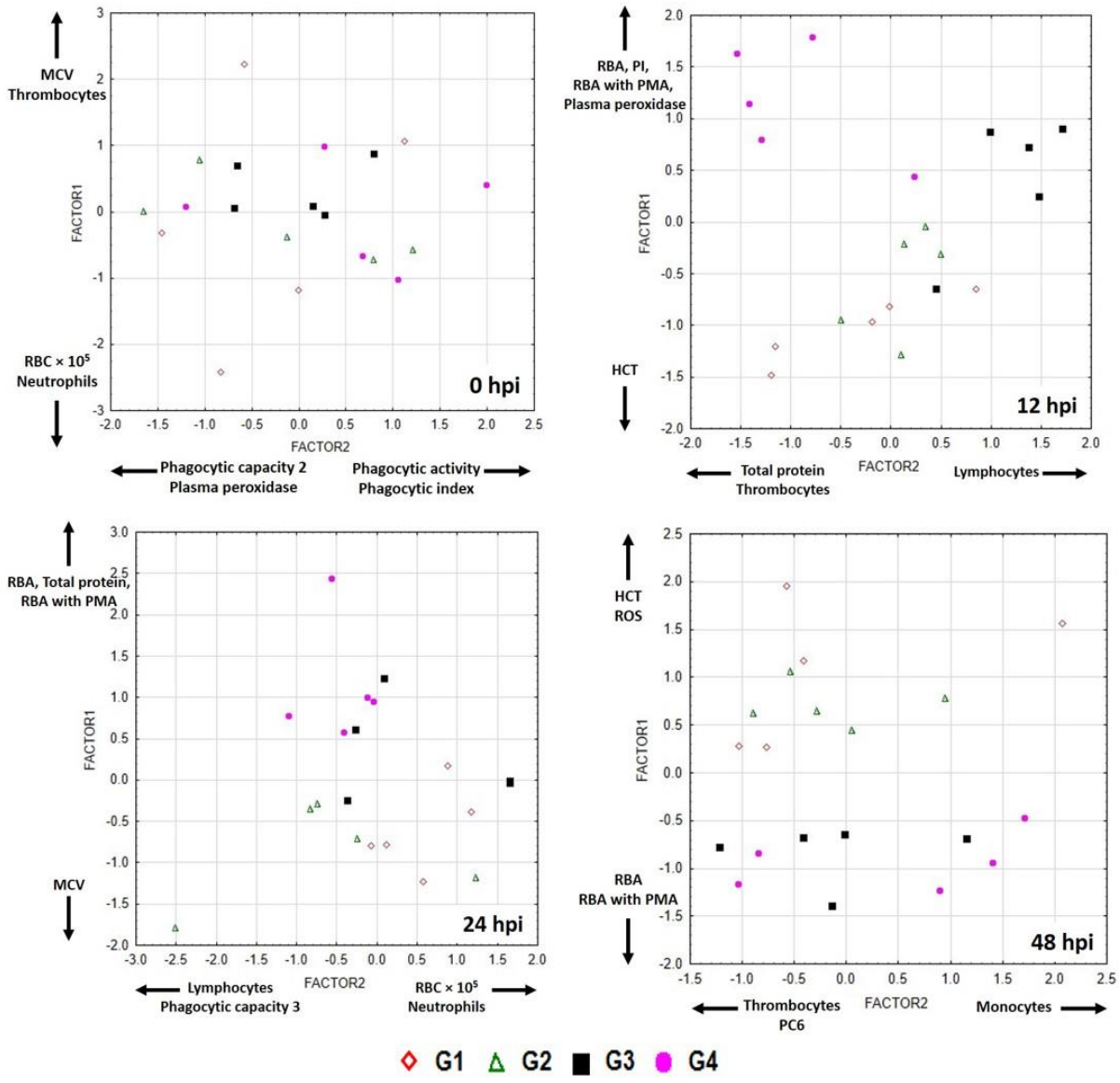


Figure 7. Plot of 20 fish (n = 5 fish per group) in the first plane of PCAs (24 variables, raw data) ran on data obtained at 0, 12, 24 and 48 hours post-infection. Each group of fish is identified by a symbol where G1 (open diamonds) = normal fish not subjected to a *Streptococcus agalactiae* challenge; G2 (open triangles) = *Francisella noatunensis*-infected fish not subjected to a *S. agalactiae* challenge; G3 (solid squares) = normal fish subjected to an *S. agalactiae* challenge; and, G4 (solid circles) = *F. noatunensis*-infected fish subjected to a *S. agalactiae* challenge. For each PCA plot, the variables making a major contribution to the separation of the specimens and the direction in which they act is marked on each factor.

Conclusion and Discussion

Before *S. agalactiae* challenge, the haemato-immunological parameters measured in the *Fno*-infected red tilapia were comparable to values obtained for the normal fish, except for a lower percentage of lymphocytes from the differential white blood cell count. During the *S. agalactiae* challenge, both the % Hct and RBC count were significantly lower in the *Fno*-infected fish, compared to the normal fish. Since the MCV values were normal, it suggests that the reduced % Hct was caused by the reduced number of RBC, not the size of the RBC. As the head-kidney is also the site of RBC production (Abdel-Aziz *et al*., 2010), it is possible that *Fno* infection affected erythropoiesis in the *Fno*-infected fish.

The total WBC and WBC differential counts were similar between the *Fno*-infected and normal fish before *S. agalactiae* challenge, butwere significantly higher in the *Fno*-infected fish after the *S. agalactiae* challenge. This 40-50% increase (compared to levels of both normal and *Fno*-infected only fish) may reflect the "alert" level of the WBC following the natural *Fno*-outbreak. The percentage of lymphocytes were found to be lower in the *Fno*-infected fish than in the controls before challenge, but during the challenge, it remained more or less the same level as that of the normal fish. The finding suggests that *Fno* infection in the head-kidney of these fish did not impact on the normal myelopoiesis and, probably, lymphopoiesis functions. The presence of basophils has not been reported in the peripheral blood of the hybrid red tilapia either (Hrubec *et al*., 2000), but it has been reported that this type of cell is easy overlook during microscopic identification of the cells (Ueda *et al*., 2001).

Following *S. agalactiae* challenge, plasma lysozyme and peroxidase activities of the *Fno*-infected fish were significantly higher than those of the normal fish at certain time-

points. Both of these enzymes are produced by neutrophils and monocytes (Ueda *et al*., 2001; Scapigliati *et al*., 2002), so the reason why these two enzymes increased following the challenge was possibly due to an increased in number and function of the WBC in response to the *S. agalactiae* infection. Peroxidase combines H_2O_2 with Cl^- to produce hypochlorite, which plays an important role in destroying bacteria (Secombes and Fletcher, 1992).

The total plasma protein level in this study was comparable to values described elsewhere (Hrubec *et al*, 2000). The main protein component in plasma is albumin and immunoglobulin. It is not known which component contributed the increase in the total plasma protein seen in *Fno*-infected fish, although it is probable the immunoglobulin, involved in the adaptive immunological response to *S. agalactiae*, might have increased (Harikrishnan and Balasundaram, 2010).

While it was anticipated that the *Fno*-infected fish would have a sub-normal RBA, the head-kidney macrophages from the *Fno*-infected fish was in fact significantly higher than that of the normal fish, with or without PMA stimulation. In addition, the phagocytic activities were found to be comparable to the normal fish. In fish, as in other animals, RBA is the strong defence mechanism against pathogens, which occurs after phagocytosis, and is associated with an intense oxygen consumption by the phagocytes, which causes them to produce superoxide and other reactive compounds, such as hydrogen peroxide and hydroxyl radicals; and together with lysozyme and peroxidase, the radicals kill the engulfed pathogens (Secombes and Fletcher, 1992). The results from the plasma enzymes, RBA and phagocytic activities suggest that the *Fno*-infected fish did not show any reduced capacity of their innate immune response. In humans, *Francisella tularensis* infected neutrophils have been shown to have the ability to evade RBA of the infected cells by preventing NADPH oxidase assembly and the bacteria survived well in the infected cells (McCaffrey and Allen,

2006). This mechanism does not seem to operate in the *Fno*-infected fish in this study as the RBA was increasing despite the *Fno*-infection.

It is interesting to look in more detail as to why the *Fno*-infected fish had higher mortality than the normal fish following *S. agalactiae* challenge despite comparable, or even better innate immune activity.

The first possibility is that the innate immune defence mechanisms of the fish are not adequate to protect the fish from pathogens. It was reported in Senegalese sole that increasing RBA of phagocytes of the fish was not necessarily adequate to protect the fish from *Photobacterium damselae* infection (Díaz-Rosales *et al*., 2009). In *O. niloticus*, it has been shown that the correlation between the levels of the RBA and protection against a pathogen is not always demonstrated. Two different strains of *O. niloticus* responded differently to *Vibrio parahaemiolyticus* challenge by showing significantly different levels of RBA, but yet had the same mortality rate following challenge with the bacterium (Balfry *et al*., 1997). In the present study, on the other hand, we found that normal and *Fno*-infected fish had different levels of cumulative mortality following infection with *S. agalactiae*, but with more or less similar haematological and innate immune responses, thus it can be concluded that there does not appear to be a correlation between the these parameters and protection against *S. agalactiae* in either group of fish.

The second possibility is that the combination of the *Fno* and *S. agalactiae* infections caused further decrease in erythropoiesis resulting in acute anaemic, with % Hct dropping from an average of 26.6% to 20.6% within 48 h. The bacteria *S. agalactiae* has been reported to infect several organs of Nile and red tilapia, especially their brain and kidney (Hernandez *et al*., 2009; Assis *et al*., 2017), and *S. agalactiae* were cultured from the head-kidney of the infected fish.

The third possibility is that *Fno*-infected fish had a less effective adaptive immune reponse than the normal fish. The production of antibodies is accomplished by lymphocytes, which differentiate into plasma cells after encountering their target antigen. In this study, the percentage of lymphocyte in the *Fno*-infected fish was not lower than that of the normal fish, however. Interestingly, the head-kidney of many fish, including *O. niloticus*, also contains thyroid hormone-producing cells (Geven and Klaren, 2017). As thyroid hormones and immune system interact and support each other in ways that are still unclear, possible damage of thyroid function by the *Fno* infection in the head-kidney of the fish might be related to the lower resistance against the superimposing *S. agalactiae* infection and this is an interesting point to consider in future research.

Conclusions

Hybrid red tilapia, *Oreochromis* sp., chronically infected with *Fno* have normal haematological and innate immune function and survive well under normal conditions. They, however, have more rapid and higher level of mortality, compared to the non-infected red tilapia, when experimentally infected with *S.agalactiae*, despite having normal or higher innate immune responses. Following *S. agalactiae* infection, the *Fno*-infected fish had an acute reduction in their red blood cell count. Whether this acute anaemia or other unknown mechanisms caused the rapid death of the fish is unknown, but it is likely that the seemingly normal innate immune responses in the *Fno*-infected red tilapia is not adequate to protect the fish from mass mortality by opportunistic pathogens.

References

Abdel-Aziz E-S H, Abdu SBS, Ali T E-S, Fouad HF (2010) Haemopoiesis in the head kidney of tilapia, *Oreochromis niloticus* (Teleostei: Cichlidae): a morphological (optical and ultrastructural) study. *Fish Physiol Biochem* 36, 323–336.

Amal MNA, Zamri-Saad M (2011) Streptococcosis in Tilapia (*Oreochromis niloticus*): A Review. *Pertanika J Trop Agric Sci* 34, 195 – 206.

Assis GBN, Tavares GC, Pereira FL, Figueiredo HCP, Leal CAG (2017) Natural coinfection by *Streptococcus agalactiae* and *Francisella noatunensis* subsp. *orientalis* in farmed Nile tilapia (*Oreochromis niloticus* L.). *J Fish Dis* 40, 51-63.

Austin B, Austin D (2007) Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish. Springer Netherlands, Dordrecht.

Aviles F, Zhang MM, Chan J, Delamare-Deboutteville J, Green TJ, Dang C, Barnes AC (2013) The conserved surface M-protein SiMA of *Streptococcus iniae* is not effective as a cross-protective vaccine aainst differing capsular serotypes in farmed fish. *Veterinary Microbiology* 162, 151-159.

Badran AF, Eissa IAM, Elgmal A, Dena M (2010) Efficacy of intraperitoneal injection vaccination in African catfish, *Clarias gariepinus,* Against streptococcosis. *SCVMJ* 15(1), 73-79.

Bakkemo KR, Mikkelsen H, Bordevik M, Torgersen J, Winther-Larsen HC, Vanberg C, Seppola M (2011) Intracellular localisation and innate immune responses following *Francisella noatunensis* infection of Atlantic cod (*Gadus morhua*) macrophages. *Fish & shellfish immunology* 31(6), 993-1004.

Balfry SK, Shariff M, Iwama GK (1997) Starin difference in non-specific immunity in tilapia *Oreochromis niloticus* following challenge with *Vibrio parahaemolyticus*. Dis Aquat Org 30, 77-80.

Birkbeck TH, Feist SW, Verner–Jeffreys DW (2011) Francisella infections in fish and shellfish. Journal of fish diseases 34(3), 173-187.

Birkbeck TH, Bordevik M, Froystad MK, Baklien (2007) A: Identification of *Francisella* sp. from Atlantic salmon, *Salmo salar* L., in Chile. *Journal of Fish Diseases* 30, 505-507.

Bowser PR, Wooster GA, Getchell RG, Timmons MB (1998) *Streptococcus iniae* infection of tilapia Oreochromis niloticus in a recirculation production facility. *Journal of the World Aquaculture Society 29*(3), 335-339.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248–254.

Brudal E, Lampe EO, Reubsaet L, Roos N, Hegna IK, Thrane IM, Winther-Larsen HC (2015) Vaccination with outer membrane vesicles from *Francisella noatunensis* reduces development of francisellosis in a zebrafish model. *Fish & shellfish immunology* 42(1), 50-57.

Colorn A, Diamant A, Eldar A, Kvitt H, Zlotkin A (2002) *Streptococcus iniae* infections in Red Sea cage-cultured and wild fishes. *Diseases of aquatic organisms*, 49(3), 165-170.

Colquhoun DJ, Duodu S (2011) Francisella infections in farmed and wild aquatic organisms. *Veterinary Researsh* 42(45), 1-15.

Corbel M. (1975) The immune response in fish: a review. *Journal of fish biology* 7, 539-563.

Díaz-Rosales P, Arijo S, Chabrillón M, Alarcón FJ, Tapia-Paniagua ST, Martínez-Manzanares E, Balebona MC, Moriñigo MA (2009) Effects of two closely related probiotics on

respiratory burst activity of Senegalese sole (*Solea senegalensis*, Kaup) phagocytes, and protection against *Photobacterium damselae* subsp. *piscicida*. *Aquaculture* 293, 16-21.

Domeénech A, Derenaáandez-Garayzábal JF, Pascual C, Garcia JA, Cutuli MT, Moreno MA, Dominguez L (1996) Streptococcosis in cultured turbot, *Scopthalmus maximus* (L.), associated with *Streptococcus parauberis*. *Journal of Fish Diseases* 19(1), 33-38.

Dong JJ, Wu F, Ye X, Sun CF, Tian YY, Lu MX, Chen ZH (2015) β-Defensin in Nile tilapia (*Oreochromis niloticus*): Sequence, tissue expression, and anti-bacterial activity of synthetic peptides. *Gene* 566(1), 23-31.

Eldar A, Horovitcz A, Bercovier H (1997) Development and efficacy of a vaccine against *Streptococcus iniae* infection in farmed rainbow trout. *Veterinary Immunology and Immunopathology* 56(1-2), 175-183.

Ellingsen T, Inami M, Gjessing MC, Van Nieuwenhove K, Larsen R, Seppola M, Schrøder MB (2011) Francisella noatunensis in Atlantic cod (Gadus morhua L.); waterborne transmission and immune responses. *Fish & shellfish immunology* 31(2), 326-333.

Evans AS (1976) Causation and disease: the Henle-Koch postulates revisited. *Yale Journal of Biology and Medicine* 49(2), 175-195.

Evans JJ, Klesius PH, Shoemaker CA (2004) Efficacy of *Streptococcus agalactiae* (group B) vaccine in tilapia (*Oreochromis niloticus*) by intraperitoneal and bath immersion administration. *Vaccine* 22(27-28), 3769-3773.

Figueiredo HCP, Nobrega-Netto L, Leal CAG, Pereira UP, Mian GF (2012) *Streptococcus iniae* outbreaks in Brazilian Nile tilapia (*Oreochromis niloticus* L:) farms. *Brazilian Journal of Microbiology 43*(2), 576-580.

Findlay VL, Munday BL (2000) The immunomodulatory effects of levamisole on the nonspecific immune system of Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* 23, 369-378.

Food and Agriculture Organization of the United Nations. (2014). Fishery and aquaculture statistics: Aquaculture production. FAO yearbook 2012, pp 10, 48. Rome: FAO. ISBN 978-92-5-008293-6 [online] Available at: http://www.fao.org/3/a-i3740t.pdf [Accessed 10 September 2014].

Forsman M, Sandstrom G, Sjostedt A (1994) Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. *Int J Syst Evol Micr*. 44, 38-46.

Geng Y, Wang KY, Huang XL, Chen DF, Li CW, Ren SY, Lai WM (2012) *Streptococcus agalactiae*, an Emerging Pathogen for Cultured Ya-Fish, *Schizothorax prenanti*, in China. *Transboundary and emerging diseases* 59(4), 369-375.

Geven EJW, Klaren PHM (2017) The teleost head kidney: Integrating thyroid and immune signaling. *Devel Comp Immunol* 66, 73-83.

Gibello A, Mata AI, Blanco MM, Casamayor A, Domínguez L, Fernández-Garayzabal JF (2005) First identification of *Streptococcus phocae* isolated from Atlantic salmon (*Salmo salar*). *Journal of clinical microbiology* 43(1), 526-527.

Hardi EH, Harris E, Lusiastuti AM (2013) Potential vaccine candidate of *Streptococcus agalactiae* to prevent Streptococcosis on Nile tilapia. 2013. *Journal Veteriner*, 14(4): 408-416.

Harikrishnan R, Balasundaram C, Heo MS (2010) *Lactobacillus sakei* BK19 enriched diet enhances the immunity status and disease resistance to streptococcosis infection

in kelp grouper, *Epinephelus bruneus*. Fish & shellfish immunology, 29(6): 1037-1043.

Hawke J, Soto E (2012) U.S. Patent No. 8,147,820. Washington, DC: U.S. Patent and Trademark Office. [online] Available at:http://www.google.com/patents /US8147820 [Accessed 20 July 2015].

He A, Yang J, Tang S, Wang C (2013) Cloning and molecular characterization of complement component 1 inhibitor (C1INH) and complement component 8β (C8β) in Nile tilapia (*Oreochromis niloticus*). *Fish & shellfish immunology* 35(3), 1055-1058.

Hernandez E, Figuer J, Iregui C (2009) Streptococcosis on a red tilapia, *Oreochromis* sp., farm: a case study. *Journal of Fish Diseases* 32, 247-252.

Hernandez E, Figueroa J Iregui C (2009). Streptococcosis on a red tilapia, *Oreochromis* sp., farm: a case study. *Journal of Fish Diseases*, 32(3): 247-252.

Holm S (1979) A simple sequentially rejective multiple test procedure. *Scand J Stat* 6 (2), 65-70.

Hrubec TC, Cardinante JL, Smith SA (2000) Haematology and serum chemistry reference intervals for cultured tilapia (*Oreochromis hybrid*). *Vet Clin Pathol* 29, 7-12.

Hsieh CY, Tung MC, Tu C, Chang CD Tsai SS (2006) Enzootics of visceral granulomas associated with Francisella-like organism infection in tilapia (*Oreochromis* spp.). *Aquaculture* 254, 129–138.

Hsieh CY, Wu ZB, Tung MC Tsai SS (2007) PCR and in situ hybridization for the detection and localization of a new pathogen Francisella-like bacterium (FLB) in ornamental cichlids. *Diseases of Aquatic Organisms* 75, 29–36.

Huang HY, Chen YC, Wang PC, Tsai MA, Yeh SC, Liang HJ, Chen SC (2014) Efficacy of a formalin-inactivated vaccine against *Streptococcus iniae* infection in the farmed

grouper *Epinephelus coioides* by intraperitoneal immunization. *Vaccine* 32(51), 7014-7020.

Huang LY, Wang KY, Xiao D, Chen DF, Geng Y, Wang J, He Y, Wang EL, Huang JL Xiae GY (2014) Safety and immunogenicity of an oral DNA vaccine encoding Sip of *Streptococcus agalactiae* from Nile tilapia *Oreochromis niloticus* delivered by live attenuated *Salmonella typhimurium. Fish and shellfish Immunology* 38, 34-41.

Iregui CA, Vasquez GM, Rey AL, Verjan N (2011) Piscirickettsia-like organisms as a cause of acute necrotic lesions in Colombian tilapia larvae. *Journal of Veterinary Diagnostic Investigation 23*(1), 147-151.

Jantrakajorn S, Maisak H, Wongtavatchai J (2014) Comprehensive Investigation of Streptococcosis Outbreaks in Cultured Nile Tilapia, Oreochromis niloticus, and Red Tilapia, Oreochromis sp. of Thailand. *Journal of the World Aquaculture Society*, *45*(4), 392-402.

Jeffery KR, Stone D, Feist SW, Vemer-Jeffreys DW (2010) An outbreak of disease caused by *Francisella* sp. In Nile tilapia *Oreochromis niloticus* at a recirculation fish in the UK. *Diseases of Aquatic Organisms*, 91(2), 161-165.

Kamaishi T, Fukuda Y, Nishiyama H, Kawakami H, Matsuyama T, Yoshinaga T, Oseko N (2005) Identification and pathogenicity of intracellular Francisella bacterium in three-lined grunt *Parapristipoma trilineatum*. *Fish Pathology* 40, 67–71.

Kamaishi T, Miwa S, Goto E, Matsuyama T, Oseko N (2010) Mass mortality of giant abalone Haliotis gigantea caused by a *Francisella sp. bacterium*. *Diseases of Aquatic Organisms* 89, 145–154.

Ke J, Zhao F, Luo L, Jiang L, Lu MX, Zou WM (2010) Isolation, identification and pathogenicity of pathogenic bacteria of fulminant disease of tilapia in Guangdong Province. *Journal of Guangdong Ocean University*, *3*, 008.

Klesius PH, Shoemaker CA (1999) Development and use of modified live *Edwardsiella ictaluri* vaccine against enteric septicemia of catfish. *Advances in Veterinary Medicine* 41, 523-537.

LaFrentz BR, LaPatra SE, Call DR, Cain KD (2008) Isolation of rifampicin resistant *Flavobacterium psychrophilum* strains and their potential as live attenuated vaccine candidates. *Vaccine* 26, 5582-5589.

Lau SKP, Woo PCY, Tse H, Leung KW, Wong SSY, Yuen KY (2003). Invasive *Streptococcus iniae* infections outside North America. *Journal of Clinical Microbiology*, 41(3), 1004-1009.

Li Y, Cai SH (2011) Identification and pathogenicity of *Aeromonas sobria* on tail-rot disease in juvenile tilapia *Oreochromis niloticus*. *Current microbiology 62*(2), 623-627.

Locke JB, Vicknair MR, Ostland VE, Nizet V, Buchanan JT (2010) Evaluation of *Streptococcus iniae* killed bacterin and live attenuated vaccines in hybrid striped bass through injection and bath immersion. *Diseases of Aquatic Organisms* 89(2), 117.

Magnadottir B (2010) Immunological control of fish diseases. Marine Biotechnology 12, 361-379.

Mauel MJ, Miller DL, Frazier K, Liggett AD, Styer L, Montgomery-Brock D, Brock J (2003) Characterization of a piscirickettsiosis-like disease in Hawaiian tilapia. *Diseases of Aquatic Organisms* 53, 249–255.

Mauel MJ, Soto E, Morales JA, Hawke J (2007) A Piscirickettsiosis-like syndrome in cultured Nile tilapia in Latin America with *Francisella* spp. as the pathogenic agent. *Journal of Aquatic Animal Health* 19, 27–34.

McCaffrey RL, Allen L-A H (2006) Pivotal Advance: *Francisella tularensis* LVS evades killing by human neutrophils via inhibition of the respiratory burst and phagosome escape. *J Leukoc Biol*, 80, 1224–1230.

Morgan A, Thompson K, Auchinachie N, Migaud H (2008) The effect of seasonality on normal haematological and innate immune parameters of rainbow trout *Oncorhynchus mykiss* L. *Fish Shellfish immunol* 25, 791-799.

Morgan A, Thompson K, Auchinachie N, Migaud H (2008) The effect of seasonality on normal haematological and innate immune parameters of rainbow trout *Oncorhynchus mykiss* L. *Fish & shellfish immunology* 25, 791-799.

Nguyen VV, Dong HT, Senapin S, Pirarat N, Rodkhum C (2015) *Francisella noatunensis* subsp. *orientalis*, an emerging bacterial pathogen affecting cultured red tilapia (*Oreochromis* sp.) in Thailand. *Aquaculture Research* 1-6. DOI: 10.1111/are.12802.

Nur-Nazifah M, Sabri MY, Siti-Zahrah A (2014) Development and efficacy of feed-base recombinant vaccine encoding the cell wall surface anchor family protein of *Streptococcus agalactiae* against streptococcosis in *Oreochromis* sp. *Fish and Shellfish Immunology* 37(1), 193-200.

Ostland VE, Stannard JA, Creek JJ, Hedrick RP, Ferguson HW, Carlberg JM, Westerman ME (2006) Aquatic Francisella-like bacterium associated with mortality of intensively-cultured hybrid striped bass *Morone chrysops* x *M. saxitalis*. *Disease of Aquatic Organisms* 72, 135–145.

Ottem KF, Nylund A, Karlsbakk E, Friis-Møller A, Kamaishi T (2009) Elevation of *Francisella philomiragia* subsp. *noatunensis* Mikalsen et al. (2007) to *Francisella noatunensis* comb. *nov*. [syn. *Francisella piscicida* Ottem et al. (2008) syn. nov.] and characterization of *Francisella noatunensis* subsp*. orientalis* subsp. nov., two important fish pathogens. *Journal of applied microbiology*, 106(4), 1231-1243.

Panprommin D, Vanichkul K, Panprommin N, Areechon N (2011) Effects of turmeric (*Curcuma longa* Linn.) extract on the expression of cytokine genes in Nile tilapia (*Oreochromis niloticus* Linn.). In Proceedings of the 49th Kasetsart University Annual Conference, Kasetsart University, Thailand, 1-4 February, 2011. Volume 3. Subject: Fisheries. (pp. 416-425). Kasetsart University.

Pasnik DJ, Evans JJ, Klesius PH, Shoemaker CA, Yeh HY (2009) Pathogenicity of *Streptococcus ictaluri* to channel catfish. *Journal of aquatic animal health* 21(3), 184-188.

Pasnik, DJ, Evans JJ, Panangala VS, Klesius PH, Shelby RA, Shoemaker CA (2005) Antigenicity of *Streptococcus agalactiae* extracellular products and vaccine efficacy. *Journal of fish disease* 28(4), 205-212.

Perera RP, Johnson SK, Collins MD, Lewis DH (1994) *Streptococcus iniae* associated with mortality of *Tilapia nilotica*× *T. aurea* hybrids. *Journal of Aquatic Animal Health* 6(4), 335-340.

Pirarat N, Pinpimai K, Endo M, Katagiri T, Ponpornpisit A, Chansue N, Maita M (2011).Modulation of intestinal morphology and immunity in nile tilapia (*Oreochromis niloticus*) by Lactobacillus rhamnosus GG. *Research in veterinary science* 91(3), e92-e97.

Plouffe DA, Hanington PC, Walsh JG, Wilson EC, Belosevic M (2005) Comparison of select innate immune mechanisms of fish and mammals. *Xenotransplantatio*n 12, 266-277.

Pradeep PJ, Suebsing R, Sirithammajak S, Kampeera J, Turner W, Jeffs A, Kiatpathomchai W, Withyachumanarnkul B (2017) Vertical transmission and concurrent infection of multiple bacterial pathogens in naturally infected red tilapia (*Oreochromis* spp.). *Aquaculture Res* 48, 2706-2717.

Pridgeon JW, Klesius PH, Yildirim-Aksoy M (2013) Development of live attenuated *Streptococcus agalactiae* as potential vaccines by selecting for resistance to sparfloxacin. *Vaccine* 31, 2705-2712.

Quade MJ, Roth JA (1997) A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. *Veterinary immunology and immunopathology* 58, 239-248.

Romana-Eguia MRR, Ikeda M, Basiao ZU, Taniguchi N (2004) Genetic diversity in farmed Asian Nile and red hybrid tilapia stocks evaluated from microsatellite and mitochondrial DNA analysis. *Aquaculture 236*(1), 131-150.

Ruane NM, Bolton-Warberg M, Rodger HD, Colquhoun DJ, Geary M, McCleary SJ, Fitzgerald, RD (2015) An outbreak of francisellosis in wild-caught Celtic Sea Atlantic cod, *Gadus morhua* L., juveniles reared in captivity. *Journal of fish diseases* 38(1), 97-102.

Saurabh S, Sahoo P (2008) Lysozyme: an important defence molecule of fish innate immune system. *Aquaculture Research* 39, 223-239.

Scapigliati G, Romano N, Buonocore F, Picchietti S, Baldassini MR, Prugnoli D, Galice A, Meloni S, Secombes CJ, Mazzini M, Abelli L (2002) The immune system of sea

bass, *Dicentrarchus labrax*, reared in aquaculture. Develop Comp Immunol 26, 151-160.

Secombes CJ, Fletcher TC (1992) The role of phagocytes in the protective mechanisms of fish. *Ann Rev Fish Dis* 2, 53-71.

Seesamut T, Yimtragool N (2014) Effects of Egg Woman (*Phyllanthus amarus*) Extract on Complement C3 Expression in Nile Tilapia (*Oreochromis niloticus*) by Real Time RT-PCR Technique. *Burapha Science Journal*, 19(Special), 63-70.

Shin GW, Palaksha KJ, Kim YR, Nho SW, Kim SHGJ, Park SC, Jung TS (2007) Application of immunoproteomics in developing a *Streptococcus iniae* vaccine for olive flounder (*Paralichthys olivaceus*). *Journal of chromatography B*, 849(1-2): 315-322.

Sirimanapong W, Thompson KD, Kledmanee K, Thaijongrak P, Collet B, Ooi EL, Adams A (2014) Optimisation and standardisation of functional immune assays for striped catfish (*Pangasianodon hypophthalmus*) to compare their immune response to live and heat killed *Aeromonas hydrophila* as models of infection and vaccination. *Fish Shellfish Immunol* 40, 374–383.

Sirimanapong W, Thompson KD, Ooi EL, Bekaert M, Collet B, Taggart JB, Bron JE, Green DM, Shinn AP, Adams A, Leaver MJ (2015) The effects of feeding β-glucan to *Pangasianodon hypophthalmus* on immune gene expression and resistance to *Edwardsiella ictaluri*. *Fish Shellfish Immunol* 47, 595-605.

Sitjà-Bobadilla A, Pena-Llopis S, Gomez-Requeni P, Médale F, Kaushik S, Perez-Sanchez J (2005) Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). *Aquaculture* 249, 387-400.

Soto E, Hawke JP, Fernandez D, Morales JA (2009). *Francisella* sp., an emerging pathogen of tilapia, *Oreochromis niloticus* (L.), in Costa Rica. *J Fish Dis* 32, 713-722.

Soto E, Kidd S, Mendez S, Marancik D, Revan F, Hiltchie D, Camus A (2013a) *Francisella noatunensis* subsp. *orientalis* pathogenesis analyzed by experimental immersion challenge in Nile tilapia, *Oreochromis niloticus* (L.). *Vet Microbiol* 164, 77–84.

Soto E, McGovern-Hopkins K, Klinger-Bowen R, Fox BK, Brock J, Antonio N, Waal Zv, Rushton S, Mill A, Tamaru CS (2013b) Prevalence of *Francisella noatunensis* subsp. *orientalis* in cultured tilapia on the island of Oahu, Hawaii. *J Aquat Animal Health* 25, 104–109.

Soto E, Revan F (2012) Culturability and persistence of *Francisella noatunensis* subsp. *orientalis* (syn. *Francisella asiatica*) in sea- and freshwater microcosms. *Microbial Ecology*, 63, 398–404.

Soto E, Wiles J, Elzer P, Macaluso K, Hawke JP (2011) Attenuated *Francisella asiatica* iglC mutant induces protective immunity to francisellosis in tilapia. *Vaccine* 29(3), 593-598.

Sridhar S, Sharma A, Kongshaug H, Nilsen F, Jonassen I (2012) Whole genome sequencing of the fish pathogen *Francisella noatunensis* subsp. *orientalis* Toba04 gives novel insights into Francisella evolution and pathogenecity. *BMC Genomics* 13, 598.

Suanyuk N, Kong F, Ko D, Gilbert GL, Supamattaya K (2008) Occurrence of rare genotypes of *Streptococcus agalactiae* in cultured red tilapia *Oreochromis* sp. and Nile tilapia *O. niloticus* in Thailand-Relationship to human isolates? *Aquaculture* 284(1-4), 35-40.

Suanyuk N, Sukkasame N, Tanmark N, Yoshida T, Itami T, Thune RL, Supamattaya K (2010) *Streptococcus iniae* infection in cultured Asian sea bass (*Lates calcarifer*) and red

tilapia (*Oreochromis* sp.) in southern Thailand. Songklanakarin *Journal of Science and Technology* 32(4), 341-348.

Suebsing R, Kampeera J, Tookdee B, Withyachumnarnkul B, Turner W, Kiatpathomchai W (2013) Evaluation of colorimetric loop-mediated isothermal amplification assay for visual detection of *Streptococcus agalactiae* and *Streptococcus iniae* in tilapia. Lett Appl Microbiol 57, 317-324.

Suebsing R, Kampeera J, Sirithammajak S, Withyachumnarnkul B, Turner W, Kiatpathomchai W (2015) Colorimetric Method of Loop-Mediated Isothermal Amplification with the Pre-Addition of Calcein for Detecting *Flavobacterium columnare* and its Assessment in Tilapia Farms. *Journal of aquatic animal health* 27(1), 38-44.

Sun J, Fang W, Ke B, He D, Liang Y, Ning D, Tan H, Peng H, Wang Y, Ma Y, Ke C, Deng X (2016) Inapparent *Streptococcus agalactiae* infection in adult/commercial tilapia. Scientific Reports | 6:26319 | DOI: 10.1038/srep26319.

Tran LH, Fitzimmons KM, Lightner DV (2014) Effect of tilapia in controlling acute hepatopancreatic necrosis disease (AHPND). In Browdy CL, Hargreaves H, Tung H, and Y Avnimelech. 2014. Proceeding of the Biofloc Technology and Shrimp Disease Workshop. December 9-10, 2013. Ho Chi Minh City, Vietnam. The Aquaculture Engineering Society, Copper Hill, VA, USA. pp. 136-138. www.aesweb.org/shrimp_health.php

Ueda IK, Egami MI, Sasso WS, Matushima ER (2001) Cytochemical aspects of the peripheral blood cells of *Oreochromis* (*Tilapia*) *niloticus*. (Linnaeus, 1758) (Cichlidae, Teleostei) - Part II. Brazillian *J Vet Res Anim Sci São Paulo* 38, 273-277.

Vojtech LN, Sanders GE, Conway C, Ostland V, Hansen JD (2009) Host immune response and acute disease in a zebrafish model of *Francisella* pathogenesis. *Infection and immunity* 77(2), 914-925.

Wang E, Wang K, Chen D, Wang J, He Y, Long B, Lai W (2015) Evaluation and Selection of Appropriate Reference Genes for Real-Time Quantitative PCR Analysis of Gene Expression in Nile Tilapia (*Oreochromis niloticus*) during Vaccination and Infection. *International journal of molecular sciences* 16(5), 9998-10015.

Watts M, Munday B, Burke C (2001) Immune responses of teleost fish. *Australian Veterinary Journal* 79, 570-574.

Withyachumnarnkul B, Gerdmusic C, Jatupongraksa T, Pradeep JP, Chaiyapetchara S (2014) Experience on *Penaeus monodon*/red tilapia co-culture using a biofloc system. In Browdy CL, Hargreaves H, Tung H, and Y Avnimelech. 2014. Proceeding of the Biofloc Technology and Shrimp Disease Workshop. December 9-10, 2013. Ho Chi Minh City, Vietnam. The Aquaculture Engineering Society, Copper Hill, VA, USA. pp. 143-148. www.aesweb.org/shrimp_health.php.

Yang W, Li A (2009) Isolation and characterization of *Streptococcus dysgalactiae* from diseased *Acipenser schrenckii*. *Aquaculture* 294(1), 14-17.

Yardimci B, Aydin Y (2011) Pathological findings of experimental *Aeromonas hydrophila* infection in Nile tilapia (*Oreochromis niloticus*). *Ankara Üniv Vet Fak Derg 58*, 47-54.

Zamri-Saad M, Amal MNA, Siti-Zahrah A (2010) Pathological changes in red tilapias (*Oreochromis* spp.) naturally infected by *Streptococcus agalactiae*. *Journal of comparative pathology* 143(2), 227-229.

Zerihun MA, Feist SW, Bucke D, Olsen AB, Tandstad NM, Colquhoun DJ (2011) *Francisella noatunensis* subsp. *noatunensis* is the aetiological agent of visceral granulomatosis in wild Atlantic cod *Gadus morhua*. *Diseases of aquatic organisms* 95(1), 65-71.

Zhang XY, Fan HP, Zhong QF, Zhuo YC, Yin Y, Zeng ZZ (2008) Isolation, identification and pathogenicity of *Streptococcus agalactiae* from tilapia. *Journal of Fisheries of China* 5: 772-779.

Appendix

Output

Accepted Manuscript

Streptococcus agalactiae infection kills red tilapia with chronic *Francisella noatunensis* infection more rapidly than the fish without the infection

Wanna Sirimanapong, Kim D. Thompson, Andrew P. Shinn, Alexandra Adams, Boonsirm Withyachumnarnkul

PII: S1050-4648(18)30420-0

DOI: 10.1016/j.fsi.2018.07.022

Reference: YFSIM 5422

To appear in: *Fish and Shellfish Immunology*

Received Date: 24 April 2018

Revised Date: 5 July 2018

Accepted Date: 10 July 2018

Accepted Manuscript

Streptococcus agalactiae infection kills red tilapia with chronic *Francisella noatunensis* infection more rapidly than the fish without the infection

Wanna Sirimanapong, Kim D. Thompson, Andrew P. Shinn, Alexandra Adams, Boonsirm Withyachumnarnkul

PII: S1050-4648(18)30420-0

DOI: 10.1016/j.fsi.2018.07.022

Reference: YFSIM 5422

To appear in: *Fish and Shellfish Immunology*

Received Date: 24 April 2018

Revised Date: 5 July 2018

Accepted Date: 10 July 2018

Please cite this article as: Sirimanapong W, Thompson KD, Shinn AP, Adams A, Withyachumnarnkul B, *Streptococcus agalactiae* infection kills red tilapia with chronic *Francisella noatunensis* infection more rapidly than the fish without the infection, *Fish and Shellfish Immunology* (2018), doi: 10.1016/j.fsi.2018.07.022.

Streptococcus agalactiae infection kills red tilapia with chronic *Francisella noatunensis* infection more rapidly than the fish without the infection

Wanna Sirimanapong[a*], Kim D. Thompson[b], Andrew P. Shinn[c], Alexandra Adams[d] and Boonsirm Withyachumnarnkul[e]

[a]Veterinary Aquatic Animal Research Health Care Unit, Department of Clinical Sciences and Public Health, Faculty of Veterinary Science, Mahidol University, Nakhon-pathom 73170, Thailand

[b]Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Scotland, UK

[c]Fish Vet Group Asia Limited, 21/359 Premjairard Road, Saensook, Muang Chonburi, Chonburi, 20130, Thailand

[d]Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling, Scotland, UK

[e]AquaAcademy Farm, Tha Chana, Surat Thani 84170; and Department of Anatomy, Faculty of Science, Prince of Songkla University, Hatyai, Songkla 90112, Thailand

[*]Corresponding author: Wanna Sirimanapong

Tel.: +662-441-5242 ext. 1520; Fax: +662-441-0773

E-mail: wanna.sir@mahidol.ac.th

Abstract

In this study we examined the effect that a *Francisella noatunensis* (*Fno*) infection had on hybrid red tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*) subsquently infected with *Streptococcus agalactiae.* A variety of hemato-immunological parameters (haematocrit, total red blood cell count, mean corpuscular volume, total white blood and differential cell counts, total plasma protein, plasma lysozyme and plasma peroxidase activities, and respiratory burst and phagocytic activities of head-kidney macrophages) were measured in hybrid red tilapia that had been previously exposed to an *Fno* outbreak in a tilapia grow-out farm. The head-kidneys of these apparently healthy survivors, when checked by PCR were found to be *Fno*-positive with hemato-immunological parameters that were similar to fish without an a priori infection. The only exception was the percentage lymphocyte count in the peripheral blood, which was slightly, but significantly, lower in the *Fno*-infected fish, compared to those without the infection. When experimentally infected with *S. agalactiae*, the *Fno*-infected fish died more rapidly and at a significantly higher rate than fish without the infection. During the challenge, the hemato-immunological parameters of both groups of fish were very similar, although the *Fno*-infected fish, challanged with *S. agalactiae* expressed significantly higher plasma lysozyme and peroxidase activities, and their head kidney macrophages had significantly higher respiratory burst activity compared to non-*Fno*-infected fish challanged with *S. agalactiae*. The only two parameters for which *Fno*-infected fish showed significantly lower expressions than that of their non-infected counterparts were haematocrit and total red blood cell count. The cause of the rapidity and higher rates of mortality observed in the *Fno*-infected fish when challenged with *S. agalactiae* is unknown; but it may be due to a reduced erythropoiesis capability within the head-kidney because of the presence of *Fno*.

Keywords: *Francisella noatunensis* subsp. *orientalis*; *Streptococcus agalactiae*; red tilapia; francisellosis; streptococcosis; sequential infections; bacterial pathogens

1. Introduction

Nile tilapia (*Oreochromis niloticus*) and hybrid red tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*) are important aquaculture species that are farmed globally. In many locations where they are farmed, they are naturally infected by the bacterium *Francisella noatunensis* subsp. *orientalis* (*Fno*) (previously known as *F. asiatica*), resulting in the disease francisellosis, with associated mass mortalities [1-5]. This bacterium is a Gram-negative, pleomorphic, coccobacillus, which is a facultative intracellular pathogen able to infect many fish species, including tilapia. The predominant gross pathology in *Fno*-infected fish is the presence of multiple granulomas on the surface of the kidney, spleen and liver [6, 7]. Infection in the anterior part of the kidney, or head-kidney, may result in a decrease in the immunocompetence of the fish, since this organ is the site of erythropoiesis, myelopoiesis and lymphopoiesis, where several immunocompetent cells are generated [8]. Fish can also be infected by *Fno* without any clinical manifestation of the disease [9, 10]. From a recent study conducted in Thailand, about 60% of the farmed red tilapia were found to be sub-clinically infected with *Fno*, which has been shown to be vertically transmitted [10].

One of the most common pathogens associated with mass mortality of farmed tilapia is the bacterium *Streptococcus agalactiae*, which infects both Nile and red tilapia [11]. This bacterium has also been reported to cause subclinical infections in tilapia [12, 13]. Moreover, natural co-infection of *S. agalactiae* and *Fno*, have been reported in farmed *O. niloticus* resulting in mass mortalities [10]. The site of infection of *S. agalactiae* is the liver, spleen, heart, kidney and brain, but mortality is usually associated with the infection of the brain [11], while the site of infection of *Fno* is principally within the spleen and head-kidney [14].

It is possible that an *Fno* infection in the head-kidney of tilapia might affect the fish's haematology and immune system, thus reducing its ability to effectively protect itself against attack by opportunistic pathogens.

In the current study, red tilapia surviving a natural *Fno* infection, and shown to be sub-clinically infected with the bacterium in their head-kidney, were subsequently experimentally infected with *S. agalactiae*. Haematological and innate immune responses were measured in *Fno*-infected and non-infected fish, before and after challenge with *S. agalactiae*, as well as monitoring the survival rate of the infected fish.

2. Materials and methods

2.1 Experimental animals

Three hundred hybrid red tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*), 200-300 g in weight, which had survived a natural *Fno* infection were collected from a commercial farm site in Nakhornsawan Province, Thailand. The infection, which resulted in approximately 45% mortality, occurred a few months before sample collection, by which time the mortalities had completely stopped. Ten of these fish were randomly collected and their head-kidneys screened for the presence of residual *Fno* by polymerase chain reaction (PCR), targeting the 16S rRNA sequence (F11, 5′-TAC CAG TTG GAA ACG ACT GT-3′ and F5, 5′-CCT TTT TGA GTT TCG CTCC-3′) [1]; all fish were found to be positive for the bacterium. No *S. agalactiae* infection was detected by PCR using the specific primers (F5′-CGCT¬GAGGTTTGGTGTTTACA-3′ and R5′-CACTCCTACCAACGTTCTTC-3′) [15]. This group of red tilapia was, therefore, considered to be sub-clinically infected with *Fno*. Three hundred control hybrid red tilapia of the same size were collected from another farm in the same Province that had no previous history of any disease outbreak, including infection with *Fno.* Ten of these fish were also randomly sampled and their kidneys screened for presence of *Fno* by PCR, all of which were found to be *Fno*-negative. The fish were brought to the wet lab at Mahidol University, Bangkok, and stocked in 350-L fibreglass tanks, with 30 individuals per tank; each tank was provided with a separate water supply and drainage, using a flow-through system.

Each of the two groups were then further divided into a group to be experimentally infected with *S. agalactiae* and another that would not be infected with the pathogen, resulting in four groups altogether: G1, *Fno*-free fish without *S. agalactiae* challenge; G2, *Fno*-infected fish without *S. agalactiae* challenge; G3, *Fno*-free fish with *S. agalactiae* challenge; and G4, *Fno*-infected fish with *S. agalactiae* challenge. Five tanks were used for each group with 30 fish per tank (150 fish per group). Water quality was monitored daily to ensure optimal water conditions for the fish, with the following water quality values maintained: dissolved oxygen, >4 ppm; total ammonia nitrogen, <0.5 ppm; total nitrite, <0.5 ppm; pH, 7.0-7.5 and water temperature, 28 ± 1 ^{o}C. A water exchange of 20-30%was performed daily.

2.2 Haematological and immunological parameters

One fish was randomly sampled from each tank (5 fish per group) to assess their haemato-immunological parameters at 0 hour post-infection (hpi), before performing the *S. agalactiae* challenge; thus, the fish tested were 10 *Fno*-free control fish (from G1 and G3 combined) and 10 *Fno*-infected fish (from G2 and G4 combined). The parameters measured included haematological parameters: haematocrit (Hct), total red blood cell (RBC) count, mean corpuscular volume (MCV), total white blood cell (WBC) and differential WBC count; plasma-related parameters (lysozyme activity, peroxidase activity and total protein levels); and head-kidney macrophage activity [respiratory burst (RBA) and phagocytic activities].

2.2.1 Haematology

Blood samples were collected from the caudal vein of the fish and all the parameters described determined using the protocols outlined by Sirimanapong *et al*. [16]. Briefly, 1 mL of blood was withdrawn from the tail vein of individual fish and immediately mixed with heparin (200 U/mL). The withdrawn blood, 100 µL, was immediately used to determine Hct,

total RBC count, total and differential WBC counts and MCV. The rest of the blood was centrifuged at 3,000× g for 5 min, plasma collected, and kept at -70 ^{o}C for further analysis.

The Hct values were determined by placing well-mixed blood into haematocrit capillary tubes, which were centrifuged at 3,000 ×g for 5 min; and the values calculated as percentage of packed RBC volume against the whole blood volume. For total RBC and WBC counts, blood (20 µL) was added to 4 mL Natt-Herricks's solution and mixed thoroughly before counting on a Neubauer chamber. From the values of Hct and total RBC count, MCV was determined according to the formula: MCV (fL) = (Hct × 10)/total RBC (×10^6 cells/µL). WBC differential count was carried out by preparing a smear of whole blood on microscope slides, stained by Wright–Giemsa and examined under light microscopy and the percentage of neutrophils, lymphocytes, monocytes, eosinophils, basophils and thrombocytes determined. At least 200 cells were counted for each differential count.

2.2.2 Parameters in plasma

The plasma was thawed and lysozyme activity, plasma peroxidase activity and total protein content determined. Lysozyme activity was determined according to the method previously described by Morgan *et al*. [17]. Briefly, a suspension of 0.2% (w/v) *Micrococcus lysodeikticus* acquired from Sigma, UK was prepared in 0.04 M phosphate buffer saline (PBS) and 190 µL of the bacterial mixture was placed into microtitre plate wells, and control wells contained only the buffer. Plasma samples (10 µL) were added into the wells to make a final volume of 200 µL well^{-1}. Each plasma sample tested consisted of four replicates. The wells were incubated at room temperature (32 ± 1^{o}C) and the absorbency at 540 nm read at 1 and 5 min after the incubation. One unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance at 0.001/min.

Plasma peroxidase was determined using a modification of the method outlined by Quade and Roth [18] and Sitjà-Bobadilla *et al*. [19]. The plasma sample (15 µL) and Hanks'

balanced salt solution without Ca^{2+} and Mg^{2+} (135µL) were added to the flat bottomed well (96-well plate) followed by the addition of 50 µL of 20 mM 3,3',5,5'-tetramethylbenzidine in substrate buffer (40 mM acetic acid) containing 0.1% H_2O_2. The reaction was stopped with 50 µL of 2 M H_2SO_4 and read with an ELISA reader at 450 nm.

Total plasma protein was analysed using a Bradford assay. A protein standard series (from 0 to 2 mg/mL) were prepared by diluting 6 mg bovine serum albumin in PBS. Twenty µL of sample or standard was added to the wells of a 96 well plate, to which 200 µL of Bradford solution (Sigma, UK) was then added and mixed thoroughly on a plate shaker for 30 sec. After 5 min, the plates were read at an absorbance of 595 nm on a microplate reader. A standard curve of protein concentration against absorbance at 595 nm was plotted and the level of total protein in the samples determined.

2.2.3 Head-kidney macrophage activity

Samples of head-kidney were collected to examine head-kidney macrophage activity, i.e. RBA with and without stimulation by phorbol myristate acetate (PMA), reactive oxygen species (ROS) stimulation index and phagocytosis, all of which were performed as previously described by Sirimanapong *et al*. [20]. To determine the RBA, head-kidney phagocytes were isolated and the cell suspension was placed into a 96 well tissue culture plate (100 µL/well), 10 wells per fish, and incubated at 28-30 ^{o}C for 2 h. The absorbance of the wells was measured at 610 nm using a micro-plate reader (Synergy HT; Bio Tek Instruments, Winooski, VT, USA) and the results expressed as an absorbance at 610 nm per 10^6 cells. The ROS stimulation index was calculated from the equation PMA-stimulated ROS/mean unstimulated ROS.

Phagocytic activity by head-kidney macrophages was determined by placing 100 µL of the head-kidney cell suspension onto microscopic slides, which were then placed in a moist incubation chamber for 1 h at room temperature (32 ± 1^{o}C) to allow adherence of cells

to the slide. Yeast, *Saccharomyces cerevisiae*, 10^7 yeast cells/mL in L-15 medium (Sigma, UK), was used as the phagocytic particles and 100 µL of the yeast suspension was placed into one circle of attached cells and 100 µL of the medium, as control, into the second circle. The slides were incubated for 1 h at room temperature (32 ± 1^{o}C) to allow phagocytosis to take place and then washed with L-15 medium. The cells were fixed with 100% methanol (100 µL) for 5 min and stained with Giemsa stain, and the number of phagocytes containing yeast was counted for a total 200 phagocytes. The phagocytic index (PI), phagocytic activity (PA), and phagocytic capacity (PC) were determined as described previously; PC1, PC3, PC5 and PC≥6 signify the percentage of phogocytes containing 1 or more yeast cells [21].

2.3 *Streptococcus agalactiae* challenge

A virulent strain of *S. agalactiae* serotype Ia (MUVS2017SA02), isolated from a field outbreak, was cultured in tryptone soy agar (TSA, Oxoid England) for 24 h. Three to five colonies of the bacteria were randomly selected from the plate and placed into 100 mL of sterile tryptic soy broth (TSB). The bacteria were cultured to late logarithmic growth in a shaking incubator (150 rpm, 28 ^{o}C) (Incu-shakerTM 10L, Benchmark, NJ) for 24 h and the concentration of the bacterial suspension determined using spectrophotometer at OD_{600} nm and the concentration confirmed with a plate counting method as colony forming units.

The fish in G1 and G3 were individually injected with 0.1 mL of TSB, while those in G2 and G4 were individually injected intraperitoneally with 0.1 mL TSB containing 2.4 ×10^7 cfu/mL *S. agalactiae*. At 3, 6, 12, 24 and 48 h post-injection, one fish per tank (thus, five fish per group at each time-point) was randomly sampled and determined for haemato-immunological parameters as described. Mortality was monitored twice daily for 2 weeks in seperate tanks using 24 fish per tank in five replicate tanks per group. Moribund fish were clinically assessed and tissue swabs from kidney were cultured onto TSA and the presence of *S. agalactiae* in the cultures were confirmed by PCR assay [15].

2.4 Statistical analysis

All data were analysed using one-way analysis of variance (ANOVA), general linear models, and pairwise comparison (Tukey) of the means. All statistical tests were performed using SPSS software (Version 19) under license to Mahidol University. The differences were considered statistically significant when $p<0.05$. Principal Components Analyses (PCAs) were conducted within Statistica 10 (StatSoft, USA) on the raw data obtained for 24 measured immunological parameters. An ANOVA with a *post-hoc* Tukey HSD test with both Bonferroni and Holm's correction were ran on the factor scores for the first principal component derived from each analysis. Both tests were applied to verify that the derived *p* values were genuine and controlled for Type 1 errors (i.e. false positives). Statistically significant differences were accepted at $p<0.05$.

2.5 Ethics statement

This study was approved by the Faculty of Veterinary Science-Animal Care and Use Committee, Mahidol University (FVS-ACUC; permit number MUVS-2016-05-21).

3. Results

3.1 Haemato-immunological parameters of *Fno*-free and *Fno*-infected red tilapia

Before performing the *S. agalactiae* challenge, the haemato-immunological parameters of the *Fno*-free and *Fno*-infected fish were compared and most of the parameters tested were not statistically different between the two groups (Table 1). The only parameter that was statistically different was the percentage of lymphocytes, which was significantly lower ($p<0.05$) in the *Fno*-infected fish compared to the *Fno*-free fish (i.e. approx. 85% of those compared to the *Fno*-free fish).

3.2 Survival of *Fno*-free and *Fno*-infected red tilapia following *S. agalactiae* challenge

The survival of the fish following the *S. agalactiae* challenge is presented in Figure 1, which shows that the G4 fish died rapidly with <10% survival by at the second day post-infection (dpi), while the survival of the fish in G3 at the same time point was >10%. From 11 to 14 dpi, the survival of G4 was 3.3 (± 3.5) %, while that of G3 was 46.7 (± 5.4) %. The moribund fish had an erratic swimming pattern, exophthalmos and skin lesions. Swabs taken from the head-kidney, liver and spleen, streaked on agar produced colonies of bacteria which were characterised as *S. agalactiae* from their biochemical profile. These tests included oxidase, catalase, methyl red Voges-Proskauer, bile-aesculin tolerance, hippurate hydrolysis, fermentation of sorbitol, pyrrolidonylarylamidase, arginine deamination and leucine aminopeptidase [22]. The identity of the bacteria was confirmed by PCR (see section 2.1).

3.3 Hemato-immunological parameters of the *Fno*-free and *Fno*-infected red tilapia during *S. agalactiae* challenge

Following the *S. agalactiae* challenge, several changes in the measured parameters were seen. Although a comparison was made between the four groups at each time-point, the difference between G3 vs. G4 was interesting since it revealed how the *Fno*-infected fish responded differently to the challenge to that of the *Fno*-free fish.

3.3.1 Haematology

Following the *S. agalactiae* infection, the Hct of G3 and G4 fish were generally lower than those of their respective controls, G1 and G2, with statistically significant differences obtained at several time-points: G1 vs. G3, at 3, 12 and 48 hpi; G2 vs. G4, at 12 and 48 hpi (Fig. 2A). Comparing between G3 vs. G4, the Hct of G4 fish was significantly lower than

that of G3 fish at 12 and 48 hpi, suggesting that the *Fno*-infected fish had become more anaemic than the *Fno*-free fish following infection with *S. agalactiae*.

The RBC value of G3 fish was significantly higher than that of G1 fish at 24 hpi, while the RBC values of G4 fish was significantly lower than that of G2 fish at 12 hpi. Comparing between G3 vs. G4 fish, the RBC count of G4 was significantly lower than that of G3 at 12 and 24 hpi (Fig. 2B). Therefore, *S. agalactiae* infection in *Fno*-free fish had either "no-effect" or resulted in a higher RBC count, while the infection in the *Fno*-infected fish had either "no-effect" or resulted in a lowering of the RBC count.

The values of MCV obtained for G3 fish was significantly lower than that of G1 fish at 12 and 24 hpi, while corresponding values in G4 fish did not differ to those of G2 fish at any time-point. No significant differences between G3 and G4 fish were detected at any time-point (Fig. 2C). Therefore, compared to the non-challenge fish, infection with *S. agalactiae* caused a decrease in the MCV value in the *Fno*-free fish, but not in the *Fno*-infected fish.

The total WBC count, and the differential count for lymphocytes and thrombocytes for the four groups of fish are presented in Figure 3. No differences were found in the percentage of neutrophils, eosinophils and monocytes between the groups of fish, and therefore the data for these are not presented in the figure (see Supplemental Table 1). The total WBC count of G3 fish was significantly higher than that of G1 fish at 6, 12, 24 and 48 hpi, whereas the WBC count of G4 fish was only significantly higher than that of G2 fish at 48 hpi. Comparing G3 vs. G4 fish, the latter had significantly lower levels of WBC than G3 fish at 12 hpi. The results, therefore, suggested that there is an increase in the total WBC count in both *Fno*-free and *Fno*-infected fish in response to the *S. agalactiae* infection, but this response was statistically higher in *Fno*-free fish, compared to the *Fno*-infected fish, at certain points during the challenge.

When comparing the percentage of lymphocytes in G1 vs. G3 fish, the values were significantly higher at 12 hpi and significantly lower at 48 hpi in G3 fish, while no significant

difference was seen between G2 vs. G4 fish. When comparing the percentage of lymphocytes of G3 vs. G4 fish, the values were significantly lower in G4 fish at 0 hpi, but significantly higher at 24 hpi, and overall the percentage of lymphocytes seemed very variable between these two groups during the challenge.

The percentage of thrombocytes did not differ in the four groups of fish, except at 12 hpi when the value of G4 fish was significantly higher than that of the other groups.

3.3.2 Parameters in plasma

The levels of lysozyme and peroxidase activity and total protein levels in plasma of fish over the duration of the study are shown in Figure 4. High levels of fluctuation could be seen in lysozyme activity and no significant differences at any time-point, except at 3 hpi, when the activity in G4 fish was significantly higher than that of G3 fish. For peroxidase activity, significantly higher levels were seen in G3 fish than that of G1 fish at 48 hpi, and significantly higher levels were seen in G4 fish compared to G2 fish at 12, 24 and 48 hpi. Between G3 vs. G4 fish, the peroxidase activity was significantly higher in G4 fish at 12 and 24 hpi, but significantly lower at 48 hpi. The results, therefore, suggested a general increase in the level of plasma peroxidase activity after *S. agalactiae* challenge, both in the *Fno*-free and *Fno*-infected fish, and the response in the *Fno*-infected fish was more than that of the *Fno*-free fish during 12 and 24 hpi, but had a lower response thereafter.

For plasma protein levels, significantly higher levels of protein were seen in the G3 fish than the G1 fish at 48 hpi, while significantly higher levels were seen in G4 when compared to the G2 fish at 12 hpi. Comparing G3 vs. G4 fish, the protein level in G4 fish was significantly higher than that of G3 fish at 12 hpi. The protein level was, therefore, increased by the *S. agalactiae* infection and the response in the *Fno*-infected fish was earlier (12 hpi) than that of the *Fno*-free fish (24 hpi). Again, the response of the *Fno*-infected fish seemed to decline after 24 hpi, while that of the *Fno*-free fish continued to rise at 48 hpi.

3.3.3 Head-kidney macrophage activities

Head-kidney macrophage function was also examined in the four groups of fish. For RBA activities and ROS stimulation index without PMA stimulation, these values were significantly higher in G3 fish than those measured in the G1 fish at 12 and 48 hpi, while values in the G4 fish were significantly higher than those of the G2 fish at 12, 24 and 48 hpi (Fig. 5). When values between the G3 vs. G4 fish were compared, the levels produced by macrophages from G4 fish were significantly higher than those from G3 fish at 24 and 48 hpi. The response was similar in the presence of PMA stimulation, except higher values were obtained. The results therefore suggest that the RBA increased in both *Fno*-free and *Fno*-infected fish in response to *S. agalactiae* infection and this response was significantly higher in the *Fno*-infected fish in 24-48 hpi. The ROS stimulation index was, however, comparable in all groups at all time points, except at 48 h, where the level of G4 fish was significantly lower than those of G2 fish.

Generally, the phagocytic activity of the head-kidney phagocytes was not significantly different in any of the groups of fish, except at 24 hpi when the levels in the G4 fish were significantly higher than that seen in the G3 fish (Fig. 6). The phagocytic index was also similar among all groups at all time-points. When comparing the phagocytic index between G1 and G3 fish, there was a significantly higher activity in the G3 fish than that of G1 fish at 12 hpi. For G2 vs. G4 fish, the latter had a significantly higher index at 24 hpi. Between G1 vs. G2 fish, the phagocytic index of G2 fish was significantly higher than that of G1 fish at 3 hpi, while no significant difference was seen between the G3 vs. G4 fish at any time-point.

Phagocytic capacity, which is the percentage of the fish macrophages that could phagocytise 1 (PC1) or more (PC3, PC5 and PC≥6) yeast cells, is shown in Figure 7. It can be seenthat most of the macrophages were able to phagocytise 1 yeast cell, i.e., PC1. During

the challenge, the capacity of phagocytosis of any number of yeast cells between the *Fno*-free and *Fno*-infected fish (G3 vs. G4) did not statistically differ, except the PC1 at 3 hpi. At PC≥6, the percentage of macrophages from *Fno*-infected fish was significantly higher than that of *Fno*-free fish (G1 vs. G2) at 3 hpi, however, by 24 hpi the reverse situation was seen.

Overall, the function of the head-kidney macrophages in their phagocytic abilities (Figs. 6 and 7) in the *Fno*-free and *Fno*-infected fish did not seem to be different.

Separate rounds of PCA analysis were run for each time point in this study at 0, 3, 6, 12, 24 and 48 hpi (Fig. 8). The component loadings for the 24 parameters [Hct, MCV, RBC count, WBC count, differential WBC count (monocytes, lymphocytes, eosinophils and thrombocytes), RBA (with and without PMA), ROS, lysozyme activity, phagocytic activity, phagocytic index, phagocytic capacity (PC1, PC2, PC3, PC4, PC5, PC6 and PC6+), total protein and plasma peroxidase] were used in each PCA analysis (Supplementary Tables 2 and 3). The component loadings highlight which parameters had a major effect in separating the fish through each principal axis. From this, in general, RBA, RBA with PMA and Hct were the key parameters separating specimens through the first principal component at 12-48 hpi, while total protein, thrombocytes and lymphocytes, among others were the key variables acting through the second principal component. The percentage of the variance explained by the first two factors in each graph are 36.08%, 41.79%, 35.04% and 35.82% respectively (Fig. 8). Significant differences between the groups tested with a one-way ANOVA were seen at 12 ($p = 3.042 \times 10^{-5}$), 24 ($p = 0.0004$) and 48 ($p = 2.40 \times 10^{-6}$) hpi. Bonferroni and Holm *post-hoc* multiple comparison tests between the pairs of test groups are summarised in Table 2.

4. Discussion

Before *S. agalactiae* challenge, the haemato-immunological parameters measured in the *Fno*-infected red tilapia were comparable to values obtained for the *Fno*-free fish, except

for a lower percentage of lymphocytes from the differential white blood cell count. Following *S. agalactiae* challenge, both the Hct and RBC counts were significantly lower in the *Fno*-infected fish, compared to the *Fno*-free fish. The MCV values of the *Fno*-free fish, therefore, suggest that the reduced Hct was caused by the reduced number of RBCs, not the size of the RBCs. As the head-kidney is also the site of RBC production [8], it is possible that *Fno* infection affected erythropoiesis in the *Fno*-infected fish.

The total WBC and WBC differential counts were similar between the *Fno*-infected and *Fno*-free fish before *S. agalactiae* challenge, but were significantly higher in the *Fno*-infected fish after the *S. agalactiae* challenge. This 40-50% increase of the total WBC count (compared to levels of both *Fno*-free and *Fno*-infected only fish) may reflect the "alert" level of the WBC following the natural *Fno*-outbreak. The percentage of lymphocytes were found to be lower in the *Fno*-infected fish than in the controls before challenge, but during the challenge, it remained more or less at the same level as that of the *Fno*-free fish. The finding suggests that *Fno* infection in the head-kidney of these fish did not impact on the *Fno*-free myelopoiesis and, probably, lymphopoiesis functions. The presence of basophils has not been reported in the peripheral blood of the hybrid red tilapia [23], but it has been reported that this type of cell is easily overlooked during the microscopic evaluation of cells [24].

Following *S. agalactiae* challenge, plasma lysozyme and peroxidase activities of the *Fno*-infected fish were significantly higher than those of the *Fno*-free fish at certain time-points. These enzymes are both produced by neutrophils and monocytes [24, 25], so the reason that these two enzymes increased following the challenge was possibly due to an increase in the number and function of the WBCs in response to the *S. agalactiae* infection. Peroxidase combines H_2O_2 with Cl^- to produce hypochlorite, which plays an important role in destroying bacteria [26].

The total plasma protein level in this study was comparable to values described elsewhere [23]. The main protein component in plasma is albumin and immunoglobulin. It is

not known which component contributed to the increased total plasma protein levels seen in *Fno*-infected fish, although it is probable that the immunoglobulin, involved in the adaptive immunological response to *S. agalactiae*, might have increased [27].

While it was anticipated that the *Fno*-infected fish would have a sub-normal RBA, the head-kidney macrophages from the *Fno*-infected fish was in fact significantly higher than that of the *Fno*-free fish, with or without PMA stimulation. In addition, the phagocytic activities were found to be comparable to the *Fno*-free fish. In fish, as in other animals, RBA is a strong defence mechanism against pathogens, which occurs after phagocytosis, and is associated with an intense oxygen consumption by phagocytes, which causes them to produce superoxide and other reactive compounds, such as hydrogen peroxide and hydroxyl radicals; and together with lysozyme and peroxidase, the radicals kill the engulfed pathogens [26]. The results from the plasma enzymes, RBA and phagocytic activities suggest that the *Fno*-infected fish did not show any reduced capacity of their innate immune response. In humans, *Francisella tularensis* infected neutrophils have been shown to have the ability to evade RBA of the infected cells by preventing NADPH oxidase assembly and the bacteria survived well in the infected cells [28]. This mechanism does not seem to operate in the *Fno*-infected fish in this study as the RBA was increasing despite the *Fno*-infection.

Principle component analysis (PCA) was additionally used to simultaneously investigate any differences in all the measured parameters, i.e. assessing all 24 variables at each time point. The first PCA analysis showed a high degree of overlap between the four groups of fish at 0 hpi, suggesting that the immune parameters did not affect a significant shift in the immune system between the experimental groups. There were, however, significant differences in the immune profiles of fish between G3 and G4 at 12 hpi. At 12, 24 and 48 hpi, significant difference was seen between the non-*Streptococcus* challenged groups, G1 and G2, and the two *Streptococcus*-challenged groups, G3 and G4, at 12, 24 and 48 hpi.

It is interesting to look in more detail as to why the *Fno*-infected fish had a higher rate of mortality than the normal fish following *S. agalactiae* challenge despite comparable, or even better, innate immune activity.

The first possibility is that the innate immune defence mechanisms of the fish are not adequate to protect the fish from pathogens. It has been shown for Senegalese sole (*Solea senegalensis*) that increased RBA by their phagocytesis not necessarily protect the fish from *Photobacterium damselae* infection [29]. In *O. niloticus*, it has been shown that the correlation between the levels of RBA and protection against a pathogen is not always demonstrated. Two different strains of *O. niloticus* responded differently to *Vibrio parahaemolyticus* challenge by showing significantly different levels of RBA, but yet had the same mortality rate following challenge with the bacterium [30]. In the current study, however, it was found that normal and *Fno*-infected fish had different levels of cumulative mortality following infection with *S. agalactiae*, but had similar haematological and innate immune responses. Data from the current study did not show any solid evidence to explain why the *Fno*-infected fish had higher rates of mortality than the normal fish upon *S. agalactiae* challenge.

The second possibility is that the combination of the *Fno* and *S. agalactiae* infections resulted in a further decrease in erythropoiesis resulting in acute anemia, with Hct dropping from an average of 26.6% to 20.6% within 48 h. The bacteria *S. agalactiae* has been reported to infect several organs of Nile and red tilapia, especially their brain and kidney [14, 31], while in the current study *S. agalactiae* were cultured from the head-kidney of infected fish.

The third possibility is that *Fno*-infected fish had a less effective adaptive immune reponse than the normal fish. The production of antibodies is accomplished by lymphocytes, which differentiate into plasma cells after encountering their target antigen. In this study, the percentage of lymphocytes in the *Fno*-infected fish was not lower than that of the normal fish. The lack of specific antibody production, if any, in this case cannot be due to an

unavailability of lymphocytes. Interestingly, the head-kidney of many fish, including *O. niloticus*, also contains thyroid hormone-producing cells [32]. As thyroid hormones and the immune system interact and support each other in ways that are still unclear, possible damage to thyroid function by *Fno* infection in the head-kidney of the fish might be related to the lower resistance against the superimposing *S. agalactiae* infection and this is an interesting point to consider in future research.The impact of other factors imposing additional stress on the host may alter the host's susceptibility to infection can not be ruled out. Reductions in vaccine efficiency have, for example, been seen in fish co-infected with parasites [33, 34].

5. Conclusions

Hybrid red tilapia, *Oreochromis* sp., chronically infected with *Fno* have normal haematological and innate immune functions and survive well under normal conditions. They, however, have more rapid and higher levels of mortality, compared to the non-infected red tilapia, when experimentally infected with *S. agalactiae*, despite having normal or higher innate immune responses. It is unclear as to why the *Fno*-infected red tilapia were more susceptible to the *S. agalactiae* infection as their innate immune responses appeared similar to the non *Fno*-infected tilapia. The *Fno*-infected fish had an acute reduction in their red blood cell count. Whether the anaemic condition could contribute to the rapid death of the fish requires further study, however, this study showed that the *Fno*-infected fish had no defect in their innate immunological responses, and thus defective innate immunological function could be ruled out as the possible cause.

Acknowledgements

This work has been financial supported by The Thailand Research Fund (TRF) and Mahidol University. The experimental facilities were provided by Veterinary Aquatic Animal Research Health Care Unit, Faculty of Veterianary Science, Mahidol University. The authors

would like to thank all the staff within the Veterinary Aquatic Animal Research Health Care Unit for their assistance with components of this study.

References

[1] E. Soto, J. Hawke, D. Fernandez, J. A Morales, *Francisella* sp., an emerging pathogen of tilapia, *Oreochromis niloticus* (L.), in Costa Rica, Journal of Fish Diseases 32(8) (2009) 713-722.

[2] V.V. Nguyen, H.T. Dong, S. Senapin, N. Pirarat, C. Rodkhum, *Francisella noatunensis* subsp. *orientalis*, an emerging bacterial pathogen affecting cultured red tilapia (*Oreochromis* sp.) in Thailand, Aquaculture Research 47(11) (2016) 3697-3702.

[3] K. Ottem, A. Nylund, E. Karlsbakk, A. Friis-Møller, T. Kamaishi, Elevation of *Francisella philomiragia* subsp. *noatunensis* to *Francisella noatunensis* comb. nov. [syn. *Francisella piscicida* Ottem et al. (2008) syn. nov.] and characterization of *Francisella noatunensis* subsp. *orientalis* subsp. nov., two important fish pathogens, Journal of Applied Microbiology 106(4) (2009) 1231-1243.

[4] K.R. Jeffery, D. Stone, S.W. Feist, D.W. Verner-Jeffreys, An outbreak of disease caused by *Francisella* sp. in Nile tilapia *Oreochromis niloticus* at a recirculation fish farm in the UK, Diseases of Aquatic Organisms 91(2) (2010) 161-165.

[5] S. Sridhar, A. Sharma, H. Kongshaug, F. Nilsen, I. Jonassen, Whole genome sequencing of the fish pathogen *Francisella noatunensis* subsp. *orientalis* Toba04gives novel insights into *Francisella* evolution and pathogenecity, BMC Genomics 13(1) (2012) 598.

[6] E. Soto, D. Fernandez, R. Thune, J.P. Hawke, Interaction of *Francisella asiatica* with tilapia (*Oreochromis niloticus*) innate immunity, Infection and Immunity 78(5) (2010) 2070-2078.

[7] E. Soto, S. Kidd, S. Mendez, D. Marancik, F. Revan, D. Hiltchie, A. Camus, *Francisella noatunensis* subsp. *orientalis* pathogenesis analyzed by experimental immersion

challenge in Nile tilapia, *Oreochromis niloticus* (L.), Veterinary Microbiology 164(1-2) (2013) 77-84.

[8] E.-S.H. Abdel-Aziz, S.B. Abdu, T.E.-S. Ali, H.F. Fouad, Haemopoiesis in the head kidney of tilapia, *Oreochromis niloticus* (Teleostei: Cichlidae): a morphological (optical and ultrastructural) study, Fish Physiology and Biochemistry 36(3) (2010) 323-336.

[9] E. Soto, K. McGovern-Hopkins, R. Klinger-Bowen, B.K. Fox, J. Brock, N. Antonio, Z. Waal, S. Rushton, A. Mill, C.S. Tamaru, Prevalence of *Francisella noatunensis* subsp. *orientalis* in cultured tilapia on the island of Oahu, Hawaii, Journal of Aquatic Animal Health 25(2) (2013) 104-109.

[10] P.J. Pradeep, R. Suebsing, S. Sirithammajak, J. Kampeera, W. Turner, A. Jeffs, W. Kiatpathomchai, B. Withyachumanarnkul, Vertical transmission and concurrent infection of multiple bacterial pathogens in naturally infected red tilapia (*Oreochromis* spp.), Aquaculture Research 48(6) (2017) 2706-2717.

[11] M. Amal, M. Zamri-Saad, Streptococcosis in tilapia (*Oreochromis niloticus*): a review, Pertanika Journal of Tropical Agricultural Science 34(2) (2011) 195-206.

[12] R. Suebsing, J. Kampeera, B. Tookdee, B. Withyachumnarnkul, W. Turner, W. Kiatpathomchai, Evaluation of colorimetric loop-mediated isothermal amplification assay for visual detection of *Streptococcus agalactiae* and *Streptococcus iniae* in tilapia, Letters in Applied Microbiology 57(4) (2013) 317-324.

[13] J. Sun, W. Fang, B. Ke, D. He, Y. Liang, D. Ning, H. Tan, H. Peng, Y. Wang, Y. Ma, Inapparent *Streptococcus agalactiae* infection in adult/commercial tilapia, Scientific Reports 6 (2016) 26319.

[14] G. Assis, G. Tavares, F. Pereira, H. Figueiredo, C. Leal, Natural coinfection by *Streptococcus agalactiae* and *Francisella noatunensis* subsp. *orientalis* in farmed Nile tilapia (*Oreochromis niloticus* L.), Journal of Fish Diseases 40(1) (2017) 51-63.

[15] X. Ye, J. Li, M. Lu, G. Deng, X. Jiang, Y. Tian, Y. Quan, Q. Jian, Identification and molecular typing of *Streptococcus agalactiae* isolated from pond-cultured tilapia in China, Fisheries Science 77(4) (2011) 623-632.

[16] W. Sirimanapong, K.D. Thompson, K. Kledmanee, P. Thaijongrak, B. Collet, E.L. Ooi, A. Adams, Optimisation and standardisation of functional immune assays for striped catfish (*Pangasianodon hypophthalmus*) to compare their immune response to live and heat killed *Aeromonas hydrophila* as models of infection and vaccination, Fish & Shellfish Immunology 40(2) (2014) 374-383.

[17] A.L. Morgan, K. Thompson, N. Auchinachie, H. Migaud, The effect of seasonality on normal haematological and innate immune parameters of rainbow trout *Oncorhynchus mykiss* L, Fish & Shellfish Immunology 25(6) (2008) 791-799.

[18] M.J. Quade, J.A. Roth, A rapid, direct assay to measure degranulation of bovine neutrophil primary granules, Veterinary Immunology and Immunopathology 58(3-4) (1997) 239-248.

[19] A. Sitjà-Bobadilla, S. Peña-Llopis, P. Gómez-Requeni, F. Médale, S. Kaushik, J. Pérez-Sánchez, Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*), Aquaculture 249(1-4) (2005) 387-400.

[20] W. Sirimanapong, K.D. Thompson, E.L. Ooi, M. Bekaert, B. Collet, J.B. Taggart, J.E. Bron, D.M. Green, A.P. Shinn, A. Adams, The effects of feeding β-glucan to *Pangasianodon hypophthalmus* on immune gene expression and resistance to *Edwardsiella ictaluri*, Fish & Shellfish Immunology 47(1) (2015) 595-605.

[21] V. Findlay, B. Munday, The immunomodulatory effects of levamisole on the nonspecific immune system of Atlantic salmon, *Salmo salar* L, Journal of Fish Diseases 23(6) (2000) 369-378.

[22] N.B. Buller, Bacteria and fungi from fish and other aquatic animals: a practical identification manual, Cabi 2014.

[23] T.C. Hrubec, J.L. Cardinale, S.A. Smith, Hematology and plasma chemistry reference intervals for cultured tilapia (*Oreochromis* hybrid), Veterinary Clinical Pathology 29(1) (2000) 7-12.

[24] I.K. Ueda, M.I. Egami, W.d.S. Sasso, E.R. Matushima, Cytochemical aspects of the peripheral blood cells of *Oreochromis* (Tilapia) *niloticus* (Linnaeus, 1758) (Cichlidae, Teleostei): part II, Brazilian Journal of Veterinary Research and Animal Science 38(6) (2001) 273-277.

[25] G. Scapigliati, N. Romano, F. Buonocore, S. Picchietti, M. Baldassini, D. Prugnoli, A. Galice, S. Meloni, C.J. Secombes, M. Mazzini, The immune system of sea bass, *Dicentrarchus labrax*, reared in aquaculture, Developmental & Comparative Immunology 26(2) (2002) 151-160.

[26] C.J. Secombes, T. Fletcher, The role of phagocytes in the protective mechanisms of fish, Annual Review of Fish Diseases 2 (1992) 53-71.

[27] R. Harikrishnan, C. Balasundaram, M.-S. Heo, *Lactobacillus sakei* BK19 enriched diet enhances the immunity status and disease resistance to streptococcosis infection in kelp grouper, *Epinephelus bruneus*, Fish & Shellfish Immunology 29(6) (2010) 1037-1043.

[28] R.L. McCaffrey, L.A.H. Allen, *Francisella tularensis* LVS evades killing by human neutrophils via inhibition of the respiratory burst and phagosome escape, Journal of Leukocyte Biology 80(6) (2006) 1224-1230.

[29] P. Díaz-Rosales, S. Arijo, M. Chabrillón, F. Alarcón, S. Tapia-Paniagua, E. Martínez-Manzanares, M. Balebona, M. Moriñigo, Effects of two closely related probiotics on respiratory burst activity of Senegalese sole (*Solea senegalensis*, Kaup) phagocytes, and protection against *Photobacterium damselae* subsp. *piscicida*, Aquaculture 293(1-2) (2009) 16-21.

[30] S. Balfry, M. Shariff, G.K. Iwama, Strain differences in non-specific immunity of tilapia *Oreochromis niloticus* following challenge with *Vibrio parahaemolyticus*, Diseases of Aquatic Organisms 30(1) (1997) 77-80.

[31] E. Hernández, J. Figueroa, C. Iregui, Streptococcosis on a red tilapia, *Oreochromis* sp., farm: a case study, Journal of Fish Diseases 32(3) (2009) 247-252.

[32] E.J. Geven, P.H. Klaren, The teleost head kidney: Integrating thyroid and immune signalling, Developmental & Comparative Immunology 66 (2017) 73-83.

[33] M.L. Martins, C.A. Shoemaker, D. Xu, P.H. Klesius, Effect of parasitism on vaccine efficacy against *Streptococcus iniae* in Nile tilapia, Aquaculture 314(1-4) (2011) 18-23.

[34] J.F. Urban Jr, N.R. Steenhard, G.I. Solano-Aguilar, H.D. Dawson, O.I. Iweala, C.R. Nagler, G.S. Noland, N. Kumar, R.M. Anthony, T. Shea-Donohue, Infection with parasitic nematodes confounds vaccination efficacy, Veterinary parasitology 148(1) (2007) 14-20.

Table 1. Haemato-immunological parameters of hybrid red tilapia compared between *Fno*-free and *Fno*-infected fish.

	Normal	***Fno*-infected**
Haematology		
Haematocrit (%)	29.0 ± 3.1	27.2 ± 3.6
Total RBC count (× 10^6 cells/μL)	1.68 ± 0.48	1.58 ± 0.31
Mean corpuscular volume (fL)	189.8 ± 71.0	178.3 ± 41.2
Total WBC count (× 10^4 cells/μL)	1.41 ± 0.40	1.43 ± 0.19
WBC differential count (%)		
Neutrophils	21.5 ± 4.5	24.3 ± 6.0
Eosinophils	3.1 ± 1.9	3.5 ± 2.8
Monocytes	2.3 ± 0.8	3.0 ± 1.9
Lymphocytes	42.7 ± 3.3	36.4 ± 7.5*
Thrombocytes	30.4 ± 6.9	32.8 ± 7.1
Innate immune response		
Lysozyme activity (unit/mL)	257.9 ± 110.8	266.2 ± 165.3
Peroxidase activity (450-nm absorbance)	3.7 ± 0.9	3.6 ± 0.7
Total protein (mg/mL)	322 ± 88	320 ± 30
Respiratory burst activity		
Reactive oxygen species index	1.88 ± 0.35	1.97 ± 0.39
Phagocytic activity		
Phagocytic activity (%)	90.3 ± 1.7	89.8 ± 4.1
Phagocytic index (yeast cells/phagocyte)	3.5 ± 0.2	3.5 ± 0.3
Phagocytic capacity (%)		
1-yeast cell/phagocyte	14.4 ± 2.9	16.2 ± 4.0
2-yeast cell/phagocyte	20.3 ± 5.3	15.9 ± 3.7
3-yeast cell/phagocyte	13.8 ± 4.1	12.6 ± 2.7
4-yeast cell/phagocyte	12.1 ± 4.2	13.5 ± 2.7
5-yeast cell/phagocyte	6.0 ± 1.6	6.2 ± 0.8
6-yeast cell/phagocyte	4.5 ± 1.2	5.1 ± 0.9
>6-yeast cell/phagocyte	5.5 ± 1.6	5.5 ± 1.4

Fno, *Francisella noatunensis*; RBC, red blood cell; WBC, white blood cell. *p<0.05; ** (610-nm absorbance for 10^6 cells); Values represent the mean value of 10 fish ± SD

Table 2. Bonferroni and Holm *post-hoc* tests ran on the factor scores for the first principal component derived from each analysis testing for statistical significance ($p<0.05$). Where G1 = *Fno*-free fish that were not challenged with *S. agalactiae*; G2 = *Fno*-infected fish that were not challenged with *S. agalactiae*; G3 = *Fno*-free fish that were challenged with *S. agalactiae*; and, G4 = *Fno*-infected fish that were challenged with *S. agalactiae. Fno, Francisella noatunensis*; NS, not significant.

	12 hpi		**24 hpi**		**48 hpi**	
Treatment pair	**Bonferroni**	**Holm**	**Bonferroni**	**Holm**	**Bonferroni**	**Holm**
G1 vs G2	NS	NS	NS	NS	NS	NS
G1 vs G4	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$
G1 vs G3	$p<0.01$	$p<0.01$	NS	NS	$p<0.01$	$p<0.01$
G2 vs G4	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$
G2 vs G3	$p<0.01$	$p<0.01$	NS	$p<0.05$	$p<0.01$	$p<0.01$
G4 vs G3	NS	$p<0.05$	NS	NS	NS	NS

Supplementary Table 1. Differential white blood cell counts of *Fno* (*Francisella noatunensis*) -free and *Fno*-infected red tilapia

	no *S. agalactiae* challenge		***S. agalactiae* challenge**	
	G1 (*Fno*-free)	**G2 (*Fno*-infected)**	**G3 (*Fno*-free)**	**G4 (*Fno*-infected)**
Monocytes (%)				
3 hpi	4.60 ± 1.34	3.00 ± 2.24	3.60 ± 3.85	4.40 ± 3.29
6 hpi	1.60 ± 1.52	2.40 ± 3.29	1.60± 0.89	1.00 ± 1.41
12 hpi	2.60 ± 1.34	1.40 ± 0.89	3.20 ± 4.92	1.20 ± 0.84
24 hpi	1.60 ± 1.14	2.00 ± 2.35	2.00 ± 2.00	2.60 ± 0.89
48 hpi	3.60 ± 3.65	3.20 ± 2.77	2.40 ± 0.89	2.20 ± 1.48
Lymphocytes (%)				
3 hpi	32.40 ± 5.90	33.20 ± 5.90	34.40 ± 4.56	39.60 ± 6.84
6 hpi	32.00 ± 5.05	32.40 ± 5.18	34.80 ±11.10	34.80 ± 5.02
12 hpi	23.00 ± 5.05^{a}	28.00 ± 6.28^{ab}	34.00 ± 4.00^{b}	23.60 ± 7.77^{ab}
24 hpi	28.00 ± 2.92^{ab}	33.00 ± 9.85^{a}	23.80 ± 3.03^{b}	32.80 ± 5.63^{a}
48 hpi	37.80 ± 6.34^{a}	29.00 ± 6.16^{b}	28.00 ± 3.54^{b}	30.40 ± 3.29^{b}
Neutrophils (%)				
3 hpi	22.80 ± 6.42	18.80 ± 7.29	20.40 ± 3.58	18.40 ± 4.10
6 hpi	22.80 ± 8.67	20.00 ± 4.24	22.80 ± 1.10	20.00 ± 4.00
12 hpi	27.40 ± 5.81	24.00 ± 6.20	20.60 ± 2.51	20.00 ± 6.00
24 hpi	25.40 ± 4.22	25.40 ± 9.53	24.00 ± 5.48	19.60 ± 5.73
48 hpi	20.00 ± 3.39	25.00 ± 2.00	21.40 ± 4.10	22.80 ± 5.22
Eosinophils (%)				
3 hpi	6.40 ± 2.97	3.60 ± 1.67	4.40 ± 1.67	6.40 ± 3.29
6 hpi	4.00 ± 3.46	2.80 ± 1.79	2.80 ± 1.79	2.20 ± 2.28
12 hpi	7.40 ± 1.34	5.60 ± 4.34	3.00 ± 1.87	6.40 ± 6.99
24 hpi	6.60 ± 3.13^{ab}	3.20 ± 2.39^{a}	8.40 ± 4.77^{b}	6.60 ± 2.61^{ab}
48 hpi	4.80 ± 4.15	5.00 ± 3.87	9.00 ± 7.35	9.00 ± 4.80
Thrombocytes (%)				
3 hpi	33.80 ± 7.69	41.40 ± 5.18	37.20 ± 6.57	31.20 ± 9.65
6 hpi	39.60 ± 12.16	42.40 ± 9.63	38.00 ± 9.80	42.00 ± 10.58
12 hpi	39.60 ± 4.28^{a}	41.00 ± 2.83^{a}	39.20 ± 4.15^{a}	48.80 ± 4.82^{b}
24 hpi	38.40 ± 3.85	36.40 ± 10.04	41.80 ± 8.38	38.40 ± 6.66
48 hpi	33.80 ± 8.26	37.80 ± 4.27	39.20 ± 9.12	35.60 ± 8.41

Different superscripts indicate statistical significance (p<0.05); comparison was made between the values of the same row. Values represent the mean value of 5 fish ±SD.

Supplementary Table 2. The component loadings and the percentage of the variance explained by each variable (n = 24) for each principal components analysis (PCA). PCA 1 (*t* = 0 hour post-infection (p.i.); 4 groups; n = 5 replicates per group); PCA 2 (*t* = 3 hpi.); PCA 3 (*t* = 6 hpi.). Each analysis considers four groups of samples: G1 - *Fno*-free fish not subjected to a *Streptococcus agalactiae* challenge; G2 - *Fno*-infected fish not subjected to a *S. agalactiae* challenge; G3 *Fno*-free fish subjected to an *S. agalactiae* challenge and G4 - *Fno*-infected fish subjected to a *S. agalactiae* challenge. Values above ± 0.70 are shown in a bold font. *Fno, Francisella noatunensis*

Variable	PCA 1 (*t* = 0 hpi.)			PCA 2 (*t* = 3 hpi.)			PCA 3 (*t* = 6 hpi.)		
	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3
HCT	0.484	-0.323	0.520	0.345	0.004	0.480	-0.017	0.488	-0.175
MCV	**0.793**	-0.299	-0.253	0.109	**-0.766**	0.342	-0.442	0.447	0.593
RBC × 10^5 cell/ml	**-0.708**	0.108	0.333	0.022	**0.783**	0.012	0.449	-0.169	**-0.752**
WBC × 10^4 cell/ml	-0.452	-0.281	0.411	0.090	0.157	-0.392	0.232	-0.393	-0.308
Respiratory burst activity (RBA)	-0.646	-0.325	0.483	-0.039	-0.630	-0.581	-0.454	0.418	0.024
Respiratory burst activity (RBA with PMA)	-0.554	-0.349	0.378	0.154	**-0.784**	-0.383	-0.303	0.369	-0.345
Reactive oxygen species (ROS)	0.157	-0.087	-0.188	**0.763**	-0.176	0.259	0.344	-0.234	-0.663
Lysozyme activity	-0.233	0.351	-0.341	-0.233	-0.563	0.158	-0.141	**-0.733**	-0.032
Total protein	-0.591	-0.216	-0.354	-0.377	0.346	0.262	0.313	0.100	0.290
Plasma peroxidase	0.176	-0.477	0.522	-0.664	-0.172	-0.151	-0.278	-0.635	-0.254
Monocytes	-0.193	0.512	0.225	-0.051	0.376	-0.160	0.201	-0.506	0.408
Lymphocytes	0.017	-0.297	0.403	-0.187	-0.155	-0.553	-0.641	-0.515	0.133
Neutrophils	**-0.777**	0.163	-0.072	-0.316	0.139	0.050	-0.272	-0.244	0.215
Eosinophils	-0.222	0.239	-0.297	-0.036	0.678	-0.342	-0.020	-0.233	0.060
Thrombocytes	**0.700**	-0.039	-0.271	0.385	-0.335	0.544	0.528	0.614	-0.288
Phagocytic activity (PA)	-0.175	**0.715**	0.316	-0.682	0.131	0.305	0.495	-0.096	-0.153
Phagocytic index (PI)	0.066	**0.889**	0.264	**-0.939**	-0.050	0.192	**0.896**	0.025	0.234
Phagocytic capacity 1 (PC1)	-0.518	-0.330	-0.318	**0.744**	0.092	-0.571	-0.668	-0.288	-0.407
Phagocytic capacity 2 (PC2)	-0.034	-0.485	-0.478	0.688	0.214	0.306	-0.507	0.516	-0.090
Phagocytic capacity 3 (PC3)	0.352	0.031	0.665	0.177	0.366	0.267	-0.420	0.023	-0.563
Phagocytic capacity 4 (PC4)	0.518	0.095	0.480	-0.161	-0.157	0.367	-0.037	-0.175	0.665
Phagocytic capacity 5 (PC5)	-0.319	0.168	0.214	-0.159	-0.551	-0.045	0.483	-0.400	0.022
Phagocytic capacity 6 (PC6)	-0.595	0.052	-0.207	-0.339	-0.089	0.623	0.501	-0.572	0.273
Phagocytic capacity 6+ (PC6+)	0.309	0.663	-0.115	**-0.800**	0.034	-0.260	0.631	0.629	0.064
% Total variation	0.216	0.145	0.133	0.203	0.168	0.130	0.195	0.176	0.132
Cumulative percentage	21.59	36.08	49.38	20.34	37.16	50.2	19.49	37.09	50.24

Supplementary Table 3. The component loadings and the percentage of the variance explained by each variable (n = 24) for each principal components analysis (PCA). PCA 4 (t = 12 hpi.); PCA 5 (t = 24 hpi.) and PCA 6 (t = 48 hpi.). Each analysis considers four groups of samples: G1 - *Fno*-free fish not subjected to a *Streptococcus agalactiae* challenge; G2 - *Fno*-infected fish not subjected to a *S. agalactiae* challenge; G3 - *Fno*-free fish subjected to an *S. agalactiae* challenge and G4 - *Fno*-infected fish subjected to a *S. agalactiae* challenge. Values above ± 0.70 are shown in a bold font. *Fno*, *Francisella noatunensis*

Variable	PCA 4 (t = 12 hpi.)			PCA 5 (t = 24 hpi.)			PCA 6 (t = 48 hpi.)		
	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3	Factor 1	Factor 2	Factor 3
HCT	**-0.819**	0.296	-0.147	-0.341	0.373	0.422	**0.768**	-0.249	0.073
MCV	-0.288	-0.441	0.006	-0.513	-0.475	-0.236	-0.073	-0.173	0.546
RBC × 10^5 cell/ml	-0.367	0.563	-0.104	0.204	0.627	0.406	0.471	0.082	-0.427
WBC × 10^4 cell/ml	0.614	0.555	0.053	0.585	-0.286	0.391	-0.607	0.014	0.178
Respiratory burst activity (RBA)	**0.785**	-0.193	-0.431	**0.860**	-0.109	-0.269	**-0.745**	0.080	0.203
Respiratory burst activity (RBA with PMA)	**0.778**	-0.381	-0.323	**0.871**	-0.243	-0.253	**-0.778**	0.003	0.216
Reactive oxygen species (ROS)	-0.168	-0.388	0.364	-0.198	-0.485	-0.163	**0.708**	0.060	0.184
Lysozyme activity	0.086	0.079	-0.240	-0.430	0.026	0.115	0.571	-0.028	0.171
Total protein	0.614	-0.597	-0.032	**0.745**	-0.095	-0.275	-0.606	-0.076	0.135
Plasma peroxidase	**0.812**	-0.437	-0.236	0.458	-0.226	-0.037	-0.676	0.069	0.299
Monocytes	-0.280	-0.031	**-0.735**	0.544	-0.290	0.259	0.332	**0.729**	-0.094
Lymphocytes	0.025	0.687	-0.350	-0.295	-0.557	-0.371	0.489	-0.153	0.330
Neutrophils	-0.444	-0.070	0.596	-0.481	0.607	-0.414	-0.010	0.568	-0.324
Eosinophils	0.026	-0.294	-0.421	0.444	0.571	-0.142	-0.295	0.592	0.181
Thrombocytes	0.547	-0.571	0.493	0.364	-0.244	0.733	-0.291	**-0.828**	-0.194
Phagocytic activity (PA)	0.509	0.418	0.134	0.456	0.321	-0.130	0.309	-0.164	0.416
Phagocytic index (PI)	**0.715**	0.517	0.262	0.370	0.287	-0.145	0.229	-0.484	0.680
Phagocytic capacity 1 (PC1)	-0.671	-0.263	-0.251	-0.015	0.274	0.461	-0.267	0.430	-0.288
Phagocytic capacity 2 (PC2)	-0.238	-0.346	0.242	0.364	-0.259	-0.435	-0.198	0.177	-0.379
Phagocytic capacity 3 (PC3)	-0.174	-0.377	0.108	-0.332	-0.572	0.246	0.445	0.400	0.429
Phagocytic capacity 4 (PC4)	-0.511	-0.162	-0.357	-0.174	0.331	-0.759	-0.066	-0.389	-0.290
Phagocytic capacity 5 (PC5)	0.482	0.161	-0.431	0.352	-0.056	-0.067	0.361	-0.259	-0.486
Phagocytic capacity 6 (PC6)	0.393	0.220	-0.325	-0.220	-0.306	0.224	-0.216	-0.705	-0.159
Phagocytic capacity 6+ (PC6+)	0.461	0.408	0.670	0.288	0.357	0.174	0.030	0.237	**0.893**
% Total variation	0.263	0.155	0.129	0.211	0.139	0.121	0.215	0.143	0.136
Cumulative percentage	26.26	41.79	54.7	21.11	35.04	47.14	21.49	35.82	49.4

Figure legends

Figure 1. Survival rate of *Fno*-free and *Fno*-infected red tilapia following *S. agalactiae* challenge, compared to that of the non-challenge fish. Values are means ± standard deviation with n = 24 fish per tank in five replicate tanks per group. *Fno, Francisella noatunensis.*

Figure 2. Haematocrit (2A), total red blood cell count (2B) and mean corpuscular volume (2C) of normal and *Fno*-infected red tilapia, at time 3, 6, 12, 24 and 48 hours following *S. agalactiae* challenge. Values are means ± standard deviation with n = 5 per group. Statistical comparison was made among the values of the same time-point; different superscripts indicate statistical significance (p<0.05). *Fno, Francisella noatunensis.*

Figure 3. Total white blood cell count (3A) and percentage of lymphocytes (3B) and thrombocytes (3C) of *Fno*-free and *Fno*-infected red tilapia, at time 3, 6, 12, 24 and 48 hours following *S. agalactiae* challenge. Values are means ± standard deviation with n = 5 per group. Statistical comparison was made among the values of the same time-point; different superscripts indicate statistical significance (p<0.05). *Fno, Francisella noatunensis.*

Figure 4. Activities of plasma lysozyme (4A) and peroxidase (4B) and level of plasma protein (4C) of *Fno*-free and *Fno*-infected red tilapia, at time 3, 6, 12, 24 and 48 hours following *S. agalactiae* challenge. Values are means ± standard deviation with n = 5 per group. Statistical comparison was made among the values of the same time-point; different superscripts indicate statistical significance (p<0.05). *Fno, Francisella noatunensis.*

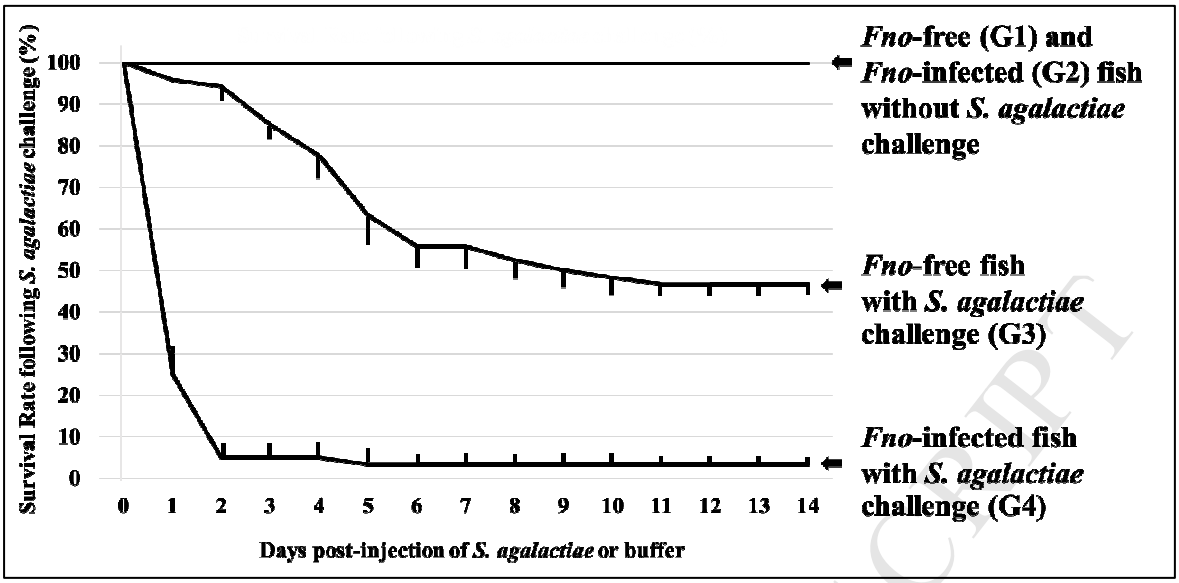
Figure 5. Respiratory burst activities (RBA), with (5A) and without (5B) phorbol myristate acetate (PMA) stimulation and reactive oxygen species (ROS) stimulation index (5C) of *Fno*-free and *Fno*-infected red tilapia, at time 3, 6, 12, 24 and 48 hours

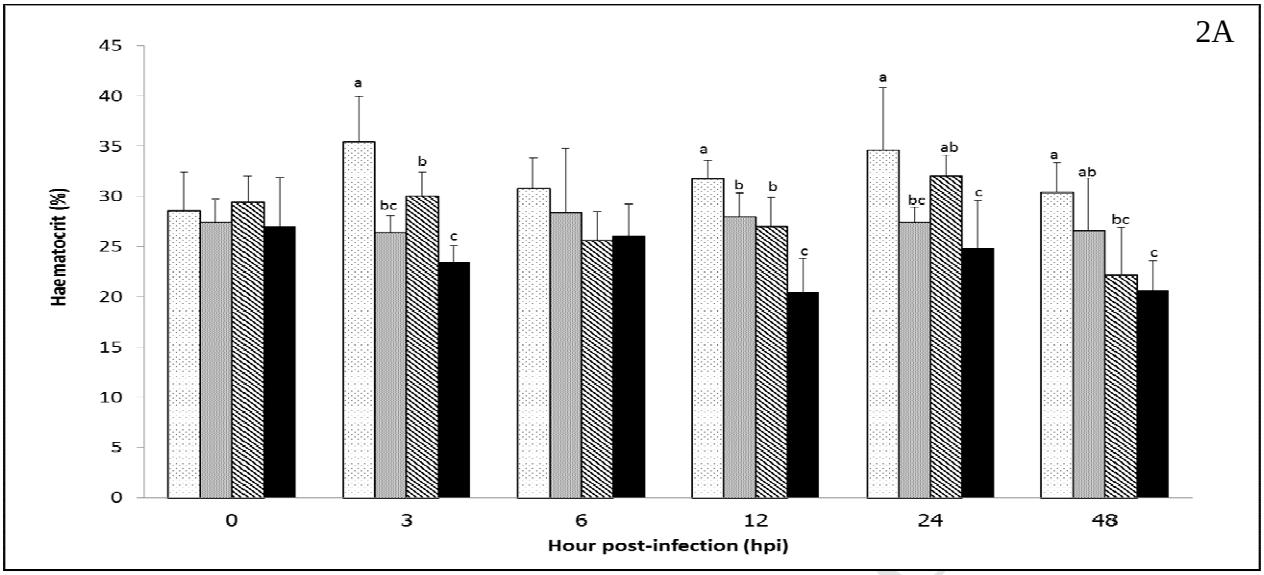
following *S. agalactiae* challenge. Values are means ± standard deviation with *n* = 5 per group. Statistical comparison was made among the values of the same time-point; different superscripts indicate statistical significance ($p<0.05$). *Fno, Francisella noatunensis.*

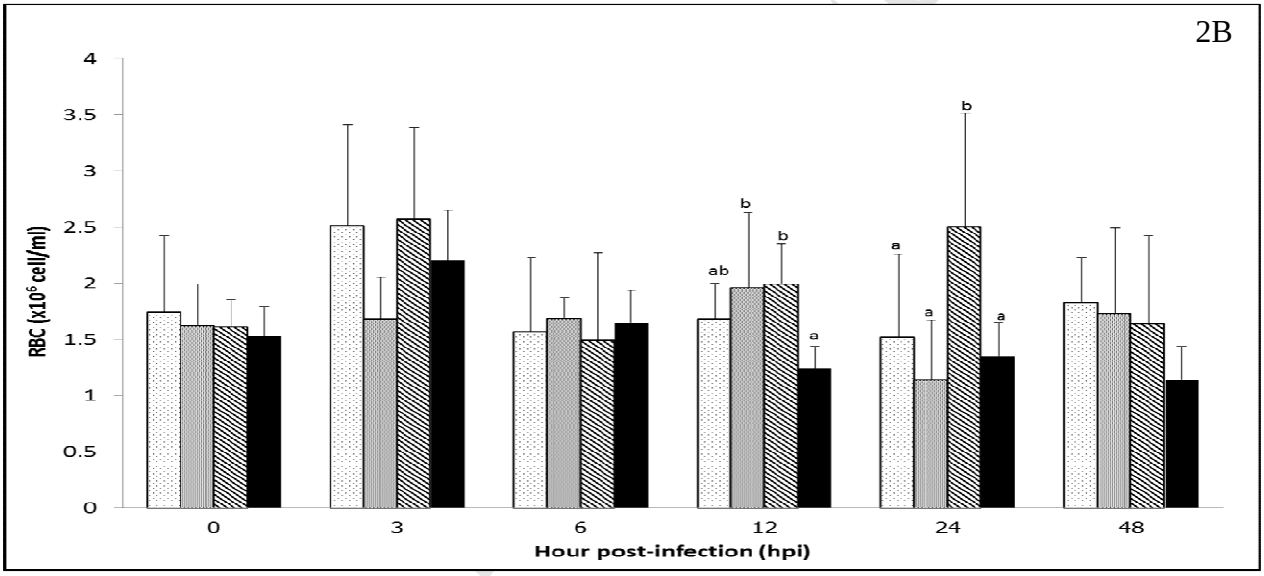
Figure 6. Phagocytic activity (6A) and phagocytic index (6B) of *Fno*-free and *Fno*-infected red tilapia, at time 3, 6, 12, 24 and 48 hours following *S. agalactiae* challenge. Values are means ± standard deviation with *n* =5 per group.Statistical comparison was made among the values of the same time-point; different superscripts indicate statistical significance ($p<0.05$). *Fno, Francisella noatunensis.*

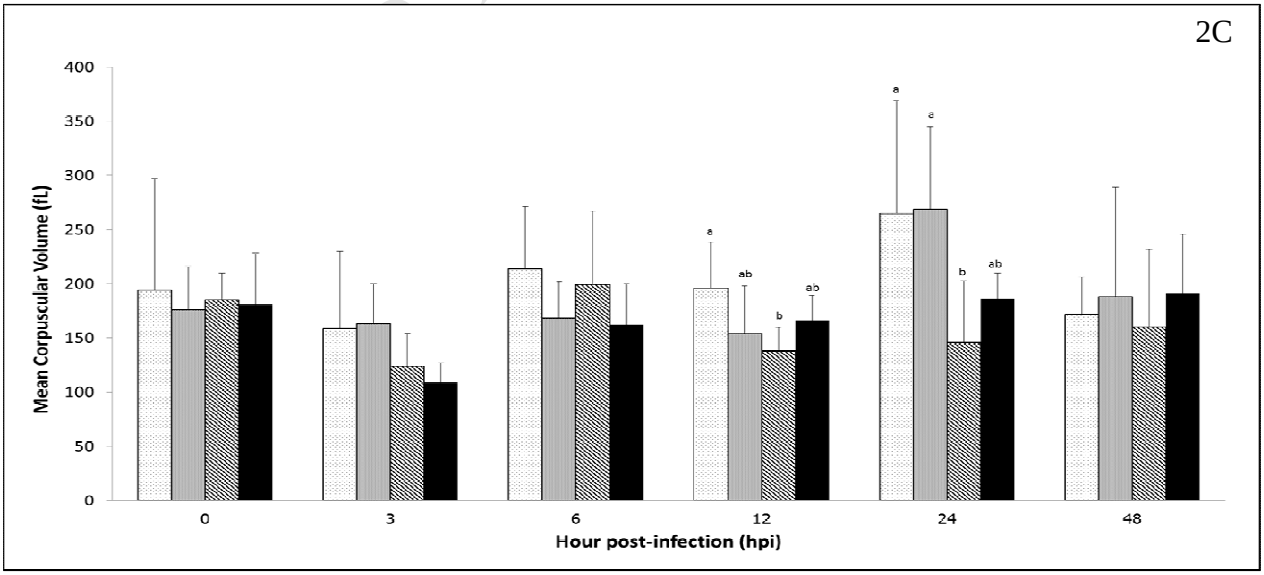
Figure 7. Phagocytic capacity of *Fno*-free and *Fno*-infected red tilapia, at time 3, 6, 12, 24 and 48 hours following *S. agalactiae* challenge. Values are means ± standard deviation with *n* =5 per group.Statistical comparison was made among the values of the same time-point; different superscripts indicate statistical significance ($p<0.05$). PC1 (7A), PC3 (7B), PC5 (7C) and PC≥6 (7D) signify the percentage of phogocytes containing 1 yeast cell or more. *Fno, Francisella noatunensis.*

Figure 8. Principal components analysis of the 24 hemato-immunological parameters measured from each group of fish (n = 5) at 0, 12, 24 and 48 hours post-infection. For each PCA plot, the first two principal components (Factor 1 vs Factor 2) accounting for the most variation between the specimens are shown. The variables making a major contribution to the separation of the specimens through each Factor and the direction in which they act are marked. *Fno, Francisella noatunensis.*

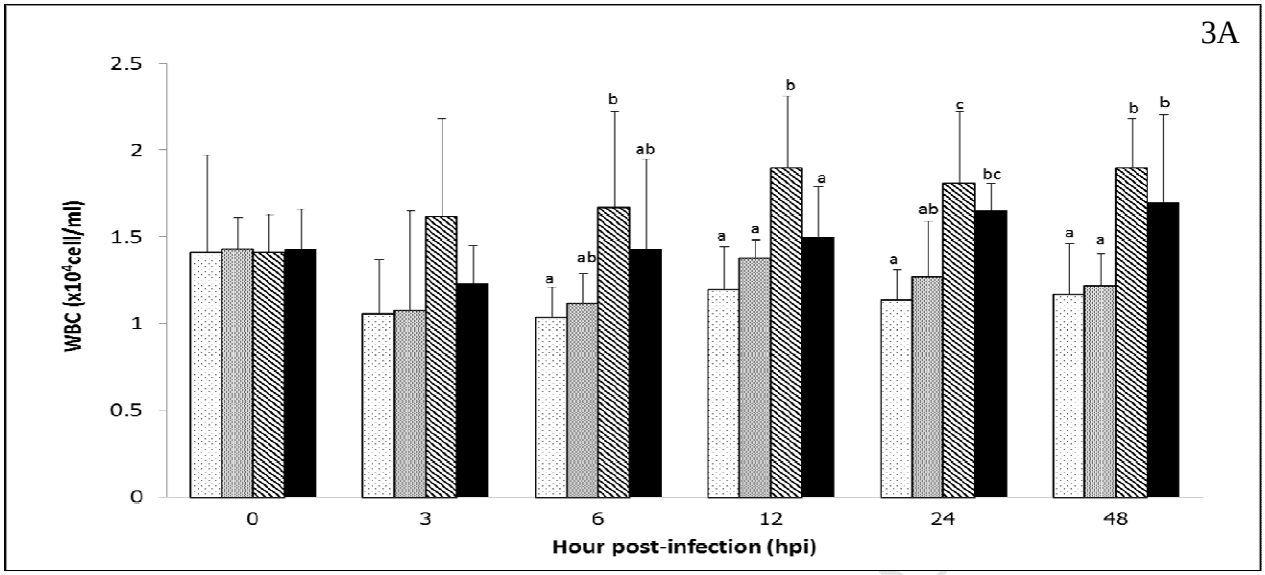


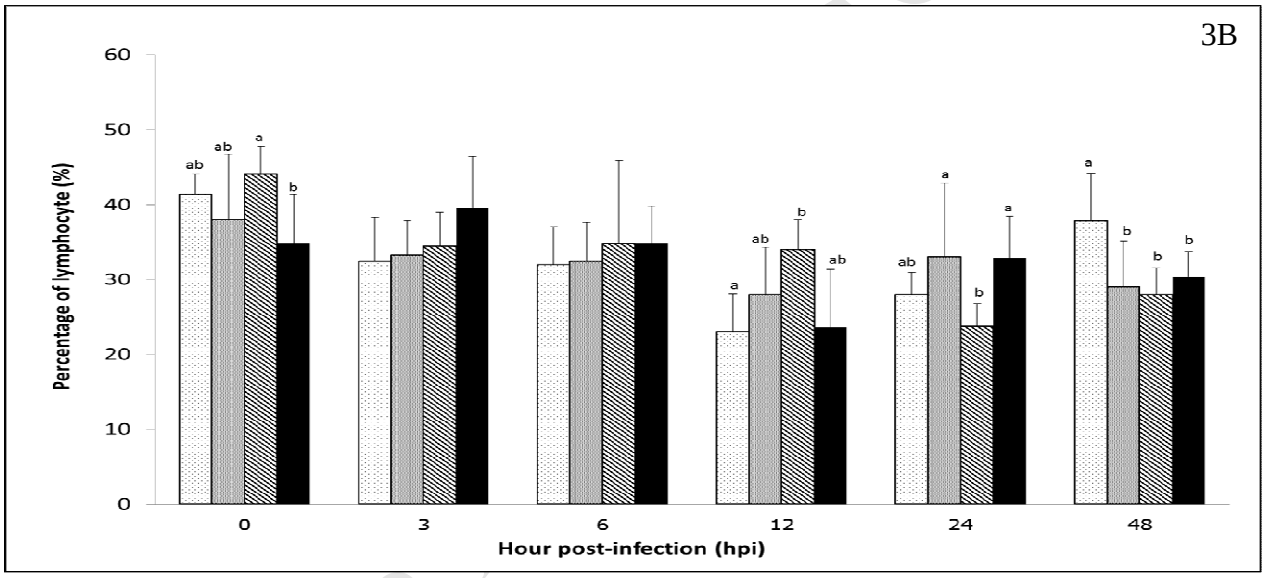


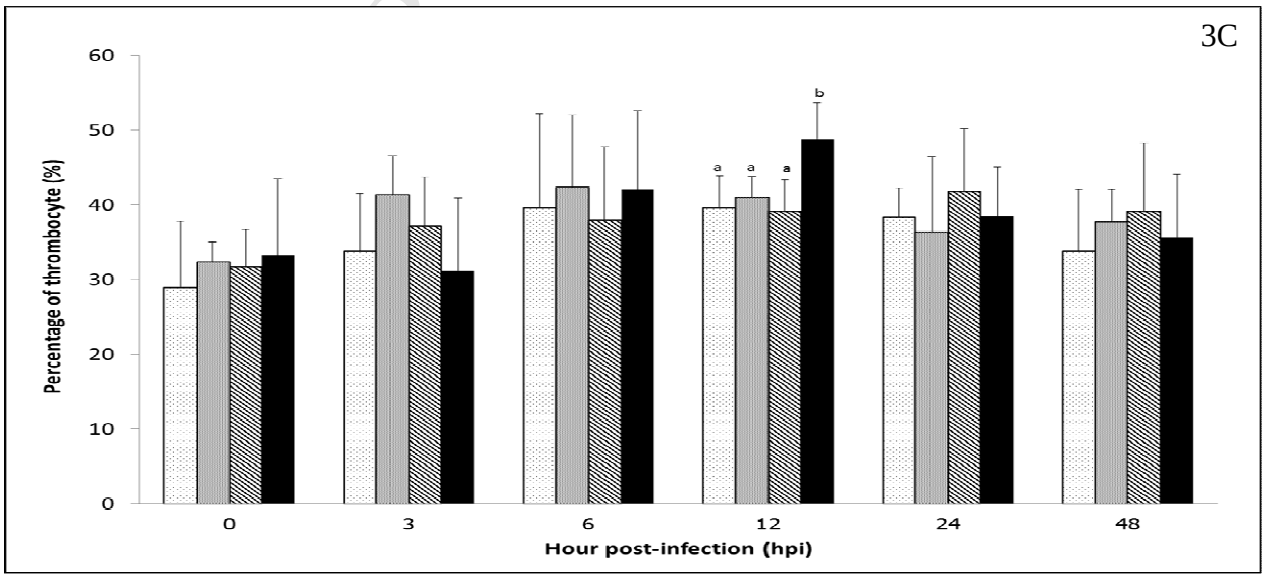




Fno-free fish (G1)
Fno-free fish + *S. agalactiae* (G3)
Fno-infected fish (G2)
Fno-infected fish + *S. agalactiae* (G4)





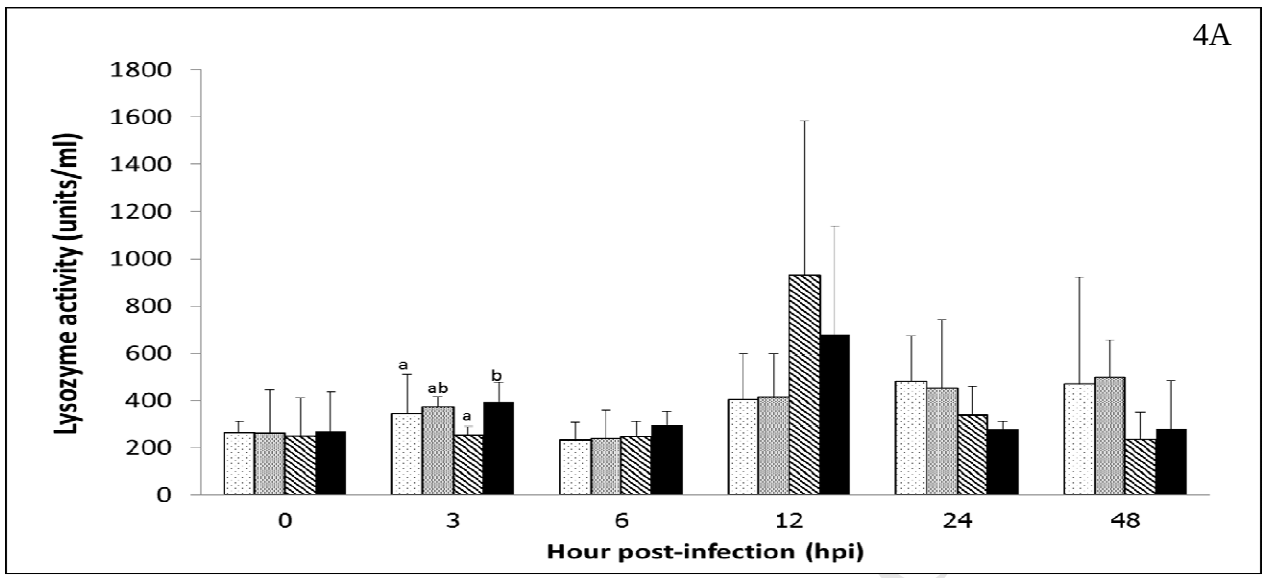


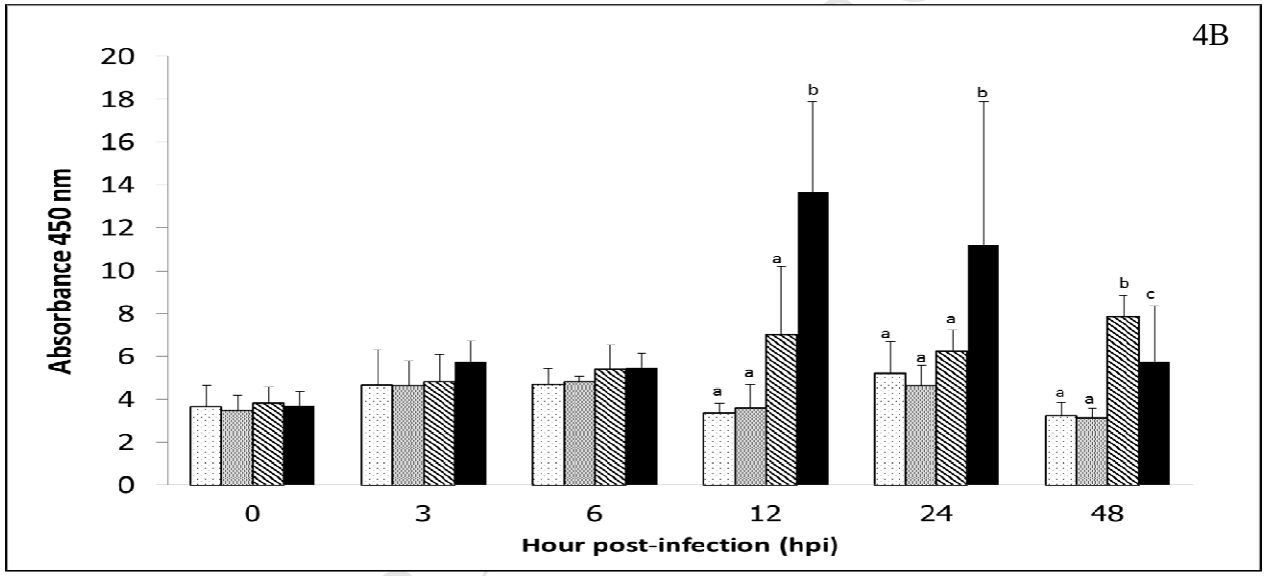
Fno-free fish (G1)

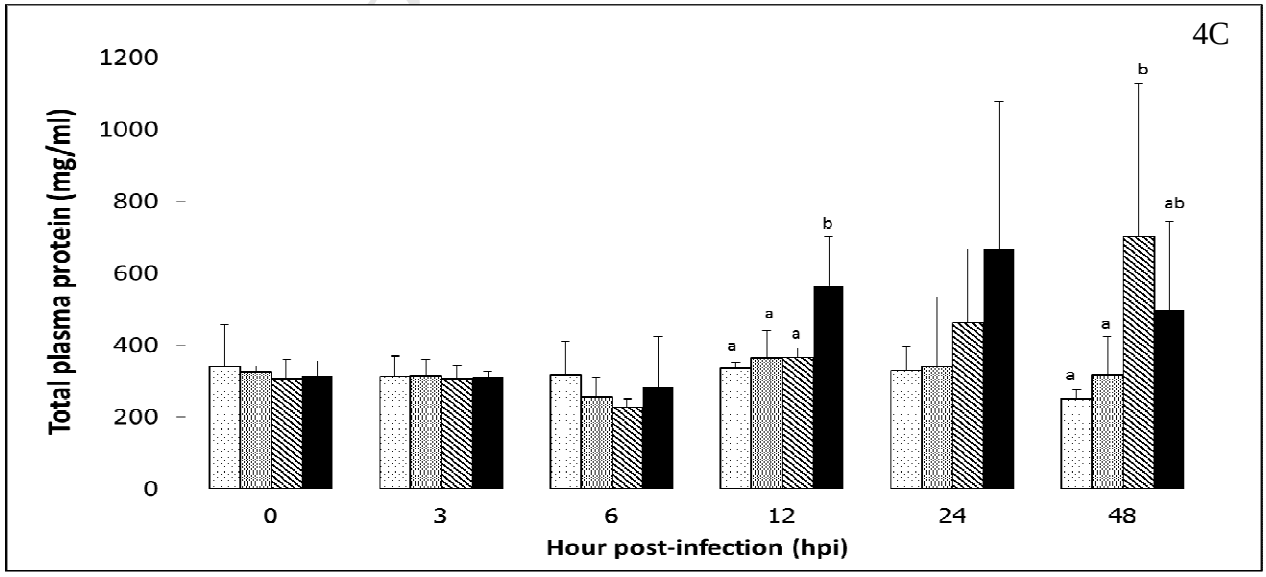
Fno-free fish + *S. agalactiae* (G3)

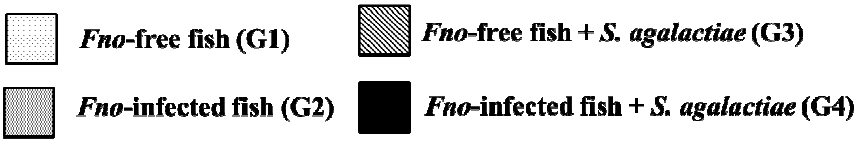
Fno-infected fish (G2)

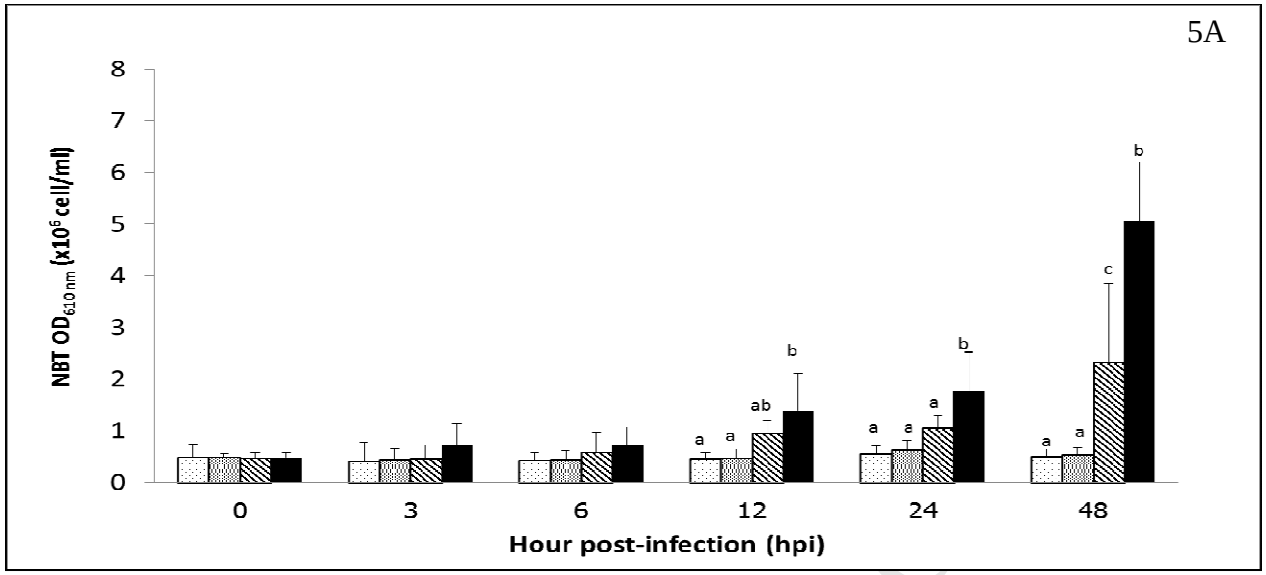
Fno-infected fish + *S. agalactiae* (G4)

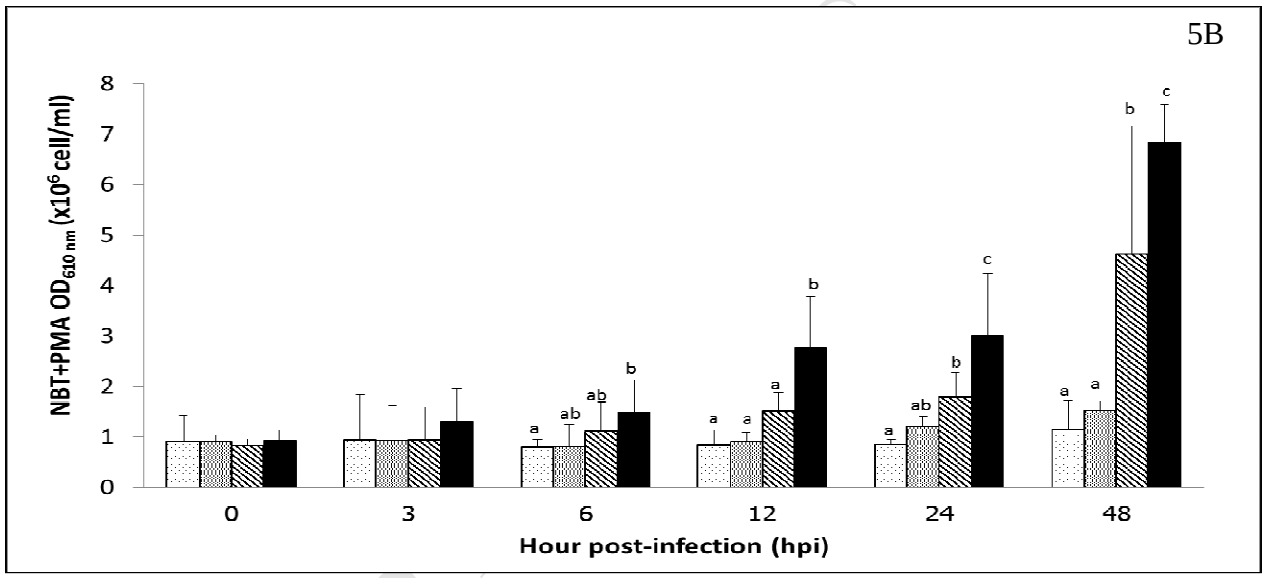

4A
Lysozyme activity (units/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
a
ab
a
b
0
3
6
12
24
48
Hour post-infection (hpi)

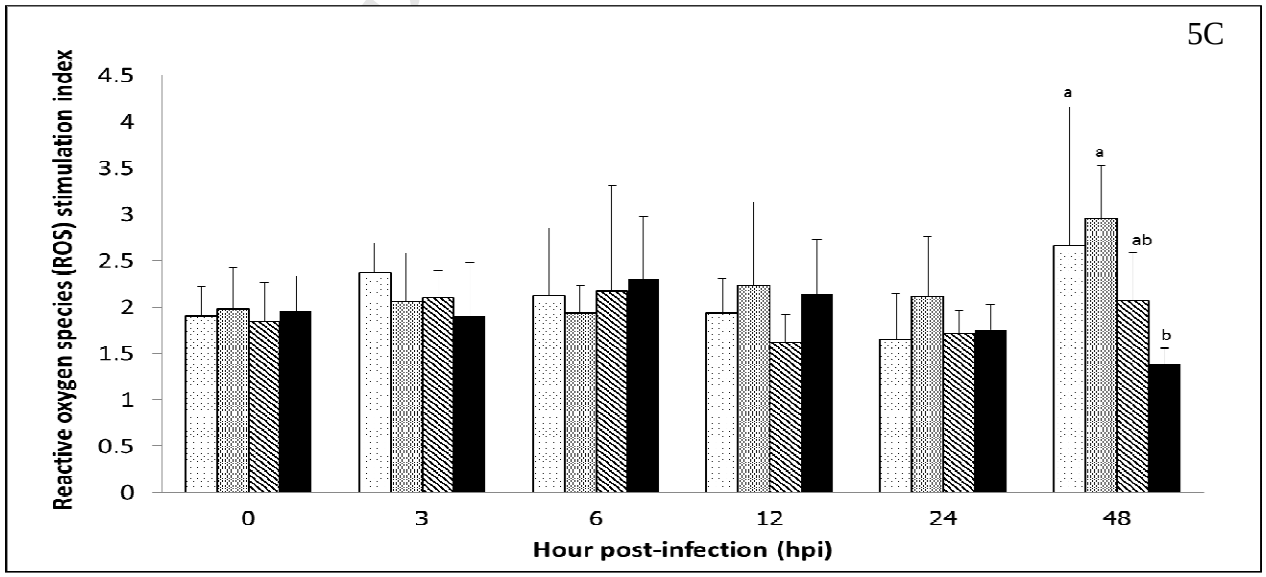

4B
Absorbance 450 nm
20
18
16
14
12
10
8
6
4
2
0
a
a
a
b
a
a
a
b
a
a
b
c
0
3
6
12
24
48
Hour post-infection (hpi)


4C
Total plasma protein (mg/ml)
1200
1000
800
600
400
200
0
a
a
a
b
a
a
b
a
ab
0
3
6
12
24
48
Hour post-infection (hpi)


Fno-free fish (G1)
Fno-free fish + *S. agalactiae* (G3)
Fno-infected fish (G2)
Fno-infected fish + *S. agalactiae* (G4)





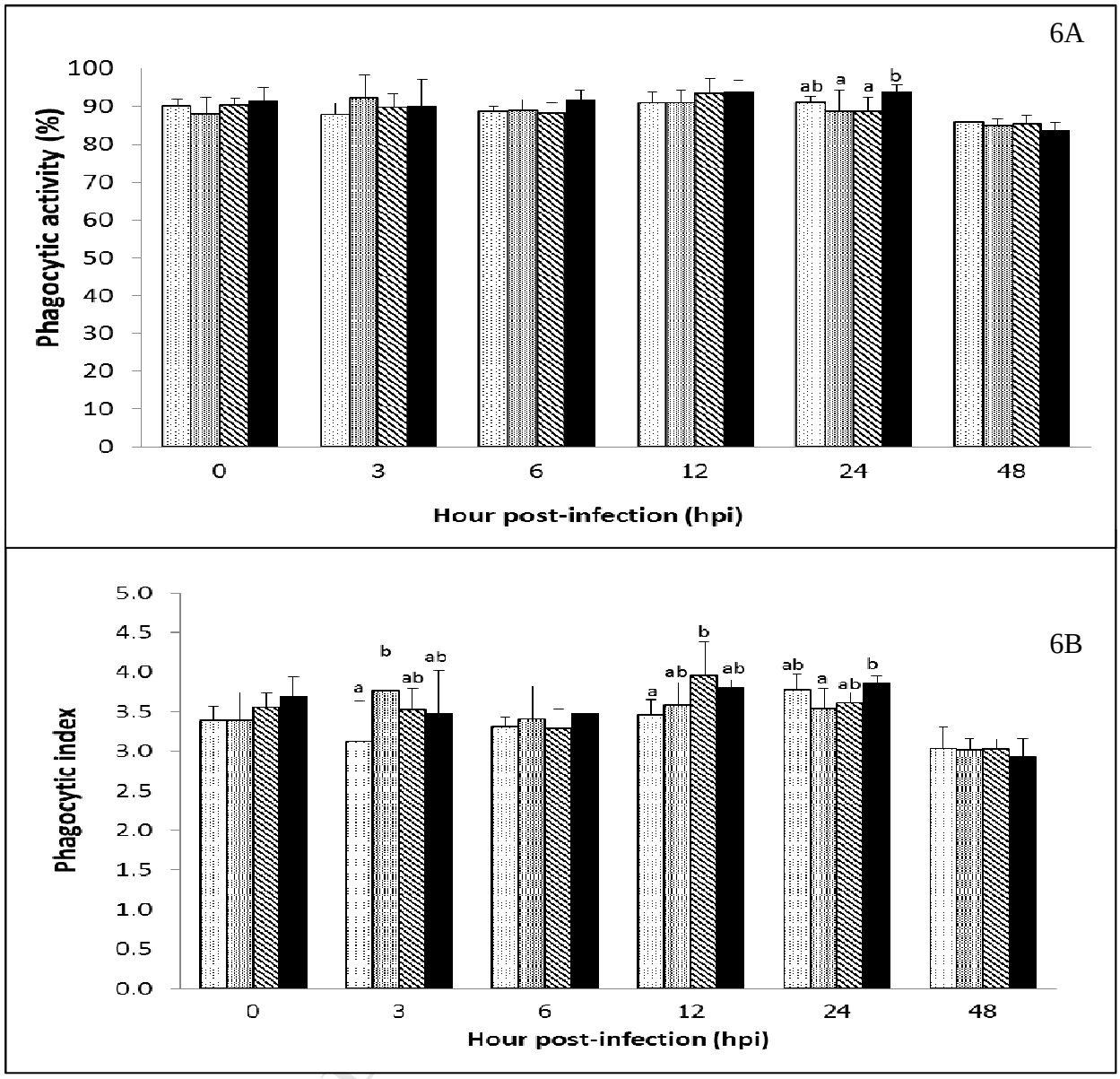


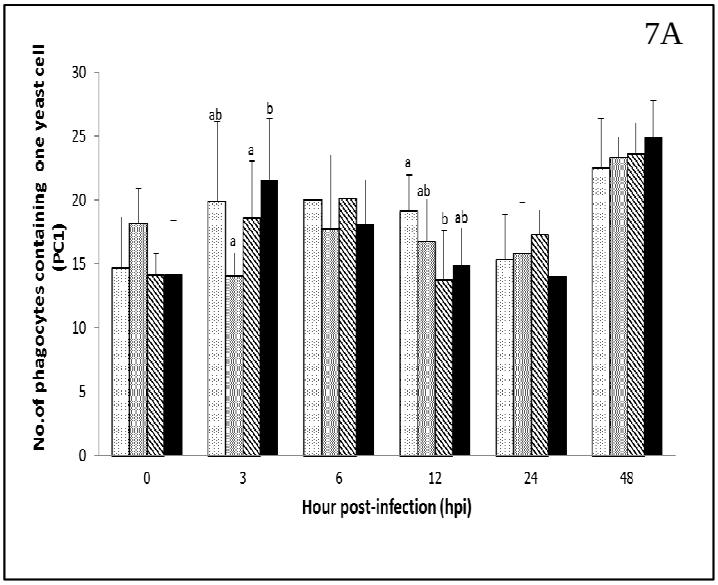
Fno-free fish (G1)

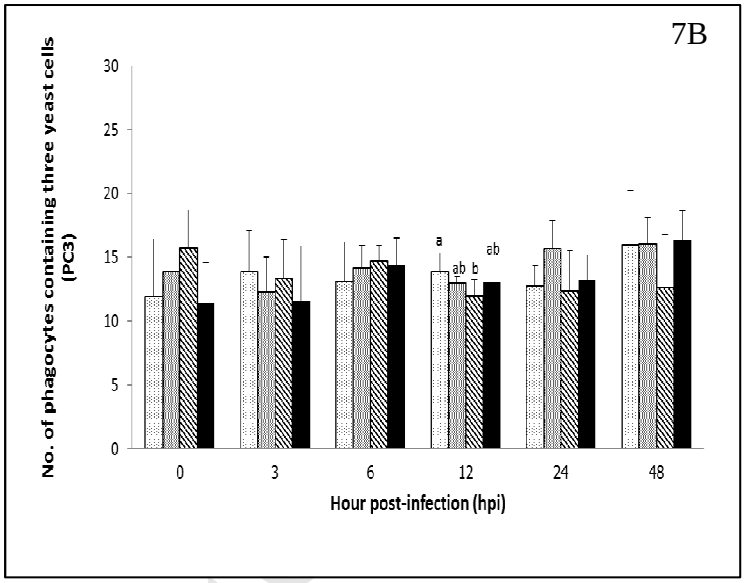
Fno-free fish + *S. agalactiae* (G3)

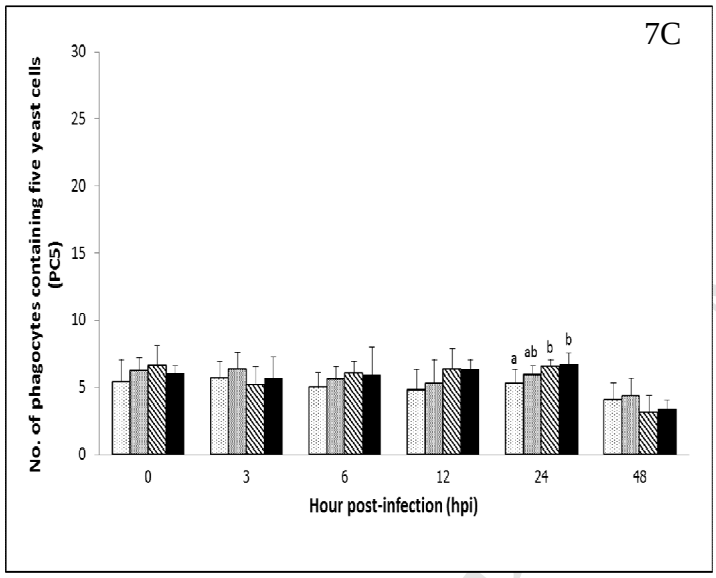
Fno-infected fish (G2)

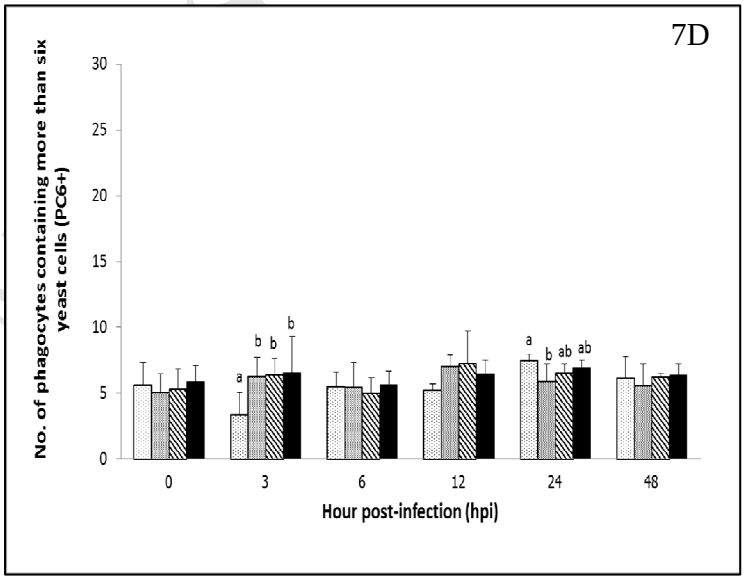
Fno-infected fish + *S. agalactiae* (G4)

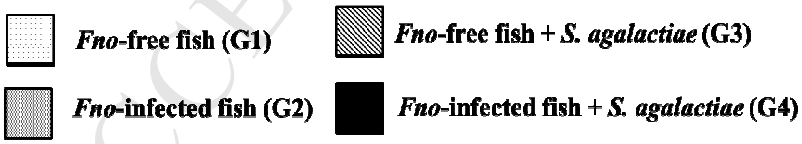

6A
Phagocytic activity (%)
100
90
80
70
60
50
40
30
20
10
0
ab
a
a
b
0
3
6
12
24
48
Hour post-infection (hpi)
6B
Phagocytic index
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
ab
ab
a
ab
b
ab
ab
a
ab
b
0
3
6
12
24
48
Hour post-infection (hpi)
Fno-free fish (G1)
Fno-infected fish (G2)
Fno-free fish + S. agalactiae (G3)
Fno-infected fish + S. agalactiae (G4)

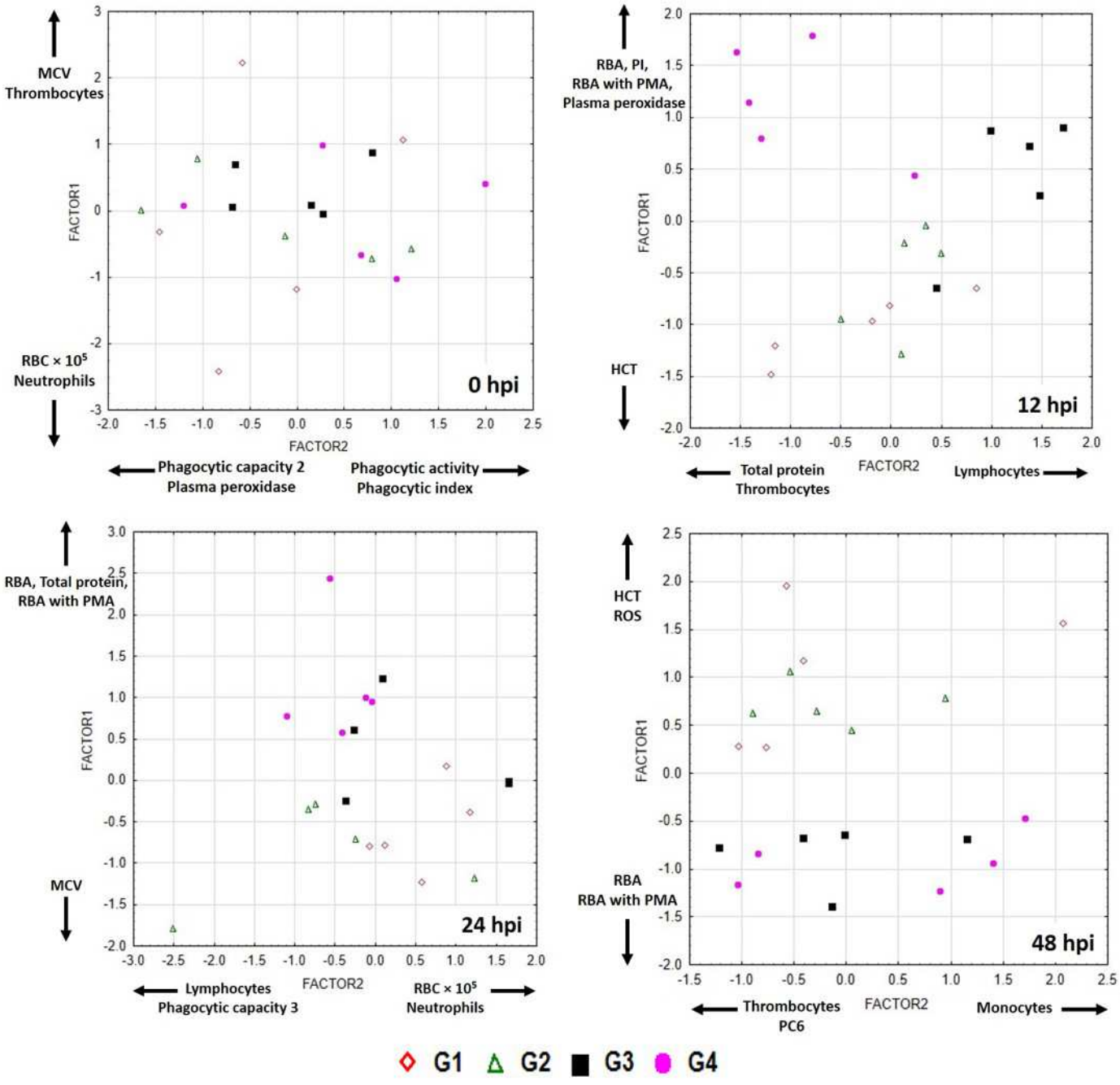

7A
No.of phagocytes containing one yeast cell (PC1)
Hour post-infection (hpi)


7B
No. of phagocytes containing three yeast cells (PC3)
Hour post-infection (hpi)


7C
No. of phagocytes containing five yeast cells (PC5)
Hour post-infection (hpi)


7D
No. of phagocytes containing more than six yeast cells (PC6+)
Hour post-infection (hpi)


Fno-free fish (G1)
Fno-free fish + *S. agalactiae* (G3)
Fno-infected fish (G2)
Fno-infected fish + *S. agalactiae* (G4)


MCV
Thrombocytes
RBC × 10⁵
Neutrophils
FACTOR1
FACTOR2
0 hpi
Phagocytic capacity 2
Plasma peroxidase
Phagocytic activity
Phagocytic index
RBA, PI,
RBA with PMA,
Plasma peroxidase
HCT
12 hpi
Total protein
Thrombocytes
Lymphocytes
RBA, Total protein,
RBA with PMA
MCV
24 hpi
Lymphocytes
Phagocytic capacity 3
RBC × 10⁵
Neutrophils
HCT
ROS
RBA
RBA with PMA
48 hpi
Thrombocytes
PC6
Monocytes
G1
G2
G3
G4

Highlights

1. The immune parameters of *Francisella noatunensis*-infected red tilapia were normal.

2. When infected fish were challenged with *Streptococcus agalactiae*, mortalities were high.

3. During the course of infection, however, the immunological responses of the fish were normal.

4. A reduction in the red blood cell count and haemotcrit were the only abnormalities.

5. An *F. noatunensis*-induced reduction in erythropoiesis in the head-kidney is implicated.