Molecular immune response of channel catfish immunized with live theronts of
Ichthyophthirius multifiliis

De-Hai Xu^{1*}, Qi-Zhong Zhang², Craig A. Shoemaker¹, Dunhua Zhang¹ and Gabriel S. A.
Moreira³

¹ United States Department of Agriculture, Agricultural Research Service, Aquatic Animal
Health Research Unit, 990 Wire Road, Auburn, AL 36832-4352, USA

² Institute of Hydrobiology, Jinan University, Key Laboratory of Eutrophication and Red
Tide Prevention of Guangdong Higher Education Institutes, Guangzhou 510632, PR China.

³ Laboratory of Parasitology, College of Animal Science and Food Engineering, University
of Sao Paulo (USP), Av. Duque de Caxias Norte, 225, 13635-900, Pirassununga, SP,
Brazil.

ABSTRACT: The parasite *Ichthyophthirius multifiliis* (Ich) has been reported in various freshwater fishes worldwide and results in severe losses to both food and aquarium fish production. The fish surviving natural infections or immunized with live theronts develop strong specific and non-specific immune responses. Little is known about how these immune genes are induced or how they interact and lead to specific immunity against *I. multifiliis* in channel catfish *Ictalurus punctatus*. This study evaluated the differential expression of immune-related genes, including immunoglobulin, immune cell receptor, cytokine, complement factor and toll-like receptors in head kidney from channel catfish at different time points after immunization with live theronts of *I. multifiliis*. The immunized fish showed significantly higher anti-Ich antibody expressed as immobilization titer and ELISA titer than those of control fish. The vast majority of immunized fish (95%) survived theront challenge. Expression of IgM and IgD heavy chain genes exhibited a rapid increase from 4 hours (h4) to 2 days (d2) post immunization. Expression of immune cell receptor genes (CD4, CD8- α , MHC I, MHC II β , TcR- α , and TcR- β) showed up-regulation from h4 to d6 post immunization, indicating that different immune cells were actively involved in cellular immune response. Cytokine gene expression (IL-1 β a, IL-1 β b, IFN- γ and TNF- α) increased rapidly at h4 post immunization and were at an up-regulated level until d2 compared to the bovine serum albumin control. Expression of complement factor and toll-like receptor genes exhibited a rapid increase from h4 to d2 post immunization. Results of this study demonstrated differential expression of genes involved in the specific or non-specific immune response post immunization and that the vaccination against Ich resulted in protection against infection by *I. multifiliis*.

Keywords: Immunization; serum antibody; survival; channel catfish; *Ichthyophthirius multifiliis*
Immune-related genes; qPCR

1. Introduction

Ichthyophthiriasis caused by the parasite *Ichthyophthirius multifiliis* (Ich) has been reported in various freshwater fishes worldwide and results in severe losses to both food and ornamental fish production [1,2]. In the US, 42% producers of channel catfish *Ictalurus punctatus* suffered more than 2000 lb losses per producer due to the parasite in 2002 [3,4], and Ich infections in fingerling fish affected close to four percent of total channel catfish producers in 2009 [5]. The life cycle of the parasite includes 3 stages: an infective theront, a parasitic trophont and a reproductive tomont [1,2]. There are some treatments or combinations of treatments available for the parasite [1]. However, it is difficult to control the parasite using chemotherapy after its theront penetrates into fish skin and gills [6].

Immune protection has been successfully demonstrated in fish surviving natural and experimental Ich infections or following immunization with Ich antigens [1,7,8,9]. To date, one of the most effective ways to immunize against Ich is by intraperitoneal (IP) injection of live theronts. The fish immunized with live theronts developed strong specific and non-specific immune responses [8,9,10]. Both systemic and mucosal immune responses are involved in protection against *I. multifiliis* [9,11,12]. Systemic and mucosal antibodies were demonstrated to agglutinate the parasites *in vitro* [7,9,10]. There are three major types of immunoglobulins in teleost fish: IgM, IgD and IgT/IgZ, but only IgM and IgD have been identified in channel catfish [13,14,15]. IgD includes a membrane bound form (IgD2) and a secreted form (IgD3) [13].

Functions of IgD in immune responses are largely unknown and need further study. The fish infected with theronts also induced localized cellular infiltration, including lymphocytes, granulocytes and macrophages [10,16].

Sigh et al. [12] detected an increase in gene expression of IgM, major histocompatibility complex (MHC) and complement component C3 in rainbow trout after exposure to Ich. In a study of the initial response of rainbow trout fry to infection with *I. multifiliis*, Heinecke and Buchmann [17] found that IL-1 β , IL-6, and IL-8 were up-regulated after fish were exposed to the Ich theronts. Von Gersdorff Jørgensen et al. [18] found that complement factors C3, C5 and factor B were up-regulated in liver and head kidney of rainbow trout following immunization with a live vaccine against Ich. Olsen et al. [15] also noted that significant up-regulation of genes encoding IgM, IgT, C3, and IFN- γ were induced by immunization with Ich theronts in rainbow trout. The gene expressions of toll-like receptors TLR-1 and TLR-2 were observed to be significantly up-regulated in the head kidney of channel catfish after infection by Ich [19]. Little is known about how these immune genes are induced, how they interact and how they lead to specific immunity against *I. multifiliis* in channel catfish. The objective of this study was to evaluate molecular immune response and investigate differential expression of immune-related genes in head kidney from channel catfish over a time course after immunization with live theronts of *I. multifiliis*.

2. Materials and methods

2.1 Fish and parasite

Channel catfish (Industry pool strain) were obtained from disease-free stock from the USDA-ARS Catfish Genetic Research Unit, Stoneville, MS and reared to experimental size in indoor

tanks at the USDA, Aquatic Animal Health Research Unit, Auburn, AL. *Ichthyophthirius multifiliis* (ARS 15 strain) originally isolated from infected tropical pet fish was maintained by serial transmission on channel catfish held in 50-l glass aquaria as described by Xu et al. [9].

2.2 Water quality

Dissolved oxygen (DO) and temperature were measured using an YSI 85 oxygen meter (Yellow Springs Instrument, Yellow Springs, OH). The pH, hardness, ammonia and nitrite were determined using Hach CEL/890 Advanced Portable Laboratory (Loveland, CO). During the trial, a light:dark period of 12:12 h was maintained and supplemental aeration was supplied by air stones. The water quality was measured daily throughout the trial and expressed as mean $\pm$ standard error: dissolved oxygen 5.8 ± 0.4 mg l⁻¹, pH 7.0 ± 0.2 , ammonia 0.27 ± 0.3 mg l⁻¹, hardness 95.5 ± 7.9 mg l⁻¹, nitrite concentration below detectable level and water temperature 22.1 ± 0.4 °C, measured daily. All water parameters were within acceptable levels for catfish.

2.3 Theront preparations

Fish heavily infected with mature trophonts were anesthetized with 300 mg l⁻¹ tricaine methanesulfonate (MS 222), rinsed with water and the skin was gently scraped to dislodge the parasites. Trophonts were harvested by filtering through a 0.45 μ m filter to remove fish skin. The isolated trophonts were placed in Petri dishes and allowed to attach. After replacing the water in the Petri dishes with fresh dechlorinated water to remove contaminating mucus, trophonts were incubated for 18 h at 24 °C. Theronts were harvested by pouring through a sieve with a pore size of 22 microns. For IP injection, theronts were concentrated by centrifugation at

100 × g for 3 min and suspended in phosphate-buffered saline (pH 7.2, PBS). Theronts were counted in five 10 µl samples of theront solution with the aid of a Sedgewick-Rafter cell, where average counts were used for concentration calculation. A 5% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) solution was prepared in PBS to be used in the mock-immunized control.

2.4 Experimental design and immunization procedure

A total of 110 channel catfish with an average length of 15.1 ± 1.3 cm (mean ± SEM) and weight of 30.1 ± 7.2 g were used in this trial. All fish treatment protocols were approved by Institutional Animal Care and Use Committee at the Aquatic Animal Health Research Unit. Ten catfish were sampled to verify that the fish were free from parasites and detectable serum antibody against Ich prior to initiation of the trial. All fish were negative for Ich infection (*i.e.* no visible attached theronts under microscopy) and no anti-Ich antibody was detected in fish serum prior to study.

Fish were then distributed into 4 replicated groups and immunized by IP injection as follows: groups 1 and 3 with live theronts at a dose of 15,000 theronts (0.1 ml in volume) per fish; groups 2 and 4 with 0.1 ml of 5% BSA as mock-immune control. There were 15 fish per tank in the groups 1 (immune) and 2 (control) to collect tissues for immune gene expression analysis. There were 10 fish per tank in groups 3 (immune) and 4 (control) to evaluate immune protection and fish survival 21 days post immunization.

2.5. Blood and tissue sampling

Five fish were sampled from groups 1 and 2 at h4 (4 hours), d1 (1 day), d2, d6, d10 and d20 post immunization to collect serum for measuring antibody and tissues for evaluating immune

related genes. The blood was collected after fish were anesthetized with 100 mg l⁻¹ MS-222.

After blood collection, fish were euthanized by 300 mg l⁻¹ MS-222 and head kidney was collected and immediately immersed in RNAlater (Invitrogen, Carlsbad, CA). All samples were incubated overnight at 4°C and then transferred to – 20°C until RNA extraction.

2.6. Anti-Ich antibodies in serum of channel catfish

Serum anti-Ich antibodies were determined by both theront immobilization assay and enzyme-linked immunosorbent assay (ELISA). The immobilization assay was conducted by placing theronts in serum serially diluted in 96 well flat bottomed microtitre plates (Corning Costar, Corning, NY) as described by Xu et al. [9]. A solution of 50% (v:v) PBS diluted with tank water was used as a diluent. Immobilization titer was the inverse of the highest dilution of serum in which all theronts were immobilized. Anti-Ich antibodies were measured in two replicates for each serum sample with ELISA as described by Xu et al. [20]. An optical density (OD) reading in a sample from experimental fish was considered positive when its value was two times greater than the OD readings in the control. The ELISA titer was the inverse of the highest dilution of serum in which a sample was positive.

2.7 Challenge and parasite infection level

At d21 post immunization, fish in groups 3 and 4 were exposed to 40,000 theronts per fish for 1 h with aeration after tank water was lowered to 10 L in each tank. Water flow (0.5 L min⁻¹) was resumed at 1 h post exposure and the fish were monitored for Ich infection and mortality for 3 weeks after theront challenge. Five days post theront challenge, numbers of white spots on the

body surface of each fish were counted as described previously [9]. The infection levels were evaluated using a semi-quantitative scale: no infection (0 trophonts fish⁻¹), light infection (< 50 trophonts fish⁻¹), mild infection (50-100 trophonts fish⁻¹) and heavy infection (>100 trophonts fish⁻¹).

2.8 Total RNA extraction and cDNA synthesis

Total RNA from head kidney tissue was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The isolated RNA samples were treated with DNase (Life Technologies, Carlsbad, CA) to minimize contamination of genomic DNA. Quantity and quality of RNA samples were determined on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE) and by electrophoresis on 1.0% agarose gel. The first strand cDNAs used for quantitative PCR were synthesized using 2 µg of total RNA, AMV reverse transcriptase, and Oligo-dT primer provided by the cloned AMV first strand cDNA synthesis kit (Invitrogen).

2.9 Primer design and quantitative PCR

Sixteen immune related genes were selected in this study based on published reports of immune genes affected by parasite and bacterial infection, including genes of immunoglobulin, immune cell receptor, cytokine, complement factor and toll-like receptor [18,19,21,22,23]. The gene-specific primers were designed with the aid of Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and are listed in Table 1. Channel catfish 18S ribosomal RNA gene primers [23] were included as an internal calibrator to normalize

cDNA quantities. Primers for immune genes were synthesized by Sigma Aldrich (St. Louis, MO). Quantitative PCR (qPCR) was performed in triplicate for each cDNA sample in MicroAmp[®] Optical 96-well reaction plates (Applied Biosystems, Foster City, CA) using Applied Biosystems (ABI)7500 Real-Time PCR System. All qPCR reactions were performed using Platinum[®] SYBR[®] Green qPCR SuperMix-UDG with ROX (Invitrogen) in a total volume of 12 µl. The qPCR mixture consisted of 0.5 µl of cDNA (input RNA of 5 ng), 1 µl of 5 µM gene-specific forward primer, 1 µl of 5 µM gene-specific reverse primer, 6 µl of 2× SYBR Green SuperMix and 3.5 µl of DEPC-treated water. The qPCR thermal cycling parameters were 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Specificity of amplified products was assessed using melting curve analysis (ABI 7500 Real-time PCR Software V2.0) and by gel electrophoresis using separate end-point PCR reactions. The PCR products were also gel-excised, purified and sequenced to confirm that they match target gene sequences. Primer efficiency was set ranging from 90–100% calculated using $E = (10^{(-1/\text{slope})} - 1) * 100$ and a primer pair would be redesigned if it did not meet the requirement.

2.10 Data analysis

The relative transcriptional levels of different genes were determined by subtracting the cycle threshold (Ct) of the sample by that of the 18S ribosomal RNA, the calibrator, using the formula: $\Delta Ct = Ct(\text{sample}) - Ct(\text{calibrator})$. The relative expression level of a specific gene in theront immunized fish was compared to that of mock-immunized fish to obtain $2^{\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct(\text{control}) - \Delta Ct(\text{immune})$ described by Pfaffl [24]. All data were analyzed with SAS software [25]. Mean days to death (MDD) were calculated by the Lifetest procedure (Kaplan-Meier method). Serum antibody titers from different immunized groups were analyzed with

Duncan's multiple range test. Probabilities of 0.05 or less were considered statistically significant.

3. Results

3.1 Effects of theront immunization on anti-Ich antibody responses

The ELISA titers of anti-Ich antibodies between control fish and fish immunized with live theronts at d6 or less post immunization did not differ (Table 2). Fish serum antibodies increased significantly in immune fish at d10 post immunization. Serum anti-Ich antibodies were 64 fold higher in immobilization titer or 115 fold higher in ELISA titer in immune fish than control fish at d20 post immunization (Table 2).

3.2 Effects of theront immunization on catfish infection and survival

Immunization with live theronts did not completely prevent theront re-infection. At d6 post challenge, the fish immunized with live theronts had light infection (< 50 trophonts fish⁻¹, Table 3). Fish of non-immunized control showed heavy infection with more than 100 trophonts fish⁻¹. All mock-immunized control fish died from Ich challenge with MDD of 5.6 day. Immunization with live theronts by IP injection provided good protection and 95% of fish survived theront challenge (Table 3).

3.3 Expression of immunoglobulin genes

Expression of IgM and IgD heavy chain genes exhibited a pattern of rapid increase in expression from h4 to d2 post immunization then a drop to a lower level of expression in the immunized fish compared to BSA control (Fig 1). The membrane bound form of IgD (IgD2) gene in the

immunized fish showed similar expression pattern as IgM gene after fish immunization. The secreted form of IgD (IgD3) gene showed different expression pattern compared to IgM gene. The IgD3 gene in the immunized fish showed a down-regulation from d1 to d20 post immunization (Fig 1).

3.4 Expression of immune cell receptor genes

The CD4 and CD8- α gene expression increased rapidly at h4 post immunization by 7-fold, peaked at d1 (7-17 fold) and remained at high level until d6 post immunization compared to the mock immunized control (Fig 2). CD4 and CD8- α were also the only two among immune cell receptor genes that displayed down-regulation at one time-point throughout the trial at d10 post-immunization. The MHC I, MHC II β , TcR- α , and TcR- β genes showed up-regulation from h4 to d20 post immunization. The gene expression was 3-fold or higher in immunized fish from h4 to d1, then remained at high level until d20. Among them, MHC I showed the highest expression, 5-11 fold higher from h4 to d6 in immunized fish than control. The kinetics of TcR- α expression was similar to that of TcR- β and peaked at d2 post immunization. The TcR- α , however, exhibited a higher upregulated expression (3-6 fold) than control fish compared to TcR- β in immunized fish (Fig 2).

3.5 Expression of cytokine genes

All cytokine genes increased rapidly at h4 post immunization and were up-regulated until d2 compared to the control (Fig 3). These 4 cytokine genes showed 7-22 fold increase in immunized fish compared to those of control at h4 post immunization. The expression levels of IL-1 β a, IL-

1 β b and IFN- γ were then decreased in immunized fish after d6 post immunization. TNF- α expression, however, remained at a higher level in immunized fish during the trial from h4 to d20 post immunization (Fig 3).

3.6 Expression of complement factor and toll-like receptor genes

The complement component 3 (C3) gene increased rapidly to 15 fold in immunized fish compared to the level of control at h4 post immunization (Fig 4). The expression level maintained at least 3 fold higher than control during the entire trial. Complement factor D (CFD), TLR-1 and TLR-9 exhibited a rapid increase in expression from h4 to d2 post immunization then a drop to lower levels in immunized fish at d6 post immunization compared to BSA control.

4. Discussion

Previous studies demonstrated that both humoral and cellular immune responses are involved in protection of fish against Ich [2,4,9,16]. In this study, antibodies against Ich were detected in serum of immunized fish d6 post immunization by both ELISA and immobilization assays. Anti-Ich antibody level was significantly increased from d10 post immunization to d20 of the trial. The increase of anti-Ich antibody was in agreement with previous studies. Xu et al. [9,20] found that serum antibodies in immunized fish were 37-56 fold higher than that of control fish d12 post immunization. Gene expression of IgM and IgD heavy chain exhibited a different pattern compared to antibody production (as measured via ELISA and immobilization titer). IgM and IgD heavy chain mRNAs were rapidly induced from h4 to d2 post immunization with a

subsequent drop in expression in the immunized fish. Although genes encoding immunoglobulin heavy chains showed rapid mRNA up-regulation for a short period of time, anti-Ich antibodies increased slowly reaching high levels at d20 post immunization, anti-Ich antibodies were 115 fold higher measured with ELISA (titer 1152) and 64 fold higher by immobilization assay (titer 1280) in immunized fish than that of control fish. Antibody formation includes multiple steps from gene expression to protein production. One would expect a time difference between the upregulation of immunoglobulin genes to the production of functional antibody. Once antigen is presented to the B-cell, the B-cell undergoes several steps prior to proliferation and differentiation for antibody production (i.e., plasma cell). In most cases antibody levels reach a peak 7-10 days post immunization but this time line can vary dependent on the antigen [26]. The duration from Ig gene expression to antibody production is also probably affected by fish species and water temperature. The immunized fish achieved 95% survival after challenge with live Ich theronts. In previous studies in catfish, host protection was also demonstrated in fish immunized with live theronts by IP injection, which lead to 93-100% fish survival [9, 20]. In a study in rainbow trout [18], the gene encoding IgM was weakly up-regulated 24h following immunization with a live vaccine against Ich in the head kidney. In the same trial, anti-Ich antibody was detected at a low level (titer 25) in the rainbow trout plasma using a modified ELISA 4 week post immunization. The work suggests a positive correlation between expression of immunoglobulin genes and production of antibodies against the parasite.

Channel catfish express two Ig isotypes: IgM and IgD. No IgT/IgZ reported in trout and carp (*Cyprinus carpio* L.) [4,14] has been identified in catfish. Catfish IgM has been extensively studied at the functional and structural levels [27]. In contrast, much less is known about the

teleost IgD [13]. IgD presents on the surface of B-lymphocytes in either a membrane-bound form (IgD2) or in blood serum as a secreted form (IgD3) [13]. When a B-cell reaches its mature state, it co-expresses both IgM and IgD. IgD is able to signal B-cells causing activation of the immune system [28]. In this study, specific primers (IgD) were designed to target both membrane bound form (AF363448) and secreted form (AF363450) of IgD heavy chain gene of channel catfish [13]. The primers for IgD2 are specific for targeting only the membrane bound form of IgD and IgD3 specific only to the secreted form of IgD. The gene expression pattern was similar between total IgD and the membrane-bound form of IgD (IgD2). However, the expression pattern was slightly different between IgM and IgD. Expression of IgM in the immunized fish increased rapidly and reached its peak at h4 post immunization and then gradually decreased. IgD expression increased and reached its peak at d1 post immunization with a gradual decrease thereafter. The up-regulation of IgM and IgD indicates that both IgM and IgD of catfish can be induced by theront immunization and may play an active role in humoral immune response. Similar up-regulation of IgM gene was also noted in rainbow trout. Sigh et al [12] reported that Ich infection induced up-regulation of the IgM gene in the head kidney of rainbow trout as early as 4 days and kept significantly higher level d26 post infection. Ich induced production of antibodies in both the skin and head kidney [12].

Cell-mediated immune responses are executed by leukocytes, granulocytes, macrophages (monocytes), dendritic cells and lymphocytes [29]. In the early stages of Ich infection, the neutrophil was identified as the predominant cell type in carp [16]. When the trophont develops, cells migrating toward the infection sites show increase numbers of macrophages, neutrophils and resident epidermal filament cells [16]. Dan et al. [30] recently evaluated cellular immune

response by measuring respiratory burst activity of leukocytes in orange-spotted grouper (*Epinephelus coioides*) after immunization with *Cryptocaryon irritans* vaccine. *Cryptocaryon irritans* is an obligate parasite of tropical and subtropical marine fish that causes white spot disease similar to *I. multifiliis* in freshwater. The results revealed that respiratory burst activity of leukocytes was significantly higher in fish after immunization with the high-dose (30,000 theronts fish⁻¹) of *C. irritans* vaccine than fish immunized with low-dose (10,000 theronts fish⁻¹) vaccine or with PBS control. The increased activity of respiratory burst activity in the immune fish may be due to the increased number of leukocytes involved in the cellular immune response [29]. Genes of MHC I, MHC II β , CD4 (cluster of differentiation 4) and CD8- α encoded glycoproteins expressed on the immune cell surface or served as co-receptors of immune cells [31]. All immune cell receptor genes examined in this study were up-regulated h4 post immunization with live theronts and levels remained high for 6 days or longer post immunization. Results in this study indicated that different immune cells were actively involved in cellular immune response. In a previous study, Singh et al [12] reported that MHC II β gene was significantly up-regulated in the head kidney of rainbow trout from d4 to d26 post infection by *I. multifiliis*.

Interleukins (ILs) and interferons (IFNs) are a group of small proteins that belong to cytokines and produced by macrophages, lymphocytes, granulocytes, or epithelial cells [31]. Cytokines are secreted by activated immune-related cells upon induction by various pathogens, such as parasitic, bacterial, or viral components and modulate the balance between humoral and cellular immune responses [32]. In this study, cytokine genes showed up-regulation in immunized fish from h4 to d2 post immunization. Heinecke and Buchmann [17] studied the

immune responses of rainbow trout to an infection with *I. multifiliis* and found that IL-1 β was up-regulated 3h post infection and kept up-regulation during the 72h experiment with a peak at 12 h post infection. Their result indicates that IL-1 β is an initiator of the inflammatory response in rainbow trout infected with *I. multifiliis*. In a study to evaluate inflammatory responses caused by *I. multifiliis* in carp, Gonzalez et al. [33] reported that the expression levels of IL-1 β reached a maximum of a 150-fold increase at 36h post infection, and remained significantly elevated (18 fold) as compared to the uninfected controls. They also reported a significant up-regulation of cytokine TNF- α at 36 and 48 h post infection (3- and 4-fold increase respectively). Results in this study and previous studies by other researchers indicate a rapid involvement of cytokine genes in the innate immune response.

The complement system is a part of the innate immune system that helps antibodies and phagocytic cells to clear pathogens from host. Complement-mediated killing occurs when complement is activated either directly by pathogen components and/or by antibody-antigen complexes [33]. In this study, the expression of complement factor 3 was 4-15 fold higher in the immunized fish than control fish. Complement factor 3 showed upregulation in immunized fish from h4 to d20 post immunization, indicating a rapid and active involvement of innate immune response. In a similar study to evaluate immune gene expression in rainbow trout following immunization with live vaccine against *I. multifiliis*, Jørgensen et al. [18] reported that the complement factors C3 (9-fold) and C5 (21-fold) were up-regulated in the head kidney 24 h post immunization. Sigh et al. [12] also noted an up to 5-fold increase in C3 gene expression at d 4-6 in the skin and d 6-26 in the spleen, indicating that the complement system is also contributing to the immune reactions against *I. multifiliis* in rainbow trout.

Toll-like receptors are important activators of innate immunity by recognition of conserved pathogen-associated ligands [19,35]. TLR-1 is expressed on cell surfaces and recognize mainly microbial membrane components while TLR-9 expressed exclusively in intracellular vesicles to recognize microbial nucleic acids [35]. In this study, both TLR-1 and TLR-9 showed an early up-regulation at h4, peak of expression at d1 and then down-regulation at d6. In a study with channel catfish infected with *Ich* theronts, Zhao et al [19] observed an up-regulation of gene TLR-1 at h12, the peak at d3 and down-regulation at d7 in the head kidney. In the same study, TLR9 was numerically up-regulated from h6 to d1 post infection. Results in this study and previous report [19] demonstrated that TLR1 and TLR9 genes were involved in the early immune response against *Ich* infection.

When live theronts were IP injected into fish, the theronts can survive within peritoneal cavity from several days to 3 weeks [36]. The theronts attach to fish tissues and damage fish tissues by its constant rotation, shape transformation and site relocation [37]. The invasion of theronts and tissue damage in fish initiates informatory response and cellular immune response [4,15,16]. Then the GPI-anchored surface membrane proteins and secreted products from the attached trophonts stimulates humoral immune response [4,9]. It seems that immune response is initiated locally at the site of infection and as the infection progress it becomes systemic [12]. The anterior kidney and spleen are the central systemic sites of adaptive immunity against *I. multifiliis* [4]. In this study, we mainly focused on the expression of genes related to innate and adaptive immune responses in systemic immunity. Results in this study demonstrate that *I. multifiliis* vaccination improves innate and adaptive immune responses in catfish and enhance fish anti-parasite resistance.

Conclusion

This study evaluated immune response and expression of immune-related genes in head kidney from channel catfish after immunization with live theronts of *I. multifiliis*. The immunized catfish showed significantly higher antibody titers than those of mock immunized fish and 95% of immunized fish survived theront challenge. Results of this study demonstrated differential expression of genes involved in the specific or non-specific immune response post immunization and that the vaccination against Ich resulted in protection against infection by *I. multifiliis*. Results of this study may allow for development of alternative control strategies for Ich by leading to a better understanding of the immune response of catfish to this parasite.

Acknowledgments

The authors thank Dr. Jason Abernathy, USDA-ARS, Hagerman Fish Culture Experiment Station, Hagerman and Dr. Xing-Jiang Mu, College of Fisheries and Life Science, Shanghai Ocean University for valuable comments and suggestions to improve the manuscript. We gratefully acknowledge the technical assistance of Jana Mladek, Paige Mumma and the management team of the Aquatic Animal Health Research Unit aquatic facility for daily care and management of fish. We are also grateful to the FAPESP for providing a scholarship to Gabriel S. A. Moreira (Proc. no. 2015/01822-0). This research was supported by USDA/ARS CRIS Project #6010-32000-026-00D. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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506

TABLES

507 Table 1. Primers used in qPCR for detection of target genes, including the size and location of amplicons and primer sequences.

Gene	GenBank accession number	Amplicon size (bp)	Location of amplicon	Forward Primer (5'-3')	Reverse Primer (5'-3')
IgM	X79482	126	6434-6559	TGTGTGTGTGTGTGTGTGTGTGTT	CGGATGTCTTGGCTTGTGTTG
IgD	AF363448	187	5646-5832	AAAGTGGCTCCCTGAATCTGT	ACTGGTGCTCTTCTCAACTGG
IgD2	AF363448	137	7671-7807	AGTTTACAGGAGGTGGGCAA	GGCCTATGGCATGTTTACACAC
IgD3	AF363450	73	7988-8060	ACCTGTCAATCGGTGTTGGG	TCTTGCATTCCCCTCAGCAA
CD4	DQ435302	178	323-500	CCTCTGCGAACCCATCTTCA	AGGCAGGTCCAGATTCTTGC
CD8 alpha	FJ804170	125	395-520	AGAAAGCATCCCCACTCTGC	CCGACCAAAGGACCAGAGAC
MHC class I	AY008848	117	3622-3738	CAGAGGTGGTGTGTCATGCTA	CCTGGTTGGGTAACGTCTCTC
MHC class II beta	U77598	90	442-531	CTGAGGAACGGGAAGGAGAT	CAGATGGGAGTGGACCTGAT
T cell receptor alpha	JN097591	79	351-413	AGAAACGGGAGCCGTCAATTT	TTGTGAACTGTAAAGCGCGTG
T cell receptor beta	AF410786	90	48-137	ACTCCAAGCACCAACTGTCA	CCTTTAGCCACACACACCAG
Interleukin 1 beta type a	EE993591	219	630-848	AAAAATGGCCAGCCTGTATG	CAGCCCGGGTATTTAAGTGA
Interleukin 1 beta type b	BM425330	193	224-416	GCCTCTTAGTATGCGCCAAG	AACCTTGCTTGCAGGCTGT
Inteferon (IFN- γ)	BM494266	179	8-186	CAGCAGTGACTTCAGCCAAA	GCCTCAGAGTACGCCATCAT
TNF-a	CK413376	237	597-833	TTCTTAACGCGGGTGTACC	GGGGGAAGGTTCTCTCATTC
Complement factor C3	BM438508	196	100-295	AGTTGAATACCGCTGCCAAC	CTCTCCATGCGCTGAGTACA
Complement factor D	CK414026	242	526-767	AGGCAGAGGACAAAAAGCAA	TGGCTGACTTAGCTGCCAAT
TLR1	HQ677713	109	2853-2961	AGCCAAAGAAATGCCAACTG	TGAAGTCTCGTTCGTGGTGA
TLR9	HQ677720	110	5034-5143	GGAGGAACGGGACTGGATAC	AAGCACAGCCACCCTGATTA
18S rRNA	BE469353	220	236-455	ATGGCCGTTCTTAGTTGGTG	TAGGTAGCACACGCTGATCG

Table 2. Antibody titers of immobilization assay (Immob) and enzyme-linked immunosorbent assay (ELISA) against *Ichthyophthirius multifiliis* in serum of channel catfish at different time-points post immunization. Within a column for each assay, means followed by the same lower case letter are not significantly different ($P > 0.05$).

		Sampling time					
Assay	Groups	Hour 4	Day 1	Day 2	Day 6	Day 10	Day 20
Immob	Control	26.7±6.7 ^a	16.7±3.3 ^a	6.7±6.7 ^a	20±0 ^a	26.7±3.3 ^a	20±0 ^a
	Immune	18±2 ^a	26±6 ^a	8.0±4.9 ^a	64±9.8 ^b	512±78 ^b	1280±0 ^b
ELISA	Control	10±0 ^a	10±0 ^a	10±0 ^a	10±0 ^a	10±0 ^a	10±0 ^a
	Immune	10±0 ^a	10±0 ^a	10±0 ^a	12±2 ^a	74±6.5 ^b	1152±128 ^b

Table 3. Survival and mean days to death of immunized channel catfish following challenge with theronts of *Ichthyophthirius multifiliis* at the dose of 40,000 theronts per fish for one hour and observed for 21 days. Infection level was determined 6 days post challenge. Twenty fish were used for each immunization group.

Groups	Infection level*	Number of dead fish	Survival (%)	Mean days to death
Control	Heavy	20	0	5.6
Immune	light	1	95	14

* Infection level, light (< 50 trophonts fish⁻¹), mild (50-100 trophonts fish⁻¹) and heavy infection (>100 trophonts fish⁻¹).

FIGURES

Fig 1. Expression of immunoglobulin heavy chain genes in the head kidney of channel catfish at different time points post immunization of *Ichthyophthirius multifiliis*. Data are presented as relative fold change of genes from immunized fish compared with mock-immunized control fish. IgM: immunoglobulin M, IgD: both membrane bound and secreted form of immunoglobulin D, IgD2: membrane bound IgD, IgD3: secreted form of IgD.

Fig 2. Expression of immune cell receptor genes in the head kidney of channel catfish at different time-points post immunization of *Ichthyophthirius multifiliis*. Data are presented as relative fold change of genes from immunized fish compared with mock-immunized control fish. CD: cluster of differentiation, MHC: major histocompatibility complex, TcR: T cell receptor.

Fig 3. Expression of cytokine genes in the head kidney of channel catfish at different time points post immunization of *Ichthyophthirius multifiliis*. Data are presented as relative fold change of genes from immunized fish compared with mock-immunized control fish. IL: interleukin, IFN: interferon, TNF-α: tumor necrosis factor alpha.

Fig 4. Expression of complement factor and toll-like receptor genes in the head kidney of channel catfish at different time points post immunization of *Ichthyophthirius multifiliis*. Data are presented as fold changes of genes from immunized fish compared with mock-immunized control. C3: complement component 3, CFD: complement factor D, TLR: toll-like receptor.