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Short communication

Expression profiles of toll-like receptors in channel catfish (*Ictalurus punctatus*) after infection with *Ichthyophthirius multifiliis*

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ABSTRACT

Toll-like receptors (TLRs) play a crucial role in the innate immune system, but to date the roles of fish TLRs in response to parasitic infection are still poorly understood. In the present study, we used channel catfish (*Ictalurus punctatus*) and the ciliate parasite *Ichthyophthirius multifiliis* as a model to investigate whether and which fish TLRs play important roles in the immune response against parasitic pathogens by detecting the expression profiles of a complete set of TLRs in catfish at different time points after infection with *I. multifiliis*. The expression profiles of TLR1 and TLR2 were similar, and both were significantly up-regulated in the skin and head kidney at most time points after infection. Furthermore, the expression of TLR2 was also up-regulated in the gill and spleen. TLR9 was induced in the skin and gill, whereas TLR21 was induced in the head kidney and spleen after infection. For TLR19, significant up-regulation was observed in the skin and gill, but significant down-regulation was detected in the head kidney and spleen. In contrast to TLR19, TLR25 was significantly up-regulated in the head kidney and spleen at some time points. No significant changes were observed for the rest of the TLRs at most time points. The results indicated that some TLRs may play essential roles in catfish defense against *I. multifiliis* infection.

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1. Introduction

Toll-like receptors (TLRs) are type I transmembrane proteins. They contain an extracellular leucine-rich repeat (LRR) domain that recognizes conserved pathogen-associated molecular patterns (PAMPs), a transmembrane domain, and an intracellular Toll/IL-1 receptor (TIR) domain that activates downstream signaling pathways [1]. TLRs play an essential role in the activation of innate immunity by recognizing their cognate ligands, such as lipoproteins, double-stranded RNA, single-stranded RNA, lipopolysaccharide (LPS), flagellin, and genomic DNA [2].

In mammals, several parasitic components have been shown to be the ligands of host TLRs. Glycosylphosphatidylinositol (GPI) -anchored proteins isolated from *Toxoplasma gondii* [3], *Trypanosoma cruzi* [4], *Leishmania major* [5], and *Plasmodium falciparum* [6] all could be recognized by TLR2 and/or TLR4. DNA from *T. cruzi* and profilin-like protein from *T. gondii* could trigger TLR9 [7] and TLR11 [8], respectively. Fish have more TLRs than mammals, and at least 17 TLR types have been identified in different fish species to date [9], but little information is available about the roles of fish TLRs in the response to parasitic infection. Our previous studies revealed that TLR2, TLR9, and TLR21 of *Epinephelus coioides* were involved in anti-*Cryptocaryon irritans* immune defense [10,11]. However, whether and how other fish TLRs are involved in anti-parasite immune defense is still largely unknown.

Channel catfish (*Ictalurus punctatus*) is one of the most important aquaculture species worldwide. To date, all of the reported teleost TLR genes, except TLR23, have been identified and sequenced in catfish. In addition, TLR25 and TLR26, two previously unknown TLRs, were found in catfish [12,13]. Thus, this species has one of the most complete sets of TLRs known for teleosts. *Ichthyophthirius*

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multifiliis, a highly virulent ciliate parasite, is responsible for ichthyophthiriosis, or "white spot disease", which infects almost all freshwater fish species and results in large economic losses to the aquaculture industry. The life cycle of *I. multifiliis* includes three stages: an infective theront, a parasitic trophont, and a reproductive tomont [14]. Because *I. multifiliis* is naturally restricted to the surface epithelia of the skin and gill, experimental infection can be easily monitored under laboratory conditions. Therefore, it has been used as a model of cutaneous immunity in fish [15]. Coincidentally, *I. multifiliis* has been cultured and maintained by serial infections of catfish in many laboratories, including our own, which provides a stable material for detailed research [16–18].

In the present study, we used catfish and *I. multifiliis* as a model to investigate whether and which fish TLRs play important roles in the immune response against parasitic pathogens by detecting the expression profiles of all reported catfish TLRs at different time points after infection with *I. multifiliis*. To our knowledge, this is the first study to investigate the expression profiles of a complete set of TLRs in fish during parasitic infection.

2. Materials and methods

2.1. Fish

Healthy catfish with an average body weight of 43.2 g were selected from the aquaculture base at Pearl River Fisheries Research Institute, Guangdong Province, China for use in this study. 15 randomly selected catfish were examined under a microscope, and no parasites were detected on the skin or gills of these fish, and no immobilization occurred when theronts were incubated in fish serum. Catfish were acclimatized for 3 weeks prior to the experiment and fed twice a day with commercially produced food pellets. Water temperature was maintained at 23 ± 1 °C, and a 12 h light: 12 h dark photoperiod was used.

2.2. Parasites

The strain of *I. multifiliis* was originally isolated from infected grass carp (*Ctenopharyngodon idella*) and maintained by serial infections of catfish in a laboratory system using the previously described method [17] with some modifications. To collect live theronts for the infection experiment, some heavily infected catfish with visible parasites over the entire body surface were placed in several 5-L plastic beakers of dechlorinated water, and mature trophonts were released from the fish into the water. Trophonts were collected, transferred to culture dishes, and allowed to develop into theronts.

2.3. Infection procedure and tissues sampling

For the infected group, 60 catfish were exposed to *I. multifiliis* at a dose of 8000 live theronts per fish in a 60-L bucket for 2 h. Fish then were transferred to a 300-L aquarium along with the water used for the infection procedure. For the control group, 60 catfish were treated using the same method as the infected group, except no *I. multifiliis* theronts were added. Six fish from the infected group and the control group were euthanized by an overdose of MS222 (200 mg/L) at 6 h, 12 h, 24 h, 36 h, 2 d, 3 d, 5 d, and 7 d after infection with *I. multifiliis*. The skin and gills (the infection sites) and the head kidney and spleen (the systemic immune organs) were removed from each fish and snap frozen in liquid nitrogen.

2.4. Total RNA isolation and cDNA synthesis

Total RNA was extracted from each sample using Trizol Reagent (Invitrogen, USA) following the manufacturer's protocol. The concentration of RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer (GE, USA), and the purity was checked by measuring the ratio of OD 260 nm/OD 280 nm. Total RNA was incubated with RNase-free DNase I (Fermentas, USA) at 37 °C for 30 min to eliminate contaminated genomic DNA, and then it was incubated with 1 μL EDTA (25 mM) at 65 °C for 10 min cDNA was synthesized using ReverTra Ace-α-reverse transcriptase (TOYOBO, Japan) as described by the manufacturer. The sample then was diluted two times with water and used as the template for real-time RT-PCR amplification.

2.5. Real-time RT-PCR analysis

The gene-specific primers were designed using Beacon Designer 8.0 software and are listed in Table 1 β-actin was used as the internal reference. Real-time RT-PCR was performed using a Roche LightCycler480 Real-time PCR Detection System (Roche, Switzerland) with the SYBR Green Real-time PCR Master Mix kit (TOYOBO). Real-time RT-PCR was carried out in a 10-μL reaction volume containing 5 μL of SYBR Green Real-time PCR Master Mix,

Table 1
Primers used in real-time RT-PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)	Primer efficiency	Genbank accession no.
TLR1	TCACCACGAACGAGACTTCATCC	GACAGCACGAAGACACAGCATC	93	1.95	HQ677713
TLR2	GTGGTTTCTCTCAGCCTTCAG	ACGGTCAGCGATGTCTCAG	98	2.00	HQ677714
TLR3	GGCTCAATACAACCAACAC	CAGGAAGGCTAGAACCATC	188	1.98	HQ677715
TLR4[a]	CCAGTATTCCTTCCACCGTAG	AGAGTAGTCACATTCTTCACATTG	172	2.06	HQ677716
TLR5[b]	ACACTTCACTCTCCTCAA	AATCAAGCAGCGTCATAG	116	2.02	HQ677717
TLR7	CTGTCCATCTCAAGCCATCTC	GCCGTGTCAGTTCTATCGTAG	143	1.96	HQ677718
TLR8[c]	CCGTCTCTATCCTATCACTAACTC	CAGCAGCGAAGCACTCAG	161	2.03	HQ677718
TLR9	TAGCCTTAGACCTCTGTTCAAC	CACAACCATTCTCAACGATCTC	118	2.00	HQ677720
TLR18	CTGGAGAGGATTGCTATTAGAAC	AAGAGATTACGGAAACGAATGG	185	2.04	HQ677721
TLR19	CACTCACTGGAACTGTTGTATC	ACCTGTGCTCGTGTATTCTG	170	2.02	HQ677722
TLR20[d]	ATCACGGACAGCCTCTACAG	CTCCAGGAACACCAGAACAAG	150	1.94	HQ677723
TLR21	GAGCAGTGGCGTCGTCTTC	CGGTGGTGGAGGCAAAGTC	146	2.01	HQ677724
TLR22	CCTTCTGGTGTCGTTCATTTATC	TATCCGTGTTGCTGGTGTATC	117	1.97	HQ677725
TLR25	CGAAGAGAATCAGGCTAATCAAG	TGTTAATCAAGGTGTCCACAATG	123	2.04	HQ677726
TLR26	TGGACATCGTGGAGAACATC	CTTGCTGCGGAGGTAGTG	86	1.97	HQ677727
β-actin	CCGTGACCTGACTGAATACC	GCCCATCTCCTGCTCAAAG	139	2.01	DQ399027

[a] Primers amplify both TLR4-1 and TLR4-2.
[b] Primers amplify TLR5-1, TLR5-2, and TLR5S.
[c] Primers amplify both TLR8-1 and TLR8-2.
[d] Primers amplify both TLR20-1 and TLR20-2.

0.4 μL of cDNA template, 0.4 μL of each primer (10 μM), and 3.8 μL of water. Real-time RT-PCR was conducted at 95 °C for 2 min, then 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s. Each sample was tested in triplicate. Specificity of amplified products was assessed using melting curve analysis. PCR products were purified using the E.Z.N.ATM Gel Extraction Kit (OMEGA, USA), ligated into the pMD18-T vector (TaKaRa, Japan), and inserted into *Escherichia coli* competent cells (DH5α). Plasmid DNA was isolated from overnight cultures using the E.Z.N.A Plasmid Mini Kit (OMEGA) and then sequenced for sequence confirmation. A standard curve for each primer pair was generated from serial dilutions of plasmid DNA templates. The primer efficiency was calculated using the formula: $E = 10^{(-1/\text{slope})}$.

2.6. Statistical analysis

The relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method [19]. The expression level of each target gene was first normalized to β-actin. The expression level in the infected group represented as the fold change relative to the average expression level (set as 1) in the control group at the same time point. Data are shown as mean ± standard error ($N = 6$). The data were subjected to an unpaired, two-tailed *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

In the present study, we investigated the expression profiles of a complete set of TLRs in catfish in the skin, gill, head kidney, and spleen at different time points after infection with *I. multifiliis*. The results (Table 2) showed that the expression of several TLRs, such as TLR1, TLR2, TLR9, TLR19, TLR21, and TLR25, was significantly different from the control at different time points or in different tissues, whereas other TLRs, including TLR3, TLR4, TLR5, TLR7, TLR8, TLR18, TLR20, TLR22, and TLR26, showed no significant change relative to the control at most time points.

TLR1 was mainly induced in the skin and head kidney after infection, and its induction peaked at 36 h (4.16-fold) and day 3 (4.36-fold), respectively. However, in the gill and spleen, TLR1 expression showed no significant changes throughout the experiment, except in the spleen at 12 h (1.84-fold). Similarly, the expression of TLR2 in the skin was significantly up-regulated at most time points after *I. multifiliis* infection, and the expression peak was observed at day 2 (4.85-fold). Expression in the gill increased significantly only at 24 h and day 7. The expression of TLR2 was significantly up-regulated both in the head kidney and spleen at most time points. This was especially true in the head kidney, which reached a maximum 12.98-fold increase at day 2. This was consistent with our previous research in which *E. coioides* TLR2 was greatly up-regulated in the head kidney and spleen during infection with *C. irritans* [10]. *C. irritans* and *I. multifiliis* are responsible for "white spot disease" of marine and freshwater fish species, respectively. The two parasites may elicit similar immune mechanisms, and they both contain GPI-anchored proteins [20,21]. GPI-anchored proteins isolated from some parasites were recognized by mammalian TLR2 [3–6]. Although most TLRs appear to function as homodimers, TLR2 forms heterodimers with TLR1 or TLR6 for the recognition of various PAMPs. The TLR2–TLR1 dimer and the TLR2–TLR6 dimer were shown to differentially recognize the *P. falciparum* GPIs and *sn-2 lyso* derivatives, respectively [6,22]. This may partly explain the reason for the expression similarity between TLR1 and TLR2. To date, the detailed function and structure of *I. multifiliis* GPIs and their derivatives remain unknown, but they may serve as a kind of PAMP that can be recognized by fish TLRs.

Recent studies demonstrated that chicken TLR21 had similar functions to mammalian TLR9 in the recognition of CpG-ODN [23,24]. TLR9 is absent in the chicken and TLR21 is absent in the mammals [25], but both TLR9 and TLR21 have been identified in fish. It is not known if fish TLR9 and TLR21 function in a manner similar to that of mammalian TLR9. In the present study, the expression of catfish TLR9 was rapidly up-regulated in the skin at 6 h (2.17-fold), followed by significant down-regulation at 24 h (0.60-fold). A progressive increase then was observed, reaching a peak (4.56-fold) at day 3, followed by up-regulation at the last two time points. In contrast, TLR21 expression in the skin showed no significant changes at any time point except at 12 h (0.64-fold decrease) and day 7 (1.91-fold increase). In the gill, TLR9 expression was significantly up-regulated from 6 h to day 2 (except at 12 h) after infection, and it fluctuated slightly at the later time points. TLR21 was significantly down-regulated at day 2. No significant changes in TLR9 expression were observed in the head kidney and spleen at most time points, whereas significant up-regulation of TLR21 expression was observed at some time points. This finding was not entirely consistent with our previous results, in that *E. coioides* TLR9 and TLR21 were up-regulated in the skin and gill and down-regulated in the head kidney and spleen after *C. irritans* infection [11]. This difference may be due to the inherent species disparity in the structural features of TLRs. Recent studies reported that mammalian TLR9 plays an important role during infection with some parasites. TLR9-deficient mice were shown to be more susceptible than wild-type mice to infection with *T. cruzi* [7], *T. gondii* [26], and *L. major* [27]. Furthermore, DNA from *L. major* and *Trypanosoma brucei* could stimulate cytokine responses from macrophages and dendritic cells, possibly through unmethylated CpG motifs detected by TLR9 [27,28]. However, which components of *I. multifiliis* can be recognized by TLR9 and TLR21 remains unknown.

TLR19 and TLR25 are unique to fish. To date, only zebrafish and catfish TLR19 gene sequences (GenBank Accession Nos: XM_002664846 and HQ677722) have been reported, and the function of TLR19 is unknown. Therefore, the role of TLR19 in host immunity against *I. multifiliis* infection requires further investigation. Herein, significant up-regulation of TLR19 was observed in the skin and gill at some time points after infection, with a peak occurring at day 2 (2.40-fold) and 36 h (3.06-fold), respectively. In contrast, the expression levels of TLR19 were significantly down-regulated in the head kidney and spleen at some time points, although a significant up-regulation was detected in the spleen at day 3 (1.79-fold). The expression levels of the TLR25 were up-regulated significantly in the head kidney and spleen at some time points, with expression peaks at day 5 (3.00-fold) and 12 h (3.51-fold), respectively. However, in the skin and gill, TLR25 expression was not significantly affected by *I. multifiliis* infection, with the exception of a significant change in the skin at day 3 (0.67-fold decrease) and in the gill at 36 h (1.86-fold increase). TLR25 has only been identified in catfish to date, and its function remains completely unknown [12,13].

It is not surprising that the expression levels of TLR3, TLR7, and TLR8 were not significantly changed at most time points after *I. multifiliis* infection. In mammals, these TLRs are anti-viral receptors, TLR3 recognizes double-stranded RNA and TLR7 and TLR8 recognize single-stranded RNA [29,30]. Furthermore, recent studies reported fish TLR3, TLR7, and TLR8 expressions could be regulated by virus infection or viral agonists [31,32].

In mammals, glycoinositolphospholipids (GIPLs) from *T. cruzi* could induce an inflammatory response in mice in a TLR4-dependent manner. In addition, TLR4-deficient mice were more susceptible to *T. cruzi* and *T. gondii* infection than wild-type mice [33,34]. In our study, no significant changes in TLR4 expression

Table 2
The expression profiles analysis of a complete set of TLRs in catfish and at different time points after infection with *I. multifiliis*. Significant difference ($P < 0.05$) between the infected group and the control group was indicated in bold with * (significant increase) or § (significant decrease).

TLRs	Tissues	Fold changes of the infected group relative to the control							
		6 h	12 h	24 h	36 h	2 d	3 d	5 d	7 d
TLR1	Skin	**3.11 ± 0.56***	**2.03 ± 0.11***	1.02 ± 0.06	**4.16 ± 0.28***	**2.02 ± 0.19***	1.43 ± 0.27	**2.48 ± 0.43***	1.65 ± 0.39
	Gill	1.14 ± 0.21	1.01 ± 0.13	0.76 ± 0.15	0.65 ± 0.13	1.38 ± 0.21	1.24 ± 0.28	1.66 ± 0.37	0.81 ± 0.24
	Head kidney	1.64 ± 0.35	**3.11 ± 0.26***	**2.77 ± 0.42***	**2.78 ± 0.29***	0.88 ± 0.13	**4.36 ± 0.68***	1.49 ± 0.20	0.79 ± 0.14
	Spleen	0.78 ± 0.13	**1.84 ± 0.19***	1.52 ± 0.11	1.25 ± 0.17	0.86 ± 0.24	0.92 ± 0.27	0.74 ± 0.09	0.87 ± 0.19
TLR2	Skin	**2.35 ± 0.19***	0.86 ± 0.16	0.87 ± 0.06	**3.53 ± 0.18***	**4.85 ± 0.31***	**2.31 ± 0.06***	**2.54 ± 0.21***	0.75 ± 0.17
	Gill	0.78 ± 0.09	1.42 ± 0.23	**3.29 ± 0.17***	1.48 ± 0.34	0.71 ± 0.15	0.83 ± 0.12	1.11 ± 0.08	**2.25 ± 0.36***
	Head kidney	**9.32 ± 1.54***	**6.87 ± 0.87***	**8.21 ± 1.31***	**5.41 ± 0.23***	**12.98 ±0.62***	**8.53 ± 0.36***	**6.53 ± 0.24***	1.71 ± 0.35
	Spleen	**3.41 ± 0.21***	**3.78 ± 0.12***	1.39 ± 0.17	0.82 ± 0.15	1.64 ± 0.24	**5.43 ± 0.38***	1.36 ± 0.07	1.28 ± 0.25
TLR3	Skin	0.84 ± 0.14	1.36 ± 0.25	1.21 ± 0.31	1.33 ± 0.07	0.64 ± 0.08	1.42 ± 0.23	0.82 ± 0.20	0.92 ± 0.17
	Gill	0.71 ± 0.03	**0.34 ± 0.01§**	1.53 ± 0.25	1.68 ± 0.32	0.62 ± 0.02	**2.35 ± 0.13***	0.97 ± 0.26	0.85 ± 0.14
	Head kidney	**2.47 ± 0.27***	0.68 ± 0.03	**0.55 ± 0.04§**	0.60 ± 0.02	1.32 ± 0.24	0.93 ± 0.31	1.34 ± 0.12	0.88 ± 0.03
	Spleen	1.13 ± 0.26	0.85 ± 0.02	1.24 ± 0.09	1.29 ± 0.04	1.04 ± 0.15	**0.63 ± 0.03§**	0.67 ± 0.13	**2.00 ± 0.13***
TLR4	Skin	1.54 ± 0.30	0.75 ± 0.15	1.02 ± 0.11	1.45 ± 0.17	0.84 ± 0.12	**0.55 ± 0.09§**	1.27 ± 0.16	1.11 ± 0.14
	Gill	**1.76 ± 0.08***	0.89 ± 0.13	1.27 ± 0.09	1.58 ± 0.22	**0.48 ± 0.03§**	0.87 ± 0.01	1.41 ± 0.33	1.55 ± 0.42
	Head kidney	1.38 ± 0.16	1.51 ± 0.22	0.69 ± 0.13	1.36 ± 0.09	**2.87 ± 0.32***	1.01 ± 0.04	0.82 ± 0.07	1.43 ± 0.22
	Spleen	**0.61 ± 0.16§**	0.82 ± 0.09	0.89 ± 0.06	1.47 ± 0.21	1.61 ± 0.25	0.71 ± 0.13	1.31 ± 0.22	0.92 ± 0.17
TLR5	Skin	1.21 ± 0.08	1.12 ± 0.16	0.77 ± 0.15	0.85 ± 0.17	**1.48 ± 0.31***	0.86 ± 0.07	0.91 ± 0.13	1.31 ± 0.24
	Gill	0.83 ± 0.06	1.78 ± 0.12	1.41 ± 0.20	**2.16 ± 0.09***	1.33 ± 0.18	0.99 ± 0.11	1.43 ± 0.23	**1.91 ± 0.14***
	Head kidney	1.23 ± 0.08	1.09 ± 0.08	**0.43 ± 0.06§**	**0.52 ± 0.03§**	0.77 ± 0.03	1.11 ± 0.18	1.29 ± 0.15	1.42 ± 0.15
	Spleen	0.76 ± 0.12	1.42 ± 0.09	1.07 ± 0.17	0.85 ± 0.21	1.02 ± 0.12	0.71 ± 0.12	0.67 ± 0.08	**2.17 ± 0.09***
TLR7	Skin	1.66 ± 0.32	1.03 ± 0.27	1.35 ± 0.05	0.87 ± 0.05	1.62 ± 0.29	0.73 ± 0.08	0.82 ± 0.11	1.45 ± 0.17
	Gill	0.74 ± 0.04	0.77 ± 0.11	1.26 ± 0.09	**1.89 ± 0.16***	1.38 ± 0.07	1.54 ± 0.18	1.00 ± 0.12	0.86 ± 0.07
	Head kidney	0.63 ± 0.14	0.73 ± 0.05	1.21 ± 0.15	0.99 ± 0.07	0.70 ± 0.12	1.30 ± 0.08	0.81 ± 0.09	**0.62 ± 0.01§**
	Spleen	**1.76 ± 0.07***	1.20 ± 0.13	1.07 ± 0.16	0.85 ± 0.14	1.02 ± 0.06	0.71 ± 0.11	0.67 ± 0.12	1.47 ± 0.17
TLR8	Skin	1.17 ± 0.04	0.80 ± 0.14	0.84 ± 0.17	1.43 ± 0.11	1.56 ± 0.22	**2.14 ± 0.20***	0.77 ± 0.08	1.48 ± 0.02
	Gill	1.38 ± 0.02	1.41 ± 0.25	1.23 ± 0.07	0.66 ± 0.10	1.65 ± 0.12	**1.86 ± 0.36***	1.41 ± 0.20	0.75 ± 0.08
	Head kidney	1.21 ± 0.06	0.83 ± 0.22	0.62 ± 0.15	**1.85 ± 0.13***	0.84 ± 0.06	**0.66 ± 0.09§**	0.98 ± 0.09	0.76 ± 0.08
	Spleen	**0.58 ± 0.14§**	0.76 ± 0.17	1.55 ± 0.23	0.71 ± 0.12	**1.95 ± 0.07***	1.38 ± 0.26	1.04 ± 0.03	1.35 ± 0.18
TLR9	Skin	**2.17 ± 0.11***	0.89 ± 0.17	**0.60 ± 0.04§**	**2.20 ± 0.32***	**3.78 ± 0.24***	**4.56 ± 0.33***	**3.89 ± 0.37***	**2.13 ± 0.12***
	Gill	**1.88 ± 0.31***	1.56 ± 0.23	**2.34 ± 0.12***	**3.56 ± 0.26***	**5.68 ± 0.44***	0.87 ± 0.09	1.54 ± 0.13	1.33 ± 0.08
	Head kidney	1.36 ± 0.25	1.02 ± 0.21	1.25 ± 0.23	0.73 ± 0.14	0.82 ± 0.03	0.98 ± 0.22	1.58 ± 0.13	1.63 ± 0.36
	Spleen	1.41 ± 0.13	0.98 ± 0.12	1.46 ± 0.31	1.51 ± 0.38	0.74 ± 0.09	0.78 ± 0.08	**0.58 ± 0.01§**	1.29 ± 0.13
TLR18	Skin	1.45 ± 0.14	1.21 ± 0.18	0.82 ± 0.16	0.93 ± 0.11	1.33 ± 0.10	1.02 ± 0.04	1.51 ± 0.21	1.29 ± 0.17
	Gill	1.33 ± 0.21	1.56 ± 0.31	**0.44 ± 0.02§**	0.98 ± 0.13	1.67 ± 0.26	**2.56 ± 0.12***	0.68 ± 0.22	0.87 ± 0.14
	Head kidney	0.84 ± 0.15	0.68 ± 0.14	1.35 ± 0.33	**1.69 ± 0.05***	0.78 ± 0.16	1.02 ± 0.17	1.47 ± 0.25	0.71 ± 0.13
	Spleen	1.57 ± 0.24	**2.57 ± 0.12***	1.23 ± 0.28	1.37 ± 0.09	1.22 ± 0.09	**0.33 ± 0.02§**	0.68 ± 0.12	0.89 ± 0.20
TLR19	Skin	1.11 ± 0.20	**1.89 ± 0.21***	**2.34 ± 0.15***	0.78 ± 0.13	**2.40 ± 0.31***	0.88 ± 0.11	1.42 ± 0.19	1.23 ± 0.08
	Gill	0.81 ± 0.08	**1.94 ± 0.21***	0.88 ± 0.17	**3.06 ± 0.12***	1.43 ± 0.23	**2.29 ± 0.12***	0.90 ± 0.17	1.21 ± 0.10
	Head kidney	1.39 ± 0.05	**0.36 ± 0.02§**	**0.55 ± 0.01§**	0.93 ± 0.03	0.72 ± 0.14	1.37 ± 0.14	**0.24 ± 0.03§**	**0.41 ± 0.04§**
	Spleen	**0.26 ± 0.02§**	**0.51 ± 0.05§**	1.36 ± 0.11	1.13 ± 0.12	**0.67 ± 0.09§**	**1.79 ± 0.16***	0.94 ± 0.05	0.87 ± 0.23
TLR20	Skin	1.34 ± 0.22	**0.58 ± 0.03§**	0.89 ± 0.07	1.36 ± 0.19	**1.68 ± 0.08***	1.02 ± 0.01	0.81 ± 0.10	1.41 ± 0.17
	Gill	0.70 ± 0.14	1.33 ± 0.31	0.89 ± 0.05	0.68 ± 0.12	1.31 ± 0.18	1.05 ± 0.17	0.74 ± 0.12	1.43 ± 0.13
	Head kidney	1.20 ± 0.17	1.15 ± 0.13	0.78 ± 0.14	0.73 ± 0.17	**1.89 ± 0.15***	1.16 ± 0.14	0.86 ± 0.09	**0.63 ± 0.02§**
	Spleen	0.79 ± 0.05	1.09 ± 0.06	0.81 ± 0.04	0.67 ± 0.10	1.16 ± 0.12	**0.51 ± 0.03§**	1.43 ± 0.08	1.39 ± 0.05
TLR21	Skin	1.45 ± 0.23	**0.64 ± 0.05§**	0.76 ± 0.23	0.87 ± 0.34	1.36 ± 0.28	1.39 ± 0.11	1.30 ± 0.22	**1.91 ± 0.26***
	Gill	0.78 ± 0.08	1.36 ± 0.12	1.54 ± 0.12	0.89 ± 0.14	**0.45 ± 0.07§**	0.76 ± 0.07	1.07 ± 0.11	1.46 ± 0.13
	Head kidney	0.70 ± 0.09	**2.13 ± 0.33***	**1.89 ± 0.27***	**3.87 ± 0.48***	**4.23 ± 0.34***	1.05 ± 0.12	0.84 ± 0.02	1.03 ± 0.08
	Spleen	1.03 ± 0.21	**5.66 ± 0.82***	**2.06 ± 0.36***	**2.95 ± 0.14***	1.00 ± 0.15	**0.65 ± 0.13§**	1.28 ± 0.28	1.20 ± 0.14
TLR22	Skin	1.32 ± 0.13	1.45 ± 0.13	**1.76 ± 0.18***	1.56 ± 0.25	0.78 ± 0.11	1.37 ± 0.06	1.55 ± 0.27	0.93 ± 0.15
	Gill	**0.61 ± 0.09§**	1.42 ± 0.12	0.72 ± 0.14	**1.68 ± 0.08***	1.03 ± 0.07	1.44 ± 0.08	**0.55 ± 0.11§**	1.02 ± 0.27
	Head kidney	1.02 ± 0.05	1.40 ± 0.24	1.05 ± 0.11	1.09 ± 0.05	0.96 ± 0.30	**1.75 ± 0.08***	1.24 ± 0.04	1.67 ± 0.22
	Spleen	1.26 ± 0.22	1.16 ± 0.13	1.15 ± 0.08	0.77 ± 0.05	1.11 ± 0.23	1.44 ± 0.10	0.85 ± 0.14	0.74 ± 0.11
TLR25	Skin	1.43 ± 0.24	1.29 ± 0.24	0.83 ± 0.13	1.57 ± 0.20	1.08 ± 0.07	**0.67 ± 0.04§**	0.91 ± 0.06	0.96 ± 0.14
	Gill	0.70 ± 0.09	1.47 ± 0.18	0.95 ± 0.16	**1.86 ± 0.12***	1.29 ± 0.17	0.90 ± 0.15	0.84 ± 0.09	1.32 ± 0.21
	Head kidney	1.23 ± 0.11	**2.35 ± 0.09***	**2.82 ± 0.32***	1.11 ± 0.07	1.43 ± 0.21	0.83 ± 0.07	**3.00 ± 0.24***	**2.14 ± 0.13***
	Spleen	**2.12 ± 0.23 ***	**3.51 ± 0.45***	1.31 ± 0.17	**1.79 ± 0.16***	**0.56 ± 0.10§**	0.96 ± 0.20	1.28 ± 0.19	0.85 ± 0.15
TLR26	Skin	1.38 ± 0.20	0.73 ± 0.13	0.82 ± 0.09	1.40 ± 0.31	1.34 ± 0.18	0.87 ± 0.13	1.20 ± 0.26	1.32 ± 0.18
	Gill	1.08 ± 0.21	1.50 ± 0.24	1.28 ± 0.17	0.80 ± 0.16	0.91 ± 0.21	1.52 ± 0.19	0.76 ± 0.19	1.18 ± 0.20
	Head kidney	0.80 ± 0.10	**0.57 ± 0.06§**	0.92 ± 0.16	0.70 ± 0.15	0.88 ± 0.08	**1.78 ± 0.08***	1.22 ± 0.09	1.26 ± 0.08
	Spleen	0.90 ± 0.22	1.60 ± 0.33	1.09 ± 0.19	**1.75 ± 0.16***	0.90 ± 0.23	1.49 ± 0.23	1.10 ± 0.17	0.80 ± 0.14

were observed at most time points after *I. multifiliis* infection. Most fish species lack TLR4, and it has been identified only in catfish [12] and some cyprinidae species, such as zebrafish [35], grass carp [36], and rare minnow [37]. Two experiments in zebrafish showed that fish TLR4 could not recognize LPS, which is a typical ligand recognized by mammalian TLR4 [38,39].

Unlike mammalian TLR5, some teleost fish have two TLR5 isoforms: a membrane form (TLR5M) and a soluble form (TLR5S). TLR5M is orthologous to mammalian TLR5, but TLR5S lacks a transmembrane domain and a TIR domain and is not present in mammals [40]. No significant changes in TLR5 expression were observed at most time points after infection, which suggests that *I. multifiliis* infection does not necessarily induce TLR5 expression.

TLR18, TLR20, TLR22, and TLR26, which are fish-specific TLRs, have been identified in several fish species, but their functions are still largely unclear [9]. No significant expression level changes in

these TLRs were observed after *I. multifiliis* infection, except at a few time points. Like zebrafish TLR18, catfish TLR18 may correspond to TLR14 of other fish. TLR14 was classified as a TLR1 family member, thus TLR14 might also partner with TLR2 and substitute for the function of mammalian TLR6 or TLR10 [25]. Phylogenetic analysis indicated that catfish TLR20 was closely related to murine TLR11 and TLR12 [41]. Moreover, TLR11 was shown to recognize a profilin-like protein from *T. gondii* [8]. Some studies suggested that TLR22 recognizes double-stranded RNA and is mainly involved in antiviral protection [42,43]. Like TLR25, TLR26 has only been found in catfish, and its function remains completely unknown.

In summary, the present study showed the expression levels of catfish TLR1, TLR2, TLR9, TLR19, TLR21, and TLR25 were regulated by infection with *I. multifiliis*, indicating that these TLRs may play crucial roles in the immune response against *I. multifiliis* infection. Further studies in our laboratory will focus on the identification of molecules in *I. multifiliis* that can be recognized by these TLRs.

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