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Diurnal rhythm and pathogens induced expression of toll-like receptor 9 (TLR9) in *Pelteobagrus vachellii*

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ABSTRACT

Toll-like receptor 9 (TLR9) is activated by bacterial DNA and induces the production of inflammatory cytokines. In this study, the darkbarbel catfish *Pelteobagrus vachellii TLR9* cDNA was cloned and sequenced. The daily expression pattern of *TLR9* mRNA was investigated in various tissues. Furthermore, its expression was analyzed following exposure to the pathogen *Aeromonas hydrophila*. The 4249 bp cDNA includes a 3201 bp open reading frame (ORF) encoding 1067 amino acids. The predicted amino acid sequence comprises a leucine-rich domain (LRD), a toll/interleukin-1 receptor (TIR), and a transmembrane domain. *P. vachellii* TLR9 showed 42–87% amino acid sequence identity with TLR9 sequences of *Ictalurus punctatus*, *Rhincodon typus*, and *Miichthys miiuy*. The *P. vachellii TLR9* mRNA was highly expressed in intestines, head kidney, and spleen in an apparently healthy fish. Following pathogen challenge, *TLR9* expression increased significantly ($P < 0.05$) and peaked at 48 h post-exposure in the liver, at 24 in the head kidney, and at 12 h in the spleen. In addition, the pattern of *TLR9* expression over a 24-h period showed a circadian rhythm in the head kidney, spleen, and intestine, with the acrophase at 20:34, 18:45, and 3:50, respectively. This result provided the basis for further study of the rhythm of innate immunity against bacteria in catfish.

1. Introduction

Many studies in mammals demonstrated that the immune organs, such as the spleen and lymph node, as well as immune cells, including macrophages [1] and NK cells [2], display oscillation patterns in their internal clock genes [3]. Similarly, many rhythmic phenomena for immunity have been explored in fish. Ren et al. (2018) indicated that clock genes *period1b* and *period 2* exhibit rhythmic oscillations in zebrafish leukocytes, playing a vital role in immune processes [4]. Guerra-Santos et al., and Lazado et al. revealed that the humoral and non-specific immune system displayed a circadian rhythm under 12 h light/12 h dark (12L:12D) in the Nile tilapia (*Oreochromis niloticus*) [5,6], a clear phase-dependent difference was observed in protease, globulin, myeloperoxidase, alkaline phosphatase, and glutathione peroxidase (GPX) levels in the juvenile permit (*Trachinotus falcatus*) [7]. Moreover, bacterial endotoxin challenge suggested that the responsiveness of serum humoral factors to a biological insult is likely mediated by the time of day [5]. A circadian rhythm of innate immunity was identified in tilapia (*O. mossambicus*) and zebrafish (*Danio rerio*) [4,8]. However, the diurnal variation of innate immune factors in fish species remains unclear.

Toll-like receptor 9 (TLR9) belongs to the TLR family of pattern recognition receptors (PRRs) that sense invading pathogens. TLR9 recognizes unmethylated CpG DNA of bacterial genomic DNA and activates nuclear factor-kappa B (NF-κB) via an myeloid differentiation primary response 88 (MYD88) pathway in mammals [9], leading to the production of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-a), interleukin (IL)-1, and IL-6, as well as the regulatory cytokines IL-12 and IL-18 [10]. In addition, TLR9 plays a critical role in initiating adaptive immune responses in mammals. Fish *TLR9* genes have also been identified in Japanese flounder (*Paralichthys olivaceus*) [11], common carp (*Cyprinus carpio*) [12], puffer fish (*Takifugu rubripes*) [13], zebrafish (*D. rerio*) [14], and Pacific red snapper (*Lutjanus peru*) [15]. *TLR9* mRNA expression was significantly upregulated after infection with pathogens [16–19]. These results suggested that this molecule plays a key role in innate immunity. Moreover, TLR9 in mice exhibits an enhanced sensitivity to detect and respond to pathogens during the active phase because of increased expression of components of the innate immune system, in which a circadian molecular clock controls its expression and function [3]. In addition, mice immunized at

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Zeitgeber time (ZT) 19:00 for enhanced TLR9 responsiveness presented weeks later with an improved adaptive immune response [20]. Therefore, TLR9 in mice exhibited a daily rhythm of TLR9 expression, which was beneficial to the response to pathogen infection. However, there is a little knowledge concerning the daily rhythm of *TLR9* expression in fish species.

Pelteobagrus, including *Pelteobagrus fulvidraco*, *P. vachellii*, and *P. vachellii x P. fulvidraco*, has great aquaculture and scientific value. Growing demand has seen a considerable rise in its market value, with production reaching 500,000 tons in 2017. Yang et al. observed an apparent day and night feeding rhythm in *Pelteobagrus* larval and juvenile stages [21]. Qin et al. indicated that an alternative feeding time can improve growth and create metabolic alterations in the liver of *P. vachellii* [22]. Complement components, interferons, and Fcg receptor-mediated phagocytosis play key roles in the response to *Aeromonas hydrophila* infection in the spleen of *P. vachellii* [23]. Moreover, in our earlier study, the *circadian locomotor output cycle kaput* (*CLOCK*) gene expression in *P. vachellii* was observed to be under the control of circadian rhythms in the brain, liver, and intestine, with the acrophase at ZT 21:35, 23:00, and 23:23, respectively [24].

In aquaculture, feeding regimes of fish are usually followed by the workers time; however, this schedule may not be optimal for all farmed fish species. Nevertheless, it is believed that the internalization of the 24 h rhythms of light and dark make it advantageous for an organism to predict daily recurring events, even when conditions remain constant [25]. Therefore, it was necessary to investigate the circadian expression patterns of immune molecules to enhance animal welfare. This study aimed to clone the *TLR9* cDNA, and analyzes its expression.

2. Materials and methods

2.1. Animal and experiment design

2.1.1. Fish for daily expression of TLR9

A total of 120 adult darkbarbel catfish *P. vachellii* (15.26 ± 3.67 g) were reared in our laboratory. The animals were kept in six steel tanks (20 fish/tank; 300 L/tank) with an open water circulation system. The natural water temperature was 25 ± 2 °C. The natural photoperiod was set at 13:11 light/dark cycle, with lights-on at 08:00 h (ZT8). To avoid light pollution, each tank was covered at night (21:00–08:00). Before the experiment, the fish were fed a commercial diet (Neijiang Zhengda Inc., Sichuan, China) in the morning (08:00) each day. Ammonia and nitrate concentrations were measured every week, and were < 0.1 mg/L. After 30 days under these conditions, fish were sampled during the course of one day every 4 h (at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20) to study daily rhythms of gene expression. At each sampling time, fish were collected from different tanks to avoid stress. Sampling was done under the same lighting conditions that they had been held at until that moment, using a dim red light for sampling during darkness. Nine fish were taken at each time point from different tanks. The fish were anesthetized on ice and sacrificed. Intestines, spleen, and head kidney of the nine samples were taken at each ZT. In addition, tissues (heart, brain, gill, liver, and adipose tissue) of nine fish were taken at ZT0 for tissue expression analysis. All samples were immediately placed in liquid nitrogen, and then stored at −80 °C for future analysis.

2.1.2. Fish for bacterial challenge

All fish (weight 15.49 ± 2.18 g) were obtained from a local fish farm and acclimatized at 25 ± 2 °C for 30 days in a flow-through system. The dissolved oxygen level was maintained approximately at saturation by continuous aeration. During the acclimation period, fish were fed a commercial diet twice a day. Only healthy animals were used in the experiments.

The experiments were performed as described previously [23]. *A. hydrophila* was isolated from infected *P. vachellii*, confirmed by 16s rDNA sequencing, and cultured as previously described [26]. In the bacterial challenge experiments, 240 *P. vachellii* individuals were randomly assigned to two groups in triplicate (40 fish per 300 L tank). In the challenge group, each fish received an injection of 100 μL of live *A. hydrophila* in sterilized saline water (0.85 ± 10^9 colony forming units (CFU)/mL). The other 120 animals received the same amount of sterilized saline water and constituted the bacterial challenge control group. For each treatment, three animals were sampled randomly from each tank at 0, 6, 12, 24, 48, and 96 h. Animals were anesthetized with 100 mg/L tricaine mesylate (MS-222), dissected, and the liver, spleen, and head kidney tissues were collected. Tissue samples were dissected on ice, washed thoroughly with chilled saline water, dried quickly on filter paper, and immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

2.2. RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was isolated from the liver and further purified using an RNA extraction buffer (Takara Bio Inc, Shiga, Japan) according to the manufacturer's protocol. First-strand cDNA synthesis was carried out using Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase (Clontech, Mountain View, CA, USA) with an adapter primer, using reaction conditions detailed in the manufacturer's instructions. The cDNA fragment encoding the *TLR9* gene from the transcriptome of *P. vachellii* (BioProject ID: PRJNA383309, TR18997) was used to design gene-specific primers, and the full-length *TLR9* cDNA was obtained using reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) methods using a SMART RACE cDNA Amplification Kit (Clontech). Protocols for 5′-RACE and 3′-RACE were carried out as described previously with a TLR9-3′ gene-specific primer (5′-CTACAACCCGTTCCGTATCACA-3′) and a TLR9-5′ gene-specific primer (5′-GAGATTATGCGTTCAGCCAGAG-3′) [23].

2.3. Cloning, sequencing, and analyses

The PCR fragments were subjected agarose gel (1.5%) electrophoresis. The amplified cDNA fragments were cloned into the pGEM-T Easy vector following the manufacturer's instructions (Promega Corporation, Madison, WI, USA) and then transformed into *Escherichia coli*. Recombinant bacteria were identified by blue/white screening.

Plasmids containing the insert were purified using a mini-prep kit (Promega) and used as a template for DNA sequencing. The deduced amino acid sequence of the partial cDNA was analyzed with using ORF (Open Reading Frame) finder at http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi. Protein sequence similarity searches were conducted using the Clustal W Multiple Alignment program (http://www.ebi.ac.uk/clustalw/, http://www.ch.embnet.org/software/BOX_form.html). Domain identification was performed using the Motif scan program (http://smart.embl-heidelberg.de/). In addition, the leucine-rich repeat (LRR) domains were edited manually according to methods described by Matsushima et al. (2007) [27]. The LRR repeats should be coincident with “LxxLxLxxNxL” or “LxxLxLxxCxxL” (where “L” is Leu, Ile, Val, or Phe; “N” is Asn, Thr, Ser, or Cys; “C” was Cys, Ser or Asn; and “x” represents any amino acid).

The full-length amino acid sequence of TLR9 in *P. vachellii* was compared with other previously known TLR9 proteins (retrieved from NCBI GenBank) using Clustal W Multiple Alignment program. A neighbor-joining (NJ) phylogenetic tree was constructed using the MEGA software version 5.0, and bootstrapping values were obtained from 1000 replicates.

2.4. The analysis of TLR9 expression

Total RNA was extracted as described in section 2.2. The first-strand cDNA was synthesized using a cDNA first-strand synthesis kit with MMLV reverse transcriptase (Takara) with approximately 5 μg of total

RNA, and diluted 10 times. Two gene-specific primers, QTLR9-s (5′-TTGTACCAATGCCAAGACCTCC-3′) and QTLR9-α (5′-CAGGAAAACCACAGCGAACATC-3′), were designed to amplify a 218 bp product from the *TLR9* gene.

The cDNA of all samples was used as a substrate for quantitative real-time PCR (qPCR). Samples were analyzed on a LightCycler® Nano Real-Time PCR System (Roche, Indianapolis, IN, USA) with FastStart Essential DNA Green Master (Roche); the PCR temperature profile and reaction conditions were set according to the manufacturer's instructions. The primers β-actin F and β-actin R were used to amplify a 200 bp fragment of the reference gene [28]. The *TLR9* gene expression level was calculated according to the $2^{-\Delta\Delta CT}$ method [29].

2.5. Statistical analysis

All data are expressed as means ± S.D, and were analyzed using the SPSS statistical software package version 18.0 (SPSS, Inc., Chicago, IL, USA) with values corresponding to the relevant controls. $P < 0.05$ was considered statistically significant. Moreover, liver tissue was used as a control for *TLR9* gene expression analyses. Samples obtained at ZT 00:00 were used as controls for analysis of the daily variation in *TLR9* gene in different tissues. Differences in relative *TLR9* gene expression for each tissue (head kidney, spleen, and intestine) over 24 h were analyzed using one-way ANOVA (ANOVA I) with SPSS 18.0, software, followed by Tukey's Test. Moreover, the cosine function $[Y = M + A \times \cos(\Omega t + \Phi)]$ of Microsoft Office Excel (2007) was used to analyze the rhythmic expression of the *TLR9* gene in the three tissues, where M is the mesor, A is the amplitude, Ω is the angular frequency ($2\pi/24$ for the circadian rhythms), and Φ is the acrophase [30]. The significance level was fixed at $P < 0.05$ for all the statistical analyses.

3. Results

3.1. Cloning TLR9 cDNA

The darkbarbel catfish *TLR9* cDNA was obtained using RT-PCR, which yielded a 4249 bp cDNA that included a 218 bp 5′-UTR, a 3201 bp ORF encoding a 1067 amino acid protein, and a 830 bp 3′-UTR. The nucleotide and deduced amino acid sequences of darkbarbel catfish *TLR9* have been submitted to GenBank (Accession No.MK089786). The domain architecture of TLR9 is similar to that of other fish species, and consists of 22 leucine-rich repeat (LRR) domains, a leucine-rich repeat C-terminal (LRR-CT) domain, a 150-amino acid Toll-interleukin-1 receptor (TIR) domain, and a transmembrane (TM) domain. Three highly conserved motifs were identified within the TIR domain, which are shown as box 1, F/Y(DA); box 2, RDXXPG; and box 3 (FW). The two C**C motifs and conserved amino acid residues (Asp553 and Tyr555) were also identified, which are important for interactions with CpG-DNA. The results of a sequence homology search using BLASTP revealed 80% identity to the *Ictalurus punctatus* TLR9 (AEI59673.1), 64% identity to *C. carassius* (AGO57936.1), 53% identity to *Larimichthys crocea* (KKF28592.1), and 42% identity to *Lissotriton helveticus* (AIZ71806.1) (Fig. 1).

3.2. Phylogenetic studies of TLR9

The NCBI blast results indicated that TLR9 of *P. vachellii* had 87% amino acid identity with TLR9 of *Ictalurus punctatus* and 48% with TLR9 of *Boleophthalmus pectinirostris*. Therefore, TLR9 of *P. vachellii* was subclustered with *Ictalurus punctatus*, and clearly separated from TLR9 of *B. pectinirostris* and mammals (*Ovis aries, Rattus norvegicus, Felis catus*). Moreover, the clusters of TLR9 were clearly separated from the TLR7, TLR8, and TLR5 protiens in fish species (Fig. 2).

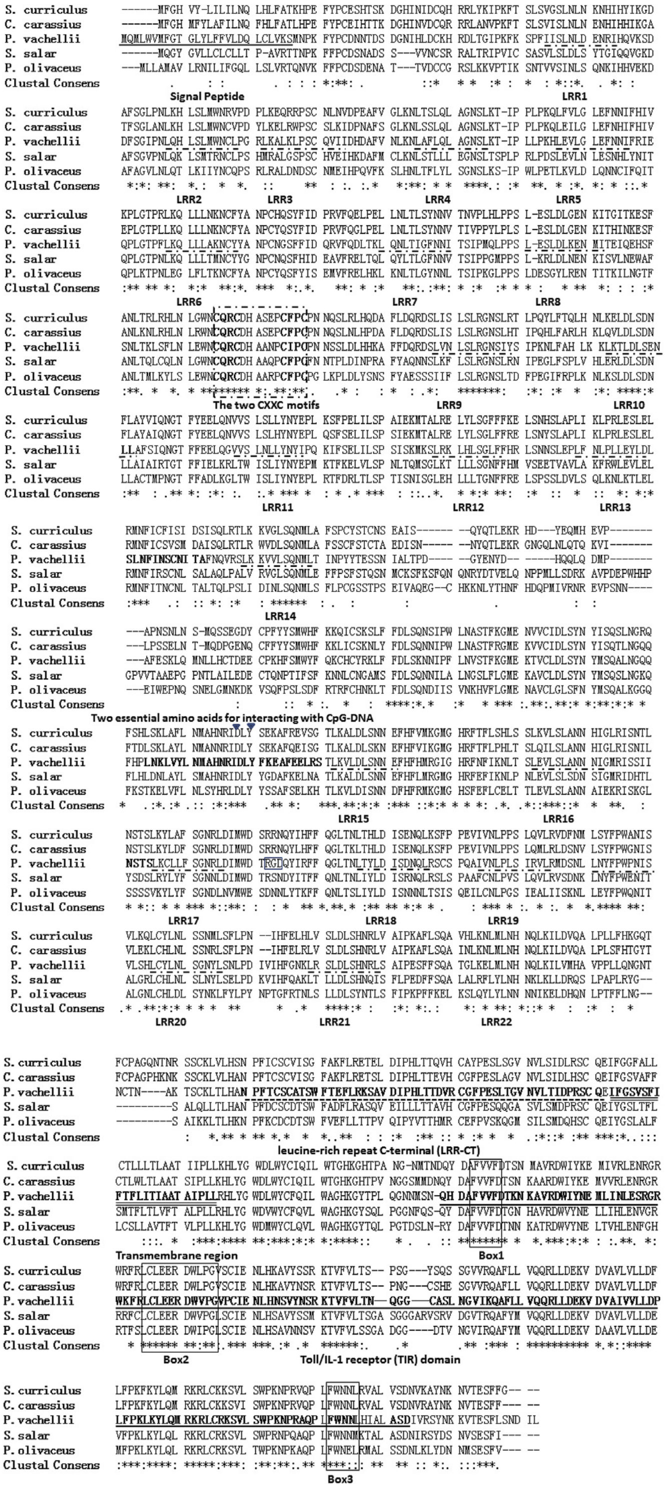

Fig. 1. Multiple sequence alignment of *P. vachellii* TLR9 with homologous sequences from other fish species. The amino sequence *P. vachellii* was aligned with that of TLR9s from *Squaliobarbus curriculus*, APB09200.1; *Carassius carassius*, AGO57936.1; *Salmo salar*, NP_001117125.1; *Paralichthys olivaceus*, BAE80690.1; using the Clustal W program.

3.3. Expression of TLR9 in different tissues

The TLR9 mRNA distribution profile in tissues was determined using qPCR (Fig. 3). The results revealed constitutive expression of *TLR9* in all tissues examined, with the most abundant expression in the spleen followed by the head kidney, and lowest in the brain and heart,

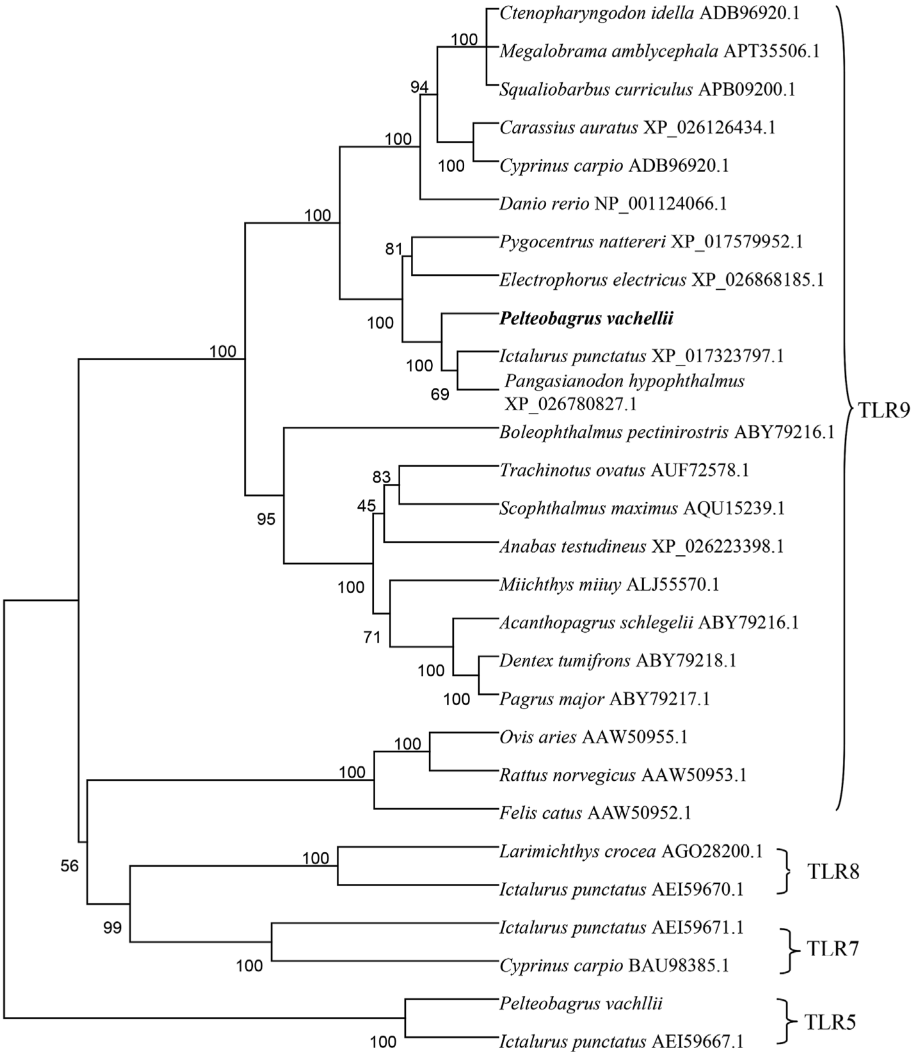

Fig. 2. Phylogenetic analysis of TLR9 of *P. vachellii* with other TLR9s. The tree was constructed using Clustal W and MEGA (v5.0).

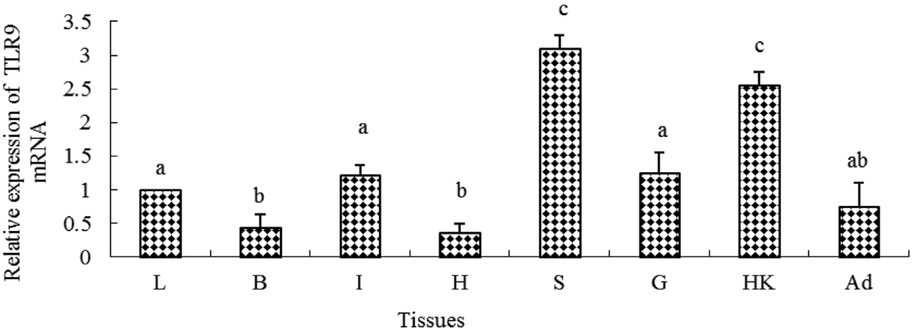

Fig. 3. Relative expression of *TLR9* in different tissues of *Pelteobagrus vachellii* analyzed by qRT-PCR. Expression levels in all tissues are presented relative to those in the liver (assigned a value of 1). A difference of $p < 0.05$ (*vs.* the expression level in liver) was considered significant and is indicated by an asterisk. L, liver; B, brain; I, intestine; H, heart; S, spleen, G, gill; HK, head kidney; Ad, adipose tissue.

respectively ($p < 0.05$; Fig. 3).

3.4. Expression of TLR9 in response to A. hydrophila challenge

Following *A. hydrophila* injection, the expression of *TLR9* was up-regulated, and transcription was highest in the liver after 48 h, with an increase in expression clearly visible from 6 to 48 h post-injection ($p < 0.05$) (Fig. 4a). In the head kidney, expression of *TLR9* was obviously increased between 6 and 96 h ($p < 0.05$), and mRNA levels peaked at 24 h (Fig. 4b). In the spleen, the *TLR9* transcript peaked at 12 h, respectively ($P < 0.05$) (Fig. 4c).

3.5. The daily expression of TLR 9

The level of *TLR9* expression varied throughout the day in the head kidney, spleen, and intestine (Fig. 5a–c). This rhythm could be fitted to a cosinor curve, having an acrophase at approximately at 20:34 in the head kidney, 18:45 in the spleen, and 3:50 in the intestine. In the head kidney, the expression of *TLR9* mRNA at ZT 20:00 was significantly higher than the other times. In the spleen, the expression of *TLR9* mRNA from ZT 16:00 to ZT 20:00 was significantly higher than from 0:00 to 16:00 ($P < 0.05$; Fig. 5). The cosinor parameters (mesor, amplitude, and acrophase) of *TLR9* expression rhythms in *P. vachellii* are shown in Table 1.

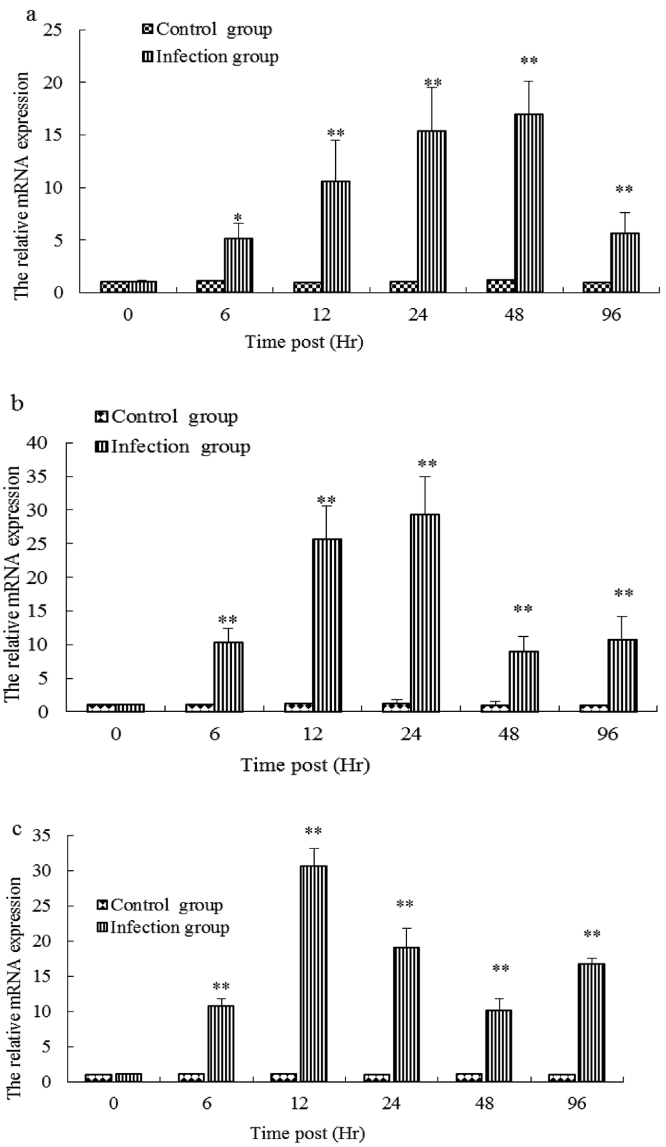

Fig. 4. Expression profiles of *TLR9* in the liver (a), head kidney (b), and spleen (c) at different time points after pathogen challenge. All data are expressed as mean ± standard deviation (n = 9). Significant differences in expression between infected and control groups at the same time point are indicated by an asterisk ($p < 0.05$).

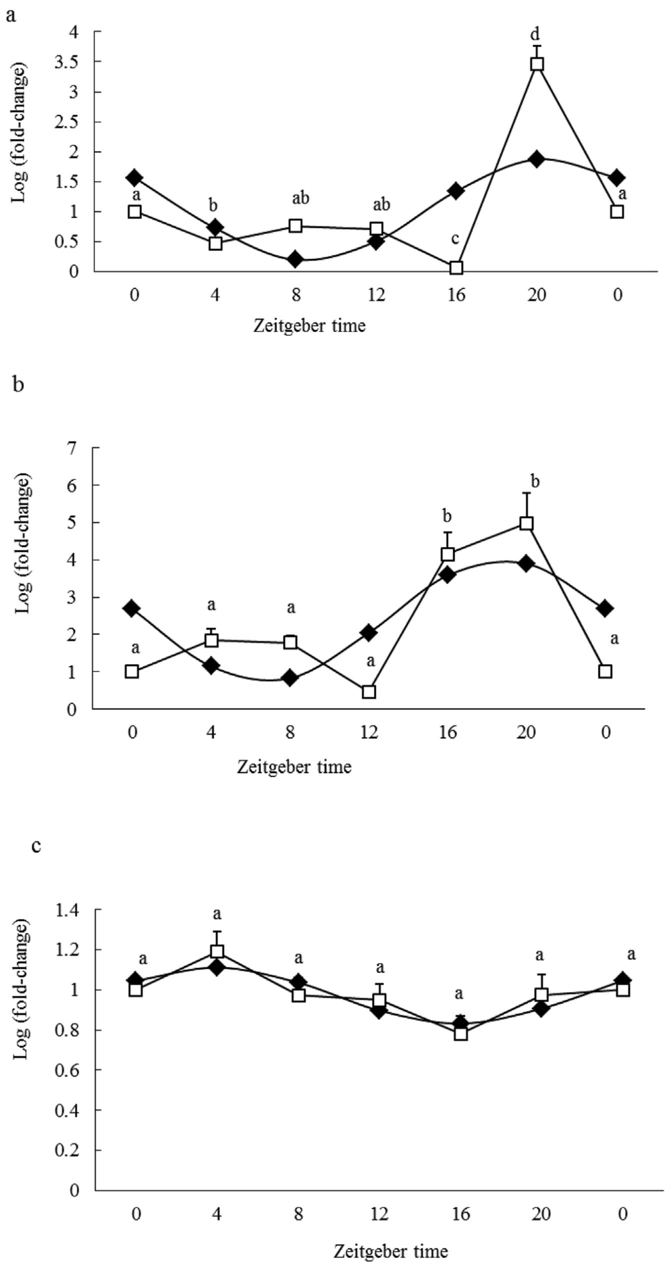

Fig. 5. Relative expression of *TLR9* mRNA in the head kidney (a), spleen (b), and intestine (c) of *P. vachellii*. Values represent the mean ± S.E. (n = 9). The zeitgeber time (ZT, in hours) is represented on the horizontal axis and the relative expression as fold-change (log10) is plotted on the vertical axis. Different letters indicate significant differences among treatments. The line with black prism indicates the circadian rhythms fitted using the cosinor method.

Table 1
Cosinor analysis of *TLR9* mRNA expression rhythms in various *P. vachellii* tissues.

Cosinor parameters	*TLR9*		
	Intestine	Spleen	Head kidney
Acrophase (h)	3:50	18:45	20:34
Mesor	0.97	2.36	1.03
Amplitude	0.13	1.61	0.84
Acrophase	1.11	3.98	1.88

4. Discussion

TLRs are type I transmembrane proteins whose extracellular domain contains leucine-rich repeat (LRR) motifs [10]. TLR9 was characterized as the receptor that recognized unmethylated CpG dinucleotides in DNA [31]. In the present study, *TLR9* from darkbarbel catfish was cloned and sequenced. Similar to TLR9 proteins from common carp and Japanese flounder [11,12], structural analysis revealed the presence of typical TLR9 domains organized in the expected manner. The putative amino acid sequence of TLR9 in *P. vachellii* was structurally characterized by three conserved domains: LRR domains, the transmembrane domain, and TIR domain. The TLR9 contains 22 LRRs, which is the functional extracellular domain (Fig. 1). The number of leucine rich repeats (LRRs) in Japanese flounder, puffer fish, common carp, and zebrafish TLR9 are 12, 14, 14, and 14, respectively [11–14]. Peter et al. stated that a mutation in the N-terminal part of TLR9 abolishes TLR9 activation, although TLR9 processing was unaffected [32]. This observation strongly implied that the N-terminal LRRs are required for receptor activity. Addition, a TIR domain with three conserved boxes, which is functionally important for TLR signaling and receptor localization, was identified in the cytoplasmic domain of *P. vachelli* TLR9, and was also identified in Japanese flounder, common carp, and zebrafish. Jault et al. reasoned that the three boxes in the TIR domain are important for TLR9 function in zebrafish [14]. These conserved sequences potentially contribute to signal transduction through the TIR domain, either by maintaining the appropriate structure of the protein, or more directly, by participating in the formation of complexes with downstream signaling proteins [33].

TLR9 was characterized as a receptor that recognized unmethylated CpG dinucleotides in DNA, and activates the NF-κB via a MYD88-dependent pathway [10]. Therefore, the C**C motifs are important for CpG binding [34], and a conserved motif of Asp and Tyr amino acids within the LRR domain is important for interacting with CpG-DNA [35]. In the TLR9 of *P. vachelli*, two C**C motifs were identified, which were separated by six amino acid residues. The amino acid residues (Asp553 and Tyr555) are also conserved in the TLR9 of *P. vachellii*, which is present in TLR9 of zebrafish (Asp546 and Tyr548), Japanese flounder (Asp562 and Tyr564), puffer fish (Asp544 and Tyr546), and common carp (Asp552 and Tyr554) [11,12,36].

The toll-like receptor family plays a role in innate immune responses against microbial pathogens, as well as the subsequent induction of adaptive immune responses [37]. Takano et al. indicated that few *TLR9*-expressing cells were found in gill, kidney, and spleen in healthy Japanese flounder, but many were found in these organs after *Edwardsiella tarda* challenge, and were coincident with lesions that had been colonized by the bacteria [12]. Pathogen challenge with *A. veronii* upregulated the expression of *TLR9* of Pacific red snapper (*L. peru*) at 24 or 48 h of exposure in the head kidney, skin, and intestine, but not in the liver [15]. Moreover, Cobia (*Rachycentron canadum*) challenged with *Photobacterium damselae* sub sp. *piscicida* showed a significant increase in *TLR9* expression at 24 h post challenge in the intestine, spleen, and liver, while in the kidney, the expression peaked at 12 h and later decreased at 24 h [16]. Similarly, in the present study, the gram-negative bacterium *A. hydrophila* effectively induced the expression of *TLR9* in the liver, spleen, and kidney of darkbarbel catfish. A similar expression pattern was found in the golden pompano (*T. ovatus*), large yellow croaker (*Pseudosciaena crocea*), and half-smooth tongue sole (*Cynoglossus semilaevis*) [17–19]. These studies suggested that upregulation of *TLR9* might play an important role in the immune defense against bacterial invasion.

Circadian rhythms, which refer to biological processes that oscillate with a period of ~24 h, are emerging as important regulators of specific immune functions [3]. Silver et al. (2012) reported that splenic *TLR* 9 mRNA expression showed a daily rhythm that peaked at ZT19, coinciding with the mouse active phase (lights off at ZT12), which indicated that the circadian molecular clock in mice controls the expression and function of *TLR9* [1]. Similarly, Ren et al. (2018) found that the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8 in zebrafish exhibited significant diurnal variation [4]. In the present study, the level of *TLR9* expression varied throughout the day in the head kidney, spleen, and intestine, which had acrophases at approximately at 20:34, 18:45, and 3:50, respectively. The possible reason was that *TLR9* is under the direct circadian control of BMAL1–CLOCK transcription factors [1]. *CLOCK* gene expression in the brain and intestine of *P. vachellii* showed an acrophase at Zeitgeber time 21:35, and 23:23, respectively [24], which was close to the acrophase of *TLR9* in head kidney and intestine. Similarly, Lazado et al. (2016) showed that innate immune defenses, including serum alkaline phosphatase, lysozyme, peroxidase, and protease, exhibited significant daily rhythms under a 12L:12D cycle [5]. Circadian oscillations of immune mediators coincide with the activity of the immune system, possibly allowing the host to anticipate and handle microbial threats more efficiently.

In summary, the full-length cDNA sequence of *TLR9* was isolated from *P. vachellii*, and *TLR9* in liver, spleen, and head kidney could response to infection by *A. hydrophila*. Moreover, the expression of *P. vachellii TLR9* showed circadian variation in neural and peripheral tissues, with a peak of expression at night.

Acknowledgments

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