International Immunopharmacology 70 (2019) 362–371

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Circadian oscillation of *TNF-α* gene expression regulated by clock gene, BMAL1 and CLOCK1, in the Japanese medaka (*Oryzias latipes*)

Teika Onoue[a], Goshi Nishi[b], Jun-ichi Hikima[b], Masahiro Sakai[b], Tomoya Kono[b,*]

[a] *Interdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki, 1-1 Gakuen kibanadai-nishi, Miyazaki 889-2192, Japan*
[b] *Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen kibanadai-nishi, Miyazaki 889-2192, Japan*

ARTICLE INFO

Keywords:
Medaka
TNF-α
Circadian oscillation
Transcriptional regulation by clock gene
TNF-α gene response upon LPS stimulation at different time points

ABSTRACT

To date, little information is available on the effects of circadian oscillation on immune regulation in lower vertebrates, such as teleost fish. In the present study, regulation of circadian oscillation of inflammatory cytokine *TNF-α* gene expression by clock gene was investigated using model fish medaka (*Oryzias latipes*). Firstly, structural analysis of clock genes was performed, which revealed that medaka BMAL1 and CLOCK1 conserve functionally important domains, such as basic helix-loop-helix (bHLH) and period-aryl hydrocarbon receptor nuclear translocator-single-minded (PAS), seen in their counterparts in other vertebrates. Expression of medaka *Bmal1*, *Clock1*, and *Per1* genes was confirmed in central and peripheral tissues. Moreover, the expression of these clock genes and *TNF-α* genes in medaka acclimated to a 12:12 light (L) - dark (D) cycle showed circadian oscillation. In addition, higher expression of *TNF-α* gene was detected in medaka embryo cells (OLHdrR-e3) overexpressing *Bmal1* and *Clock1* genes. It was suggested that this increase was mediated by transcriptional regulation by clock proteins, which target E-box sequence in the cis-element of *TNF-α* gene as was detected by luciferase reporter gene assay. Moreover, in vitro head kidney stimulation with LPS at different zeitgeber time (ZT) under LD12:12 condition affected the degree of *TNF-α* gene expression, which shows high and low responsiveness to LPS stimulation at ZT18 and ZT10, respectively. These results suggested that fish TNF-α exhibited circadian oscillation regulated by clock proteins and its responsiveness against immune-stimulation depends on time zone.

1. Introduction

Circadian rhythm is an important biological process that represents an endogenous oscillation of approximately 24 h/day. The rhythm is driven by a circadian clock, and it is known to exist in plants, animals, fungi, and cyanobacteria [1]. Circadian clock is an internal timing mechanism that enables organisms (animals) to anticipate predictable daily events, such as food intake and sleep opportunity [2]. The master circadian pacemaker in mammals exists in the suprachiasmatic nucleus (SCN), located in a part of the brain called the hypothalamus. SCN receives light input detected by photoreceptor system of retina and helps the entrainment (synchronization) of the master circadian clock and peripheral clock in tissues to external environment [3]. At a molecular level, circadian rhythm (24 h oscillation) is based upon a molecular clock generated via an intracellular transcription-translation negative-feedback loop involving several key clock proteins [4]. This loop is regulated by core clock proteins: circadian locomotor output cycles protein kaput (CLOCK), brain and muscle arnt-like protein1 (BMAL1), period (PER), and cryptochrome (CRY). BMAL:CLOCK heterodimer activates the transcription of negative regulators, PER and CRY, and PER:CRY heterodimer inhibits BMAL:CLOCK-mediated transcription. In addition, it is known that other clock proteins such as, REV-ERB (reverse erythroblastosis virus), ROR (RAR-related orphan receptor), and NFIL3 (nuclear factor, interleukin 3 regulated, also known as E4BP4) are associated with the above mentioned feedback loop. They regulate the transcription of core clock genes by binding to the transcription regulatory elements, such as E-box or RORE, in their promoter regions [5].

Recently, it has been reported that immune functions, such as translocation of leukocytes and activation of innate and adaptive immunity are strongly influenced by circadian clock in mammals [6,7]. These activities are based on time of day variation for encountering pathogens, infection, and tissue damage to the host. Immune cells, such as macrophages, NK cells, T-lymphocytes, mast cells, and eosinophils, have autonomous clocks and exhibit daily rhythms in their activity [8]. Especially in the myeloid lineage, inflammatory actions regulated by

* Corresponding author.
E-mail address: tkono@cc.miyazaki-u.ac.jp (T. Kono).

https://doi.org/10.1016/j.intimp.2019.02.004
Received 8 December 2018; Received in revised form 2 February 2019; Accepted 4 February 2019
Available online 07 March 2019

the clock proteins have been well studied to date, and it has been known that circadian oscillators rhythmically modulate basal inflammatory responses by regulating expression of inflammation-related cytokines/chemokines, such as TNF-α, IL-6, CCL2, and CXCL5. [9]. Moreover, it has been reported that differentiation of IL-17-producing T helper (Th17) cells, known as pro-inflammatory immune cells, is regulated by the circadian clocks, which suppress the role of RAR-related orphan receptor gamma (RORγt) as master regulator of Th17 cell differentiation [10]. Thus, the circadian clocks play a critical role as important gatekeepers in both innate and adaptive immune system.

In fish, it is known that various physiological phenomena exhibit periodicity, which is regulated by circadian clock. Especially, the periodic endocrine regulation of reproduction [11] and ontogeny [12,13] has been well studied to date. In addition, marine teleost (*Sparus aurata*) has a food-entrainable oscillator regulated by circadian clocks [14]. Similar to that in mammals, their rhythmic events are influenced by external factors, such as daily periodicities of light, temperature, and food intake. Therefore, these entraining agents have been investigated in most studies. Regarding rhythmic immune response, the effect of seasonality on various innate immune parameters, such as lysozyme activity and respiratory burst of macrophages, has been reported in rainbow trout, *Oncorhynchus mykiss* [15]. Recently, it has been reported that the expression of humoral and mucosal immune molecules rhythmically oscillate during a light-dark (LD) cycle in permit, *Trachinotus falcatus* [16]. In Nile tilapia (*Oreochromis niloticus*), humoral immune responses mediated by enzymes, such as alkaline phosphatase, lysozyme, peroxidase, and protease, exhibit circadian rhythmicity. Moreover, sensitivity of these humoral factors to bacterial endotoxin is different in the light phase and dark phase under LD12:12 condition [17]. However, there have been no reports that circadian oscillation of immune-related molecules is regulated by clock proteins in teleost fish.

In fish, various immune molecules and cells regulating innate and adaptive immune responses have been studied to date because fish occupy a key position in the evolution of immune system [18,19]. It is known that the immune system of fish is very similar to that of higher vertebrates, although there are some key differences [20]. Moreover, the innate immune system is an important fundamental defense mechanism for fish, as they are in constant contact with an environment containing potential pathogenic organisms, and cause induction of adaptive immune response similar to mammals [18,21]. Until now, cytokines, which are important for regulation of innate immune response, have been well studied in various fish species and their functions are becoming clearer [22]. However, existence of circadian oscillation of these molecules has not been studied to date. As mentioned above, molecular oscillators (clock proteins) rhythmically modulate the expression of inflammatory cytokines and chemokines, and maintain basal inflammatory response level in mammals [9]. Thus, circadian oscillation of the inflammatory cytokine, *TNF-α*, of medaka (*Oryzias latipes*) housed in 12:12 light-dark cycles (LD 12:12) was analyzed in the present study. Subsequently, a reporter assay was conducted to investigate the transcriptional regulation by BMAL1 and CLOCK1 via E-box present in the transcriptional regulatory region of period 1 (*Per1*) and *TNF-α*. Moreover, the responsiveness of *TNF-α* gene expression against stimulation by lipopolysaccharide was investigated at different light-dark phase (timing to reach highest/lowest level of *TNF-α* gene expression under LD 12:12 condition). The obtained results provide novel information on fish regarding timing of immuno-therapeutic intervention, such as vaccination.

2. Materials and methods

2.1. Fish maintenance and handling

The medaka fish, *Oryzias latipes* (NBRP medaka: Strain ID WS249; https://shigen.nig.ac.jp/medaka/), was supplied by Nigata University, Japan. Prior to their use in the study, the fish were acclimatized in an aerated freshwater tank at 28 °C and fed a commercial diet at 1% body weight per day for two weeks under 12:12 light–dark cycles (lights on 07:00 to 19:00 h: LD12:12) period. All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals at the University of Miyazaki.

2.2. Structural analysis of functional domains for medaka BMAL1 and CLOCK1

2.2.1. cDNA synthesis

Fish were anaesthetized with 0.05% 2-phenoxyethanol (Sigma, MO, USA) and brains were dissected out from three individuals under sterile condition. Total RNA was then extracted using ISOGEN (Nippon Gene, Tokyo, Japan) in accordance to the manufacturer's instructions. Poly(A) mRNA was purified using the quick prep micro mRNA kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and was treated with RNase-free DNase (Takara Bio, Shiga, Japan). cDNA was synthesized via reverse transcription from 2 μg mRNA using ReverTra Dash (Toyobo, Osaka, Japan) as a template for PCR.

2.2.2. Cloning and sequencing

In order to isolate the medaka *Bmal1* and *Clock1* genes, the specific primers were designed from 5′- and 3′-untranslated regions of each genes (*Bmal1* gene: XM_004084185, *Clock1* gene: XM_023955999) predicted by automated computational analysis from medaka genomic sequence (Table 1). PCR amplification was performed in a 50 μL reaction volume containing 5.0 μL dNTP mixture and 10× Gene *Taq* Universal buffer, 0.5 μL *Taq* polymerase (5 units/mL, Nippon Gene), 5.0 μL each primer set (F1 and R1 primers for each gene; 2.5 μM), 28.5 μL distilled water, and 1.0 μL medaka brain cDNA (described above; 2.2.1). The amplification regime was 3 min at 94 °C, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. The products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *E. coli* DH5α competent cells (Promega). Recombinants were identified using red-white color selection when grown on MacConkey agar (Sigma). Plasmid DNA from at least three independent clones was recovered using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and sequenced using a CEQ 8000 Automated Sequencer (Beckman Coulter, Inc., CA, USA). Sequences generated were analyzed for similarity with other known sequences using the BLAST [23] and FASTA [24] programs. The sequence of open reading frame of each mRNA was determined by above experimental procedures.

2.2.3. Functional domain analysis

Domains conserved in medaka BMAL1 and CLOCK1 were predicted by InterProScan sequence search [25] and NCBI conserved domain search program (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [26]. Multiple sequence alignments of the predicted domains, such as bHLH, PAS1, and PAS2, with those of BMAL1 and CLOCK1 in other vertebrates were generated using ClustalX version 1.81 [27] and homology analysis was performed using MatGat software version 2.02 [28].

2.3. Tissue distribution of mRNAs of clock genes

Tissues were extracted from medaka ($n = 3$) reared under LD12:12 condition at ZT12. Subsequently, cDNA was synthesized from each extracted tissue ($n = 3$ mixed) according to the method described above (Section 2.2.1). RT-PCR with primer combinations (Table 1) Orla_qBmal1-F and Orla_qBmal1-R, Orla_qClock1-F and Orla_qClock1-R, and Orla_qPer1-F and Orla_qPer1-R was performed using cDNA extracted from the brain, head kidney, spleen, liver, heart, intestine, muscle, gill, skin, and fin. Primers for medaka *β-actin* (Orla_qβ-actin-F and Orla_qβ-actin-R; Table 1) were used as internal control for RT-PCR. PCR conditions were: 1 cycle of 94 °C for 3 min, 40 (*Bmal1*, *Clock1*, and

Table 1
Primer designed for cloning and expression analysis in this study.

Gene name	Sequence (5′-3′)	Application
Orla_Bmal1-F1	TAAATGATGGGCGTGGGTCT	Cloning
Orla_Bmal1-R1	TGGTGTTAAAGCGACACTGC	Cloning
Orla_Clock1-F1	CATCCACCCCTCAGAAAAGA	Cloning
Orla_Clock1-R1	GTGGGCTCACTGACTTTCCT	Cloning
Orla_qBmal1-F	GGGAAGTTCGTCTTTGTGGA	Expression analysis
Orla_qBmal1-R	TTGATGTCGTCCTGATGGAA	Expression analysis
Orla_qClock1-F	AGCGGTGCTCATCGATCAG	Expression analysis
Orla_qClock1-R	TTGCACTCTGGCCTGTTGTC	Expression analysis
Orla_qPer1-F	TACCACCAGTGGAGTGTGGA	Expression analysis
Orla_qPer1-R	GTCCGTGTTTTTCAGGGTGT	Expression analysis
Orla_qTNFα-F	GAAGATGGCGGTTTTGGTGG	Expression analysis
Orla_qTNFα-R	GCTCTGTAGGACTCATTATTTC	Expression analysis
Orla_qβ-actin-F	CCACCATGTACCCTGGAATC	Expression analysis (internal control)
Orla_qβ-actin-R	GCTGGAAGGTGGACAGAGAG	Expression analysis (internal control)
Orla_Bmal1-F2	GAATTCTGGCCGACCAGCGGATGGAC	Overexpression plasmid construction
Orla_Bmal1-R2	CTCGAGTCACAAAGGCCAGGGCAGGT	Overexpression plasmid construction
Orla_Clock1-F2	GATATCGACATCAAGTATAGGAGATG	Overexpression plasmid construction
Orla_Clock1-R2	CCCGGGGGCTTCTGTTCAAGTGGGACT	Overexpression plasmid construction
Orla_Per1-F1	GGTACCCTTCAGCAGCACAGAAGCTG	Reporter plasmid construction (wild)
Orla_Per1-R1	CCCGGGGGCTTCTGTTCAAGTGGGACT	Reporter plasmid construction (wild)
Orla_TNFα-F1	GGTACCGGAATCCCTGATCACTTCCA	Reporter plasmid construction (wild)
Orla_TNFα-R1	CTCGAGTTGGCTTCCCTCTCAGACTC	Reporter plasmid construction (wild)
Orla_Per1-F2	AGCATTTTTGCTGCAGGTGGCCTTTTT	Reporter plasmid construction (ΔE1)
Orla_Per1-R2	TGCAGCAAAAATGCTAATGAGCGTCG	Reporter plasmid construction (ΔE1)
Orla_TNFα-F2	AGGAGCATTGGTGAGCCAGAGGCGAGG	Reporter plasmid construction (ΔE1)
Orla_TNFα-R2	CTCACCAATGCTCCTGCTCCTACACT	Reporter plasmid construction (ΔE1)
Orla_Per1-F3	CATTTTGCGCACAGGTGGCCTTTTTGCAT	Reporter plasmid construction (ΔE2)
Orla_Per1-R3	CCTGTGCGCAAAATGCTAATGAGCG	Reporter plasmid construction (ΔE2)
Orla_TNFα-F3	GAGCAGGACGTAGCCAGAGGCGAGGTTAA	Reporter plasmid construction (ΔE2)
Orla_TNFα-R3	GGCTACGTCCTGCTCCTGCTCCTACACT	Reporter plasmid construction (ΔE2)

Per1) or 28 (*β-actin*) cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, followed by 1 cycle of 72 °C for 5 min. PCR products were separated on 1.5% agarose gels and visualized by staining the gels in TBE buffer containing 100 ng/mL ethidium bromide (Sigma-Aldrich).

2.4. Expression analysis of clock and TNF-α genes in medaka reared under LD12:12 condition

2.4.1. cDNA synthesis from head kidney cells

Sampling of head kidney (HK) from medaka kept under LD12:12 condition (Section 2.1) started at ZT2 (ZT0 = 07:00) and was repeated every 4 h. Tissues were extracted from three medaka at each sampling time point for a total duration of 24 h. cDNA synthesis from sampled tissues was conducted according to the method described above (Section 2.2.1).

2.4.2. Quantitative real-time PCR of clock and TNF-α genes

qRT-PCR on cDNA specimens was performed using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, CA, USA). All real-time PCRs were performed in a reaction mixture containing 10 μL of 2 × Brilliant III Ultra-Fast SYBR Green QPCR Master Mix, 1 μL of 5 μM primer set (Orla_qBmal1, Orla_qClock1, Orla_qTNFα, or Orla_qβ-actin; Table 1), and 8 μL of HK cDNA (50 ng). Amplification was carried out as follows: 3 min at 95 °C, 40 cycles of 5 s at 95 °C, and 10 s at 60 °C. Thermal cycling and fluorescence detection was conducted using the CFX connect real time PCR system (Bio-Rad, CA, USA) in triplicates. The threshold cycle (C_T) represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal was first detected. The comparative Ct method ($2^{-\Delta\Delta Ct}$ method) [29] was used to analyze the expression levels of the medaka *Bmal1*, *Clock1*, and *TNF-α* genes. All data are given in terms of relative mRNA expressed as mean ± SE. Assessment of statistical significance was done by one-way analysis of variance (ANOVA), followed by a Tukey's test. Values were considered significant when $P < 0.01$. Moreover, curve fitting was performed using a nonlinear least squares analysis with the GraphPad Prism 7 software (GraphPad Software, San Diego, CA), as previously described [30]. Daily variations in expressions of *Bmal1*, *Clock1*, and *TNF-α* genes were fitted to a sine curve with a fixed 24 h periodicity (y = a + b sin[0.2617x + c], y: gene expression level; a = baselines; b = amplitude; x = time).

2.5. Effect of bmal1 and clock1 overexpression on the expression of TNF-α gene

2.5.1. Construction of overexpression vectors

The full-length (ORF region) of *Bmal1* and *Clock1* gene was amplified using specific primer sets (Orla_bmal1-F2 and -R2, Orla_clock1-F2 and -R2) for each gene by PCR using brain cDNA as a template (same condition as Section 2.2.2). Amplified products were digested with *Eco*RI (Nippon Gene) and *Xho*I (Nippon Gene), and then, ligated into the mammalian expression vector pcDNA4/Hismax A (Thermo Fisher Scientific) digested by same restriction enzymes. Ligated products were transformed into *E. coli* DH5α competent cells (Promega), and plasmid extraction and sequence confirmation of insert were conducted according to the method described above (Section 2.2.2). These constructs were termed as pcDNA4-Bmal1 and pcDNA4-Clock1.

2.5.2. Expression analysis of TNF-α gene in medaka embryo cells overexpressing Bmal1 and Clock1

Medaka embryo cells (OLHdrR-e3) were cultured in 96-well plates at a concentration of 1×10^4 cells per well at 33 °C prior to transfection. Before transfection, cells were washed with PBS, and then, replated with Opti-MEM (Gibco BRL, MD, USA). Transfection was performed with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. For each well, pcDNA4 empty vector (500 ng), pcDNA4-Bmal1 vector (250 ng; mixed with 250 ng empty vector), pcDNA4-Clock1 vector (250 ng; mixed with 250 ng empty vector), or pcDNA4-Bmal1 and -Clock1 vector mix (250 ng each vector) were mixed with 1.25 μL Lipofectamine™ 2000. The DNA mixtures were transfected to OLHdrR-e3 cells in 25 μL Opti-MEM, cultured at 33 °C,

and then, culture medium was completely replaced by fresh L-15 medium 36 h post-transfection. After cultivation, cDNA was synthesized from each transfected cell according to the method described above (Section 2.2.1). The expression of *TNF-α* gene was analyzed by quantitative real-time PCR as described above (Section 2.4.2). Statistical significance was validated by one-way ANOVA, followed by a Tukey's test. Values were considered statistically significant when $P < 0.01$ and $P < 0.05$.

2.6. Analysis of transcriptional activation of Per1 and TNF-α genes by BMAL1:CLOCK1 using dual luciferase reporter gene assay

2.6.1. Investigation of E-box sequence in the transcriptional regulatory region of Per1 and TNF-α genes

The 5′ upstream regions of *Per1* and *TNF-α* genes in medaka genome sequence [*Per1*: Chr. 18 (~15,138,089–15,140,432), *TNF-α*: Chr. 11 (~2,147,814–21,409,887)] were investigated to validate the presence of enhancer box (E-box; CANNTG) sequence, which is known as DNA response element, regulated by basic helix-loop-helix (bHLH) transcription factors such as BMAL1 and CLOCK1. A search for E-box sequence was carried out using web program TFBIND (http://tfbind.hgc.jp/) [31].

2.6.2. Construction of effector and reporter plasmids for reporter assay

The effector plasmids (pcDNA4-Bmal1 and pcDNA4-Clock1) were constructed according to the method described above (Section 2.5.1). For reporter constructs, two constructs of the transcriptional regulatory regions, including E-box element, were amplified by PCR (same protocol as in Section 2.2.2) using the primers (Orla_per1-F1 and -R1, Orla_TNFα-F1 and -R1) specified in Table 1 from genomic DNA. The PCR products containing transcriptional regulatory region were digested with *Kpn*I and *Xho*1 (Nippon Gene; *Per1* product) or *Kpn*I and *Sma*I (Nippon Gene; *TNF-α* product), and then, ligated with pGL3 Basic (Promega) digested with same restriction enzymes, respectively (respectively named as Per1_W and TNFα_W). Ligated products were transformed into *E. coli* DH5α competent cells (Promega), and plasmid extraction and sequence confirmation of insert were conducted according to the method described above (Section 2.2.2). Simultaneously, the site-directed mutagenesis of reporter constructs was performed using PrimeSTAR Mutagenesis Basal Kit (Takara, Shiga, Japan) and overlapping primers (Orla_per1-F2 and -R2 (ΔE1), Orla_per1-F3 and -R3 (ΔE2), Orla_TNFα-F2 and -R2 (ΔE1), Orla_TNFα-F3 and –R3 (ΔE2); Table 1) and the oligonucleotide CATTTG (ΔE1: common in both genes), TGCGCA (ΔE2: *Per1*), or GGACGT (ΔE2: *TNF-α*) was introduced in place of CACGTG (Per1_W)/CAGGTG (TNFα_W) within the putative E-box region (named as Per1_ΔE1, _ΔE2 and TNFα_ΔE1, _ΔE2, respectively). Plasmid extraction and sequence confirmation of insert were conducted according to the method described above (Section 2.2.2).

2.6.3. Luciferase reporter assay

For luciferase assay, OLHdrR-e3 cells were cultured in 96-well plates at a concentration of 1×10^4 cells per well at 33 °C prior to transfection. Before transfection, cells were washed with PBS, and then replated with Opti-MEM (Gibco). Transfection was performed with Lipofectamine™ 2000 (Invitrogen) according to manufacturer's instructions. For each well, 50 ng pGL3 vectors (Empty, Per1_W, _ΔE1, _ΔE2 and TNFα_W, _ΔE1, _ΔE2) and 125 ng pcDNA4 vectors (Empty or pcDNA4-Bmal1 and pcDNA4-Clock1 mix (62.5 ng each), pcDNA4-Bmal1, or pcDNA4-Clock1) and 10 ng of phRL-SV40 vector (Promega) were mixed with 0.25 μL Lipofectamine™ 2000. The DNA mixtures were transfected to OLHdrR-e3 cells in presence of 25 μL Opti-MEM, cultured at 33 °C, and then, culture medium was completely replaced by fresh L-15 medium 4 h post-transfection. After 24 h transfection, cells were harvested and examined for luciferase activity. OLHdrR-e3 cells were harvested according to the manufacturer's instructions using the dual-luciferase kit (Promega) using 50 μL passive lysis buffer for each well. Samples were centrifuged at 3000 rpm for 5 min to remove cell debris. Firefly and *Renilla* luciferase activities were measured using 10 μL of each sample. Statistical significance was validated by one-way ANOVA, followed by a Tukey's test. Values were considered to be statistically significant when $P < 0.01$.

2.7. Evaluation of TNF-α gene expression in response to immune-stimulation at different time points (light-dark phase)

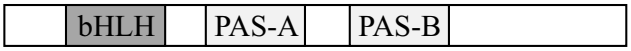
The head kidney was extracted by the method described above (Section 2.2.1) from three individual medaka kept under LD12:12 condition at ZT10 or ZT18. The cells were filtered through a 100 μm nylon mesh (Becton, Dickinson and Company, NJ, USA) in an RPMI 1640 medium (Invitrogen), containing 100 μg/mL streptomycin (Invitrogen), 100 units/mL penicillin (Invitrogen), and 5% fetal bovine serum (FBS; Invitrogen). The concentration of viable cells was determined using trypan blue staining (Life Technologies, NY, USA) and they were seeded at 2×10^6 cells/mL in wells of a 24-well plate (Nunc A/S, Roskilde, Denmark). The cells were then treated with lipopolysaccharides (LPS, *E. coli* K, Sigma) at a final concentration of 25 μg/mL and incubated for 6 h at 28 °C. Control cells (cells without stimulation) were maintained for each sample (ZT10 and ZT18). Each treatment and control had three replicates. Cells were harvested at 6 h post stimulation, and cDNA was synthesized from the cells according to the method described above (Section 2.2.1). Subsequently, quantitative real-time PCR was conducted to evaluate *TNF-α* gene expression according to the method described above (Section 2.4.2). Statistical significance was validated by one-way ANOVA, followed by a Tukey's test. Values were considered to be statistically significant when $P < 0.01$.

3. Results

3.1. Structural analysis of medaka Bmal1 and Clock1 genes

Nucleotide and amino acid sequences of cloned medaka *Bmal1* and *Clock1* cDNAs are shown in Supplementary Fig. 1. Sequence of cloned *Bmal1* gene was similar to that predicted from the genomic DNA sequence (XM_004084185). On the other hand, cloned *Clock1* gene (LC458860) showed 96.2% identity with that predicted from genomic DNA sequence (XM_023955999). Medaka BMAL1 and CLOCK1 retained the conserved functional domains, such as basic helix-loop-helix (bHLH) and two PER-ARNT-SIM (PAS-A and PAS-B), similar to other vertebrates (Fig. 1A). In BMAL1, basic residues (His97, Ile100, Glu101, and Arg105) which bind to target DNA were conserved in basic region of bHLH domain (Fig. 1B upper). Moreover, the presence of nuclear localization signal (NLS) and nuclear export signal (NES) was confirmed at N-terminal region (Glu101-Met108 and Leu129-Arg136) of the same domain. Furthermore, one serine residue (Ser110), involved in phosphorylation, was confirmed to be present at the same region. Presence of important residues (Ala174, Ala175, and Leu179) involved in dimerization was confirmed in PAS-A domain (Fig. 1B middle). Nuclear export signal (NES; Leu381-Gly390) was conserved in PAS-B domain (Fig. 1B lower). In CLOCK1, basic residues (Arg29, Glu33, and Arg37), which bind to target DNA, were conserved in basic region of bHLH domain (Fig. 1C, upper). Moreover, NLS was confirmed to be present at N-terminal region (Ala24-Arg37) of same domain. In addition, one serine residue (Ser32) involved in phosphorylation was confirmed to be present at same region. Important residues (Ala107, Leu108, and Phe112) involved in dimerization were confirmed in PAS-A domain (Fig. 1C middle). As for identity among vertebrate BMAL1/CLOCK1, the amino acid identity of whole sequences and functional domains of the genes in medaka to those in zebrafish, human, and mouse was as follows: BMAL1: whole sequence (81.7–88.3%), functional domains (92.4–100%); CLOCK1: whole sequence (58.8–61.8%), functional domains (77.5–97.8%)] (Fig. 1D).

A)



B)

```
                          basic          helix          loop          helix
Medaka bHLH        94 REAHSQIEKRRRDKMNSFIDELASLVPTCNAMSRKLDKLTVLRMAVQHMKTLRG 147
Zebrafish bHLH     78 REAHSQIEKRRRDKMNSFIDELASLVPTCNAMSRKLDKLTVLRMAVQHMKTLRG 131
Human bHLH         74 REAHSQIEKRRRDKMNSFIDELASLVPTCNAMSRKLDKLTVLRMAVQHMKTLRG 127
Mouse bHLH         74 REAHSQIEKRRRDKMNSFIDELASLVPTCNAMSRKLDKLTVLRMAVQHMKTLRG 127

Medaka PAS-A      174 AADGFLFVVGCDRGKILFVSESVFKILNYSQNDLIGQSLFDYLHPKDIAKVK 225
Zebrafish PAS-A   158 AADGFLFVVGCDRGKILFVSESVYKILNYTQNDLIGQSLFDYLHPKDIAKVK 209
Human PAS-A       154 AADGFLFVVGCDRGKILFVSESVFKILNYSQNDLIGQSLFDYLHPKDIAKVK 205
Mouse PAS-A       154 AADGFLFVVGCDRGKILFVSESVFKILNYSQNDLIGQSLFDYLHPKDIAKVK 205

Medaka PAS-B      355 VKPTEYVSRHAIDGKFVFVDQRATAILAYLPQELLGTSFYEYFHQDDINHLAE 407
Zebrafish PAS-B   339 VKPTEYVSRHAIDGKFVFVDQRATAILAYLPQELLGTSFYEYFHQDDIGHLAE 391
Human PAS-B       334 VKSMEYVSRHAIDGKFVFVDQRATAILAYLPQELLGTSCYEYFHQDDIGHLAE 386
Mouse PAS-B       334 VKSMEYVSRHAIDGKFVFVDQRATAILAYLPQELLGTSCYEYFHQDDIGHLAE 386
```

C)

```
                          basic          helix          loop          helix
Medaka bHLH        24 AKRESRNKSEKERRDQFNVLIKELGTMLPGNTRKMDKSTILQKSIDFLC 72
Zebrafish bHLH     24 AKRVSRNKSEKKRRDQFNVLIKELGTMLPGNTRKMDKSTILQKSIDYLR 72
Human bHLH         34 AKRVSRNKSEKKRRDQFNVLIKELGSMLPGNARKMDKSTVLQKSIDFLR 82
Mouse bHLH         34 AKRVSRNKSEKKRRDQFNVLIKELGSMLPGNARKMDKSTVLQKSIDFLR 82

Medaka PAS-A      105 LEALDGFFIAIMTDGNILYVSESVTSLLEHLPADLVDQNLLNFLPVGEH 153
Zebrafish PAS-A   105 LEALDGFFLVMLTDGYIIYISESVTSLLEHLPSDLMDQNLLNFLPVVDH 153
Human PAS-A       115 LEALDGFFLAIMTDGSIIYVSESVTSLLEHLPSDLVDQSIFNFIPEGEH 163
Mouse PAS-A       115 LEALDGFFLAIMTDGSIIYVSESVTSLLEHLPSDLVDQSIFNFIPEGEH 163

Medaka PAS-B      263 NEEFTSRHSMEWKFLFLDHRAPPIIGYLPFEVLGTSGYDYYHVDDL 308
Zebrafish PAS-B   262 NEEFTSRHSLEWKFLLLDHRAPPIIGYMPFEVLGTSGYDYYHVDDL 307
Human PAS-B       273 NEEFTSRHSLEWKFLFLDHRAPPIIGYLPFEVLGTSGYDYYHVDDL 318
Mouse PAS-B       273 NEEFTSRHSLEWKFLFLDHRAPPIIGYLPFEVLGTSGYDYYHVDDL 318
```

D)

BMAL1	whole	bHLH	PAS-A	PAS-B		**CLOCK1**	whole	bHLH	PAS-A	PAS-B
Zebrafish	88.3	100	96.1	98.1		Zebrafish	61.8	91.8	77.5	93.4
Human / Mouse	81.9 / 81.7	100	100	92.4		Human / Mouse	59.7 / 58.8	87.7	81.6	97.8

Fig. 1. Multiple alignment and identity of deduced amino acid sequences of medaka BMAL1 and CLOCK1 functional domains with those of other vertebrates. A) The schematic domain structure of BMAL1 and CLOCK1 in vertebrates. An alignment of functional domains in B) BMAL1 and C) CLOCK1 (upper: bHLH, middle: PAS-A, and lower: PAS-B). Boxes and dashed boxes in bHLH domain indicate basic amino acid residues involved in DNA binding, and cysteine residue involved in phosphorylation, respectively. Underline and double underline indicate sequences for nuclear localization signal and nuclear export signal, respectively. Boxes in PAS-A domain indicate hydrophobic amino acid residues involved in dimerization. D) Amino acid identity of whole sequence and each functional domain of medaka BMAL1/CLOCK1 with those of other vertebrates. The accession numbers (DDBJ, EMBL, and GenBank) of sequences retrieved in this analysis were as follow: zebrafish BMAL1, NP_571652; human BMAL1, NP_001169; mouse BMAL1, NP_031515; medaka CLOCK1, LC458860; zebrafish CLOCK1, NP_840080; human CLOCK1, NP_004889; and mouse CLOCK1, NP031741.

3.2. Expression analyses of medaka Bmal1, Clock1, Per1, and TNF-α genes

Constitutive expressions of *Bmal1*, *Clock1*, and *Per1* genes was observed in the brain, head kidney, spleen, liver, heart, intestine, muscle, gill, skin, and fin (Fig. 2). Quantitative analysis of the temporal expression patterns of *Bmal1*, *Clock1*, *Per1*, and *TNF-α* genes in the kidney of medaka raised under LD12:12 condition showed circadian oscillation in their mRNA levels. Expressions of medaka *Bmal1* and *Clock1* genes increased during light phase and decreased during dark phase (Fig. 3A, B). The acrophase of expressions of both genes was confirmed at ZT10, at which expression level had significant increased compared to basal expression ($P < 0.01$; *Bmal1*: at ZT22, *Clock1*: at ZT 18). *Per1* gene showed opposite expression pattern and reached acrophase at ZT22, which was significantly higher than basal expression at ZT14 (Fig. 3C, $P < 0.01$). Expression of *TNF-α* gene showed similar pattern as that of *Per1* and reached acrophase and baseline at ZT18 and ZT10, respectively (Fig. 3D, significant difference $P < 0.01$).

3.3. TNF-α gene expression in medaka OLHdrR-e3 cells overexpressing Bmal1 and Clock1 genes

The modulation of *TNF-α* gene expression in OLHdrR-e3 cells

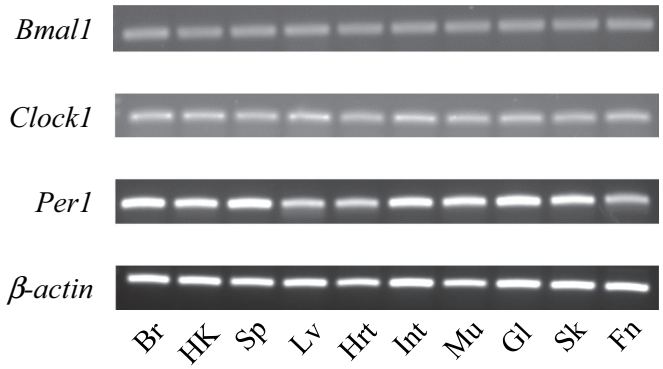


Fig. 2. Expression analyses of the *Bmal1*, *Clock1*, and *Per1* genes in tissues from medaka reared under LD12:12 condition (tissue extraction at zeitgeber time 12). RT-PCR was performed using primers specific for medaka *Bmal1*, *Clock1*, *Per1*, and *β-actin* with cDNA synthesized from a variety of tissues (Br: Brain, HK: head kidney, Sp: spleen, Lv: liver, Hrt: heart, Int: intestine, Mu: muscle, Gl: Gill, Sk: skin, and Fn: fin).

overexpressing *Bmal1* and *Clock1* genes was investigated by quantitative real-time PCR. Firstly, expression of *Bmal1* gene was significantly increased after transfection of pcDNA4-Bmal1 or both pcDNA4-Bmal1 and -Clock1 into OLHdrR-e3 compared to the control (pcDNA4-empty) (Fig. 4A, $P < 0.05$). Similarly, *Clock1* gene expression was also significantly increased in OLHdrR-e3 transfected with pcDNA-Clock1 or both pcDNA4-Bmal1 and -Clock1 (Fig. 4A, $P < 0.01$). The significant increase of *TNF-α* gene expression was confirmed in the OLHdrR-e3 cells transfected with both pcDNA4-Bmal1 and -Clock1 compared to cells transfected with pcDNA4-Bmal1, pcDNA4-Clock1, or pcDNA4-empty (Fig. 4B, significant difference $P < 0.01$).

3.4. Transcriptional activation of TNF-α gene by BMAL1:CLOCK1

To demonstrate transcriptional regulation via E-box by BMAL1 and CLOCK1, the 5′ upstream regions of the medaka *Per1* and *TNF-α* genes were examined by reporter assay. Before construction of reporter plasmid, we identified one E-box element sequence (CANNTG) in the region: ~1200–1205 bp (*Per1*)/~1995–2000 bp (*TNF-α*) (Fig. 5A and Supplementary Fig. 2). In order to investigate transactivational regulation via E-box by BMAL1 and CLOCK1, three reporter constructs containing wild type or mutated E-box (ΔE1 and ΔE2; Fig. 5B) for each gene and empty control were analyzed by a luciferase assay after co-transfection into OLHdrR-e3 cells. The reporter constructs containing wild E-box (Per1_W and TNFα_W) in *Bmal1* and *Clock1* overexpression group showed significant increase in luciferase activity compared to constructs containing the wild E-box (Per1_W and TNFα_W) in non-overexpression group for each gene (*Per1* and *TNF-α*: $P < 0.01$). Moreover, the luciferase activity of reporter constructs containing mutated E-box (ΔE1 and ΔE2 in both genes) in *Bmal1* and *Clock1*

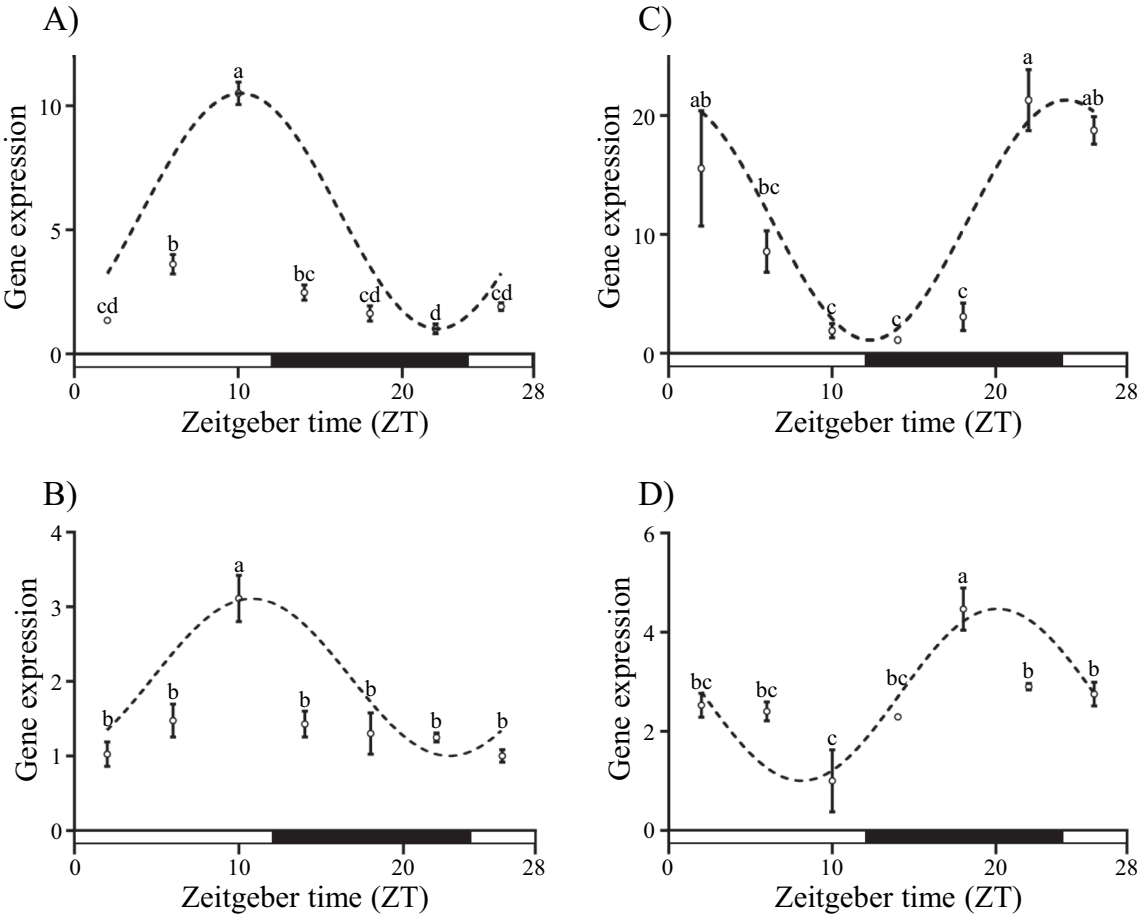


Fig. 3. Circadian oscillation of *TNF-α* gene and clock genes, such as *Bmal1*, *Clock1*, and *Per1*, in head kidney of medaka reared under 12L:12D condition. Medaka head kidneys ($n = 3$) were collected at indicated times. Relative mRNA levels were quantified by qPCR. Data are presented as $2^{-\Delta\Delta Ct}$ levels calculated relative to the baseline (lowest expression) set to 1 and normalized against the *β-actin* mRNA levels. Each data point represents the mean ± S. E. in triplicates. Times are shown in zeitgeber time (ZT), with composite block under the graph; black and white represent the dark phase and the light phase, respectively. Expression data were fitted to a sine wave with a fixed 24 h periodicity by nonlinear least squares analysis, which is indicated by broken line. Statistical differences ($P < 0.05$) tested by one-way ANOVA followed by Tukey's test at different time points are indicated by different letters. A) *Bmal1*, B) *Clock1*, C) *Per1*, and D) *TNF-α*.

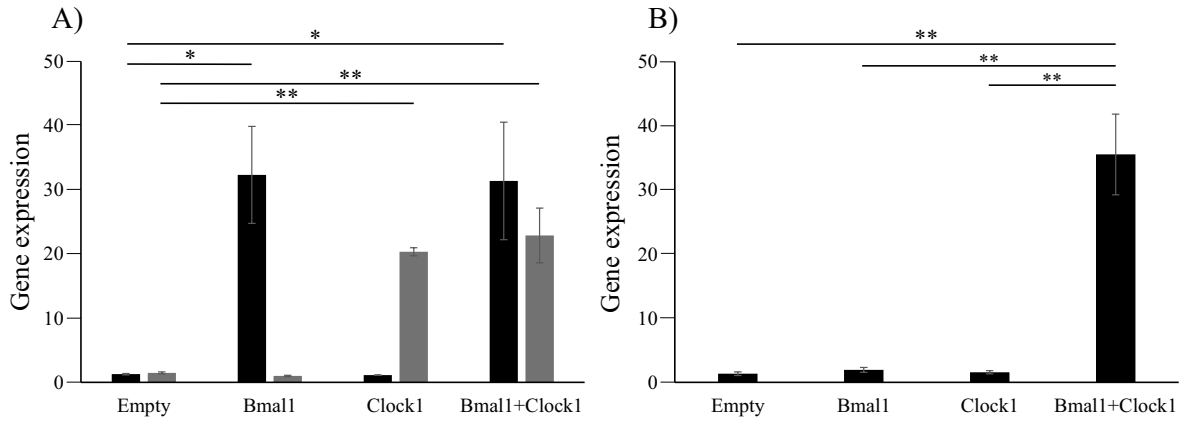


Fig. 4. *TNF-α* gene expression in medaka OLHdrR-e3 cells overexpressing *Bmal1* and *Clock1* genes. Relative mRNA levels were quantified by qPCR. Data are presented as $2^{-\Delta\Delta Ct}$ levels calculated relative to the control (transfected with empty vector) set to 1, normalized against the *β*-actin mRNA levels. Data are presented as mean ± S. E. in triplicates. Statistical differences verified by one-way ANOVA followed by Tukey's test are indicated by asterisks (*$P < 0.05$ and **$P < 0.01$). A) Overexpression level of *Bmal1* or *Clock1* genes; black bar: *Bmal1*, gray bar: *Clock1*. B) *TNF-α* gene expression. Abbreviations: Empty, pcDNA4 empty vector transfected; Bmal1, pcDN4-Bmal1 transfected; Clock1, pcDNA4-Clock1 transfected; and Bmal1 + Clock1, pcDNA4-Bmal1 and -Clock1 transfected.

overexpression group showed significant decrease compared to the wild E-box containing construct for each gene (*Per1* and *TNF-α*: $P < 0.01$) (Fig. 5C).

3.5. TNF-α gene response against LPS stimulation at different time points

qRT-PCR was conducted to investigate whether LPS stimulation at different time points (ZT10 and ZT18) impacts expression level of *TNF-α* gene. In LPS-stimulated HK cells, *TNF-α* gene expression at ZT18 was significantly higher than that at ZT10 ($P < 0.01$). Moreover, the expression level after LPS stimulation compared to the PBS control, at ZT18, was significantly higher than that at ZT10 ($P < 0.01$, Fig. 6).

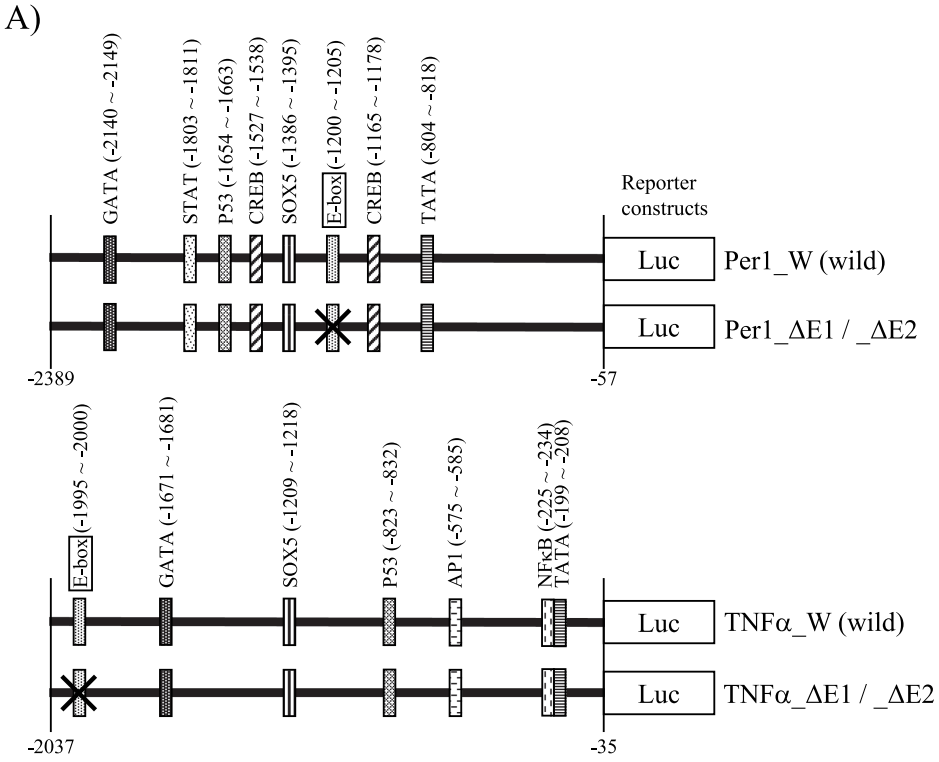
4. Discussion

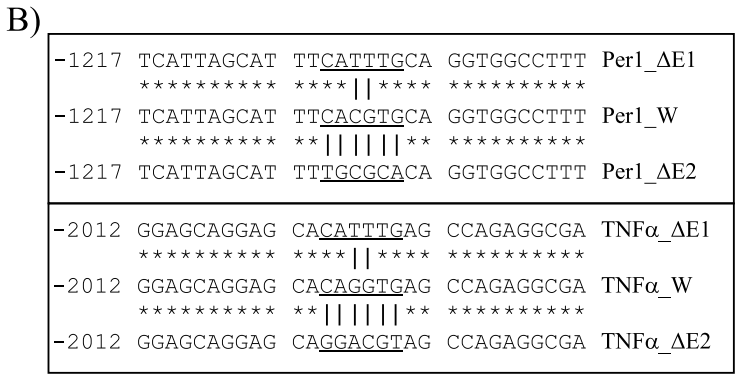
The circadian oscillation of immune-related molecules regulated by clock proteins has been widely investigated in various mammalian species [9]. However, there is little information about its regulation mechanism in lower vertebrates, including fish. Therefore, the present study showed the existence of circadian oscillation (transcriptional regulation) of pro-inflammatory cytokine *TNF-α* by clock gene (BMAL1 and CLOCK1) in medaka fish, a useful vertebrate model organism [32].

To date, *Bmal1* and *Clock1* mRNA sequences of medaka registered in nucleotide sequence databases, such as NCBI, DDBJ, and ENA, were only predicted from genomic DNA sequence [*Bmal1*: chromosome 6 (NC_019864.2), *Clock1*: chromosome 1 (NC_019859.2)]. Therefore, mRNA sequences of the clock genes were confirmed before investigation of 1) circadian oscillation of gene expression and 2) transcriptional regulation of inflammatory cytokine, *TNF-α*, by clock proteins in this study. We confirmed that the sequence of cloned *Bmal1* gene was the same as that (XP_00484233) predicted from the genomic DNA sequence, but cloned *Clock1* gene sequence (ORF region) was slightly different from *Clock 1* gene sequence (XP_023811767) predicted from genomic DNA sequence (identity: 96.2%). Medaka BMAL1 and CLOCK1 possessed conserved functional domains, such as bHLH, PAS-A, and PAS-B, which are seen in bHLH-PAS family of transcriptional regulators [33]. In addition, identity of these domains with other vertebrates was high. The bHLH domain is important for DNA binding for transcription of target gene. In general, this domain contains basic amino acid residues, and typically binds, via hydrogen bond, to a consensus sequence known as E-box (CANNTG) conserved in transcriptional regulatory region of target gene [34]. Important basic amino acid residues known for DNA binding in mammals [35] are conserved in the same position as cloned medaka BMAL1 and CLOCK1; therefore, it was speculated that medaka BMAL1 and CLOCK1 may act as transcriptional regulators. Moreover, NLS and NES, which are important for nucleocytoplasmic shuttling [36], were confirmed in medaka BMAL1 (bHLH/PAS-B) and CLOCK1 (bHLH). In addition, hydrophobic residues, which are important for heterodimerization [33,37], were conserved in PAS-A domain of medaka BMAL1 and CLOCK1. These results suggested that medaka BMAL1 and CLOCK1 harbored conserved structures that participated in crucial processes, such as heterodimerization and accumulation, in the nucleus during the transcriptional regulation of clock-controlled genes (CCGs). However, DNA binding/dimerization ability of the domains will need to be investigated in the future studies.

Expressions of clock genes such as *Bmal1*, *Clock1*, and *Per1* were evaluated in central and peripheral tissues in medaka. The present result is similar to that of tissue distribution of clock genes reported in Atlantic cod (*Gadus morhua*) [38]. Therefore, it was suggested that a peripheral clock is present in various tissues of fish, and these clock genes acted as circadian regulators of genes involved in the physiology of specific tissues seen in mammals [39]. Among the fish tissues, head kidney, analogous to the mammalian adrenal gland, is known as a major lymphoid and endocrine organ [40]. Therefore, it is important to study immuno-modulation in head kidney to understand fish immune system. In the present study, we analyzed gene expression every 4 h (from ZT2 to ZT26), and found that levels of *Bmal1* and *Clock1* genes increased in during light phase, whereas *per1* levels decreased. This result corroborated the patterns of expression previously reported in medaka tissues (heart and fin [41], liver [32]), rainbow trout (*Oncorhynchus mykiss*) neural retina [42], Atlantic cod fast skeletal muscle (*Clock* gene [38]), and Japanese flounder (*Paralichthys olivaceus*) caudal fin (*Per1* gene [43]). Moreover, *Bmal1* and *Clock1* showed similar expression patterns; however, *Cry1* and *Per1* show similar pattern to that in mammals. It is known that this expression profile was maintained with transcriptional regulation of *Per1* and *Cry1* by BMAL1 and CLOCK1 via a negative-feedback loop [4]. Therefore, it was suggested that this regulatory mechanism exists in fish as well as mammals. The expression of *TNF-α* showed circadian oscillation and its pattern was similar to that of *Per1* gene. In mammals, circadian oscillation of TNF-α was reported in hippocampal microglia of rat (mRNA expression) [44] and spleen of mouse simulated with LPS (protein secretion) [45]. Moreover, it has been reported that TNF-α response of macrophages upon LPS stimulation is regulated by a cell-intrinsic, local clock [45]. Therefore, it was indicated that the local clock could participate in regulation of *TNF-α* gene expression in fish too.

Expression of *TNF-α* gene in the head kidney of medaka reared under LD12:12 condition showed circadian oscillation. Its expression





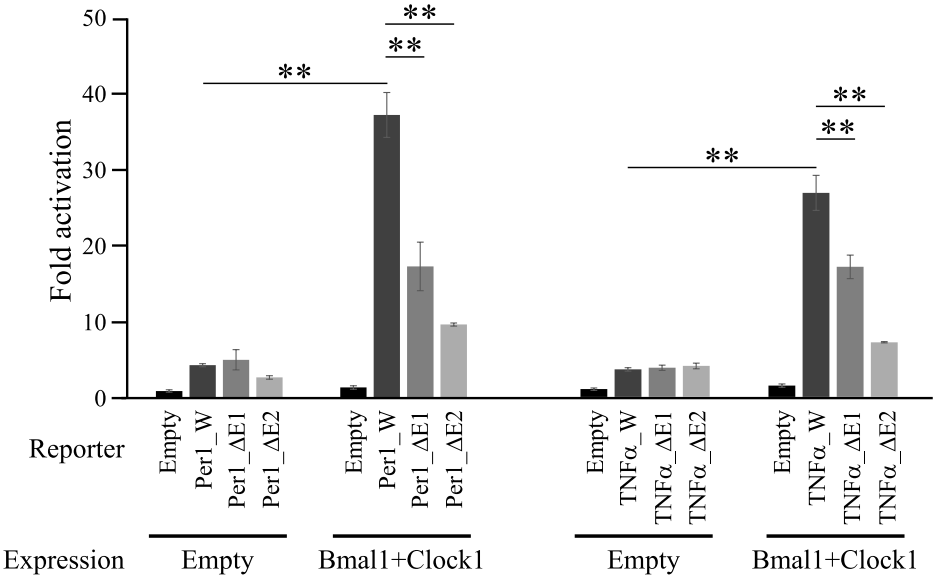


Fig. 5. The *E*-box motif found in transcription regulatory region of the medaka *Per1* and *TNF-α* genes. (A) Schematic drawings of transcription factor binding motif locations and the constructs used in the luciferase (Luc) assay. (B) The mutated E-box motifs (upper; ΔE1 and lower; ΔE2) compared to the wild type of sequences (center). E-box motif of *Per1* and *TNF-α* genes were substituted with either TT (center of E-box motif) or scrambling sequence. The number of nucleotides indicated in 5A and 5B was counted from open reading frame (refer to Supplementary Fig. 2.). (C) Transcriptional activities of wild E-box and mutants (ΔE1 or ΔE2) were evaluated in OLHdrR-e3 cells overexpressing *Bmal1* and *Clock1* genes. Fold activation is shown based on the empty control (non-overexpression) set to 1. Data are presented as mean ± S. E. in triplicates. Statistical differences verified by one-way ANOVA followed by Tukey's test are indicated by asterisks (**$P < 0.01$). Abbreviation: W, wild E-box; ΔE1 and ΔE2, mutated E-box motif.

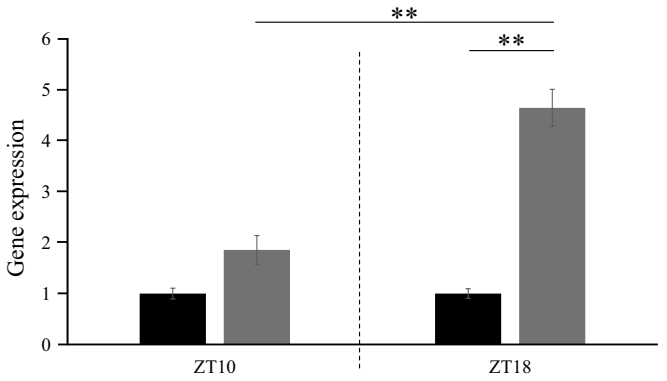


Fig. 6. The response of *TNF-α* gene to LPS stimulation at different time points. Head kidney cells were isolated at either zeitgeber time (ZT) 10 or ZT18 from medaka ($n = 3$) reared under 12L: 12D condition. Both cells were plated with 25 μg/mL LPS for 6 h and mRNA expression of *TNF-α* was evaluated by qPCR. Data are $2^{-\Delta\Delta Ct}$ levels calculated relative to the un-treated cells (PBS control) set to 1 and normalized against the *β-actin* mRNA levels. Data are presented as mean ± S. E. in triplicates. Statistical differences verified by one-way ANOVA followed by Tukey's test are indicated by asterisk ($P < 0.01$).

pattern was similar to that of *Per1* gene, which is transcriptionally regulated by BMAL1:CLOCK1 heterodimer in mammals [5]. Therefore, we hypothesized that the expression of *TNF-α* gene is regulated by some clock or clock-controlled gene (CCG). In the medaka OLHdrR-e3 cells overexpressing both *Bmal1* and *Clock1* genes, the expression of *TNF-α* gene was significantly upregulated; therefore, it was suggested that BMAL1, CLOCK1, or CCGs were associated with the transcription of *TNF-α* gene. Subsequently, the role of BMAL1 and CLOCK1 in transcriptional regulation of *Per1* and *TNF-α* genes via E-box (consensus motif CANNTG) was investigated by luciferase reporter assay. The reporter constructs containing mutated *E*-box [CATTTG (ΔE1: common in both genes), TGCGCA (per1_ΔE2), or GGACGT (TNFα_ΔE2)] showed significant decrease of luciferase activity compared to the wild E-box containing construct [CACGTG (per1_W) and CAGGTG (TNFα_W)] in the *Bmal1* and *Clock1* overexpression group. Moreover, scrambling mutation [TGCGCA (per1_ΔE2) and GGACGT (TNFα_ΔE2)] of E-box showed significantly lower activity than that of mutation (per1_ΔE1 and TNFα_ΔE1) at NN sequence position. It has been reported that E-box functions as an enhancer of transactivation of *Per1* via BMAL1 and CLOCK1 in mammals [46]. In addition, in fish, it has been revealed that BMAL1 and CLOCK1 regulate transcription of *Per4* gene in zebrafish (*Danio rerio*) PAC2 fibroblast cells by luciferase assay [47]. Therefore, it was suggested that medaka BMAL1 and CLOCK 1/CCGs (induced by BMAL1 and CLOCK1) could be associated with transactivation, via *E*-box, of *Per1* gene. There are no reports of direct transcriptional regulation of *TNF-α*, via E-box, by BMAL1 and CLOCK1 in vertebrates. However, it has been reported that CLOCK acts as a positive regulator of NF-κB-responsive genes distinct from the transactivation of circadian genes, via E-box elements [48]. Moreover, TNF-α suppresses BMAL1/CLOCK1-induced activation of E-box regulatory elements-dependent clock gene promoters in mammals [49]. Therefore, the current results suggested that BMAL1 and CLOCK1/CCGs (induced by BMAL1 and CLOCK1) could be involved in transactivation of *TNF-α* gene. Further analysis by protein-nucleotide interaction assay such as Chip or EMSA would be needed in the future study. Moreover, significant difference in degree of transactivation was confirmed among wild, ΔE1, and ΔE2 constructs of *Per1* and *TNF-α* genes. It is already known that BMAL1 and CLOCK proteins bind noncanonical E-box [except for CACGTG (canonical)] in mammals [50]. Three noncanonical E-boxes (CAGGTG, CAGCTG, and CATTTG) exist in human toll-like receptor (TLR) 9 promoter, which are associated with circadian control of *Tlr9* gene expression [51]. In the present study, *TNF-α* wild E-box (CAGGTG) and ΔE1 in both genes (CATTTG) refer to above mammalian noncanonical E-boxes. Therefore, it was suggested that these nucleotide sequences could be E-box elements which are regulated by bHLH-PAS proteins in fish as well as mammals.

It was confirmed that expression of *TNF-α* gene showed circadian oscillation, which the lowest and highest expressions at ZT10 and ZT18, respectively. Therefore, we investigated whether LPS stimulation at ZT10 or ZT18 impacts *TNF-α* gene expression. The expression of *TNF-α* gene was strongly induced in HK cells stimulated with LPS at ZT18 than that at ZT10. In mouse splenocytes, TNF-α response upon LPS stimulation is regulated by a cell-intrinsic, local clock [45]. Similar results (for *TNF-α* and *IL-1β*) have been reported in rat microglia [44]. These reports suggested that cell-intrinsic clockwork provides temporal gating of cytokine response to LPS. Furthermore, mortality caused by LPS injection varies depending on the time of administration in mammals [48,52]. With other immune-molecules, such as TLR9, CpG (TLR9 ligand) stimulation strongly induced expressions of *TNF-α*, *CD80*, and *CD86* genes and IFN-γ production at peak time of *Tlr9* gene expression during the day compared to the time of its lowest expression [51]. In fish, innate immune defenses by lysozyme and peroxidase in serum exhibit circadian rhythmicity and differential temporal sensitivity to LPS in Nile tilapia (*Oreochromis niloticus*) [17]. In addition, serum-mediated bactericidal activity against some pathogenic bacteria was significantly variable depending on timing of infection in rainbow trout [53]. Moreover, the counts of peripheral blood leukocytes such as granulocytes, lymphocytes, and monocytes showed circadian oscillation in Mozambique tilapia (*Oreochromis mossambicus*) maintained in LD12:12 condition [54]. Therefore, it was suggested that fish immune system has different responsiveness to immune-stimulation/infection during a day.

However, above reports regarding circadian oscillation of fish immune system did not mention the relationship between circadian oscillation of immune parameters and clock genes. Therefore, this is the first report suggesting that expression and response of cytokine, *TNF-α*, exhibits circadian oscillation regulated by clock proteins found in fish as well as mammals. Recently, it has been reported that the increase in antibody levels due to influenza vaccination differed between morning and afternoon administration in humans [55]. Although further studies will be needed, the present study could provide basic knowledge for development of chronotherapeutic strategies with respect to vaccination and management of infectious and inflammatory diseases in fish.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.02.004.

Acknowledgements

We are grateful to NBRP Medaka (https://shigen.nig.ac.jp/medaka/) for providing Saito (Strain ID: WA249). This work was financially supported by a Grant-in-Aid for Scientific Research (C) (17K07915) from the Japan Society for the Promotion of Science (JSPS).

References

[1] R.S. Edgar, E.W. Green, Y. Zhao, et al., Peroxiredoxins are conserved markers of circadian rhythms, Nature 485 (7399) (2012) 459–464.
[2] R.A. Hut, D.G. Beersma, Evolution of time-keeping mechanisms: early emergence and adaptation to photoperiod, Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 366 (1574) (2011) 2141–2154.
[3] S. Hughes, A. Jagannath, M.W. Hankins, R.G. Foster, S.N. Peirson, Photic regulation of clock systems, Methods Enzymol. 552 (2015) 125–143.
[4] M.H. Hastings, E.S. Maywood, A.B. Reddy, Two decades of circadian time, J. Neuroendocrinol. 20 (6) (2008) 812–819.
[5] H.A. Duong, M.S. Robles, D. Knutti, C.J. Weitz, A molecular mechanism for circadian clock negative feedback, Science 332 (6036) (2011) 1436–1439.
[6] N. Labrecque, N. Cermakian, Circadian clocks in the immune system, J. Biol. Rhythm. 30 (4) (2015) 277–290.
[7] A.M. Curtis, M.M. Bellet, P. Sassone-Corsi, L.A. O'Neill, Circadian clock proteins and immunity, Immunity 40 (2) (2014) 178–186.
[8] L.E. Hand, T.W. Hopwood, S.H. Dickson, A.L. Walker, A.S. Loudon, D.W. Ray, D.A. Bechtold, J.E. Gibbs, The circadian clock regulates inflammatory arthritis, FASEB J. 30 (11) (2016) 3759–3770.

[9] K. Man, A. Loudon, A. Chawla, Immunity around the clock, Science 354 (6315) (2016) 999–1003.
[10] X. Yu, D. Rollins, K.A. Ruhn, J.J. Stubblefield, C.B. Green, M. Kashiwada, P.B. Rothman, J.S. Takahashi, L.V. Hooper, TH17 cell differentiation is regulated by the circadian clock, Science 342 (6159) (2013) 727–730.
[11] H. Migaud, A. Davie, J.F. Taylor, Current knowledge on the photoneuroendocrine regulation of reproduction in temperate fish species, J. Fish Biol. 76 (1) (2010) 27–68.
[12] V. Di Rosa, E. Frigato, J.F. Lopez-Olmeda, F.J. Sanchez-Vazquez, C. Bertolucci, The light wave length affects the ontogeny of clock gene expression and activity rhythms in zebrafish larvae, PLoS One 10 (7) (2015) e0132235.
[13] M. Mogi, S. Uji, H. Yokoi, T. Suzuki, Early development of circadian rhythmicity in the suprachiamatic nuclei and pineal gland of teleost, flounder (*Paralichthys olivaeus*), embryos, Develop. Growth Differ. 57 (6) (2015) 444–452.
[14] L.M. Vera, P. Negrini, C. Zagatti, E. Frigato, F.J. Sanchez-Vazquez, C. Bertolucci, Light and feeding entrainment of the molecular circadian clock in a marine teleost (*Sparus aurata*), Chronobiol. Int. 30 (5) (2013) 649–661.
[15] A.L. Morgan, K.D. Thompson, N.A. Auchinachie, H. Migaud, The effect of seasonality on normal haematological and innate immune parameters of rainbow trout *Oncorhynchus mykiss* L, Fish Shellfish Immunol. 25 (6) (2008) 791–799.
[16] C.C. Lazado, I. Lund, P.B. Pedersen, H.Q. Nguyen, Humoral and mucosal defense molecules rhythmically oscillate during a light-dark cycle in permit, *Trachinotus falcatus*, Fish Shellfish Immunol. 47 (2) (2015) 902–912.
[17] C.C. Lazado, P.V. Skov, P.B. Pedersen, Innate immune defenses exhibit circadian rhythmicity and differential temporal sensitivity to a bacterial endotoxin in Nile tilapia (*Oreochromis niloticus*), Fish Shellfish Immunol. 55 (2016) 613–622.
[18] D.A. Plouffe, P.C. Hanington, J.G. Walsh, E.C. Wilson, M. Belosevic, Comparison of select innate immune mechanisms of fish and mammals, Xenotransplantation 12 (4) (2005) 266–277.
[19] Z. Pancer, M.D. Cooper, The evolution of adaptive immunity, Annu. Rev. Immunol. 24 (2006) 497–518.
[20] C. Uribe, H. Folch, R. Enriquez, G. Moran, Innate and adaptive immunity in teleost fish: a review, Vet. Med. 56 (2011) 486–503.
[21] B. Magnadottir, Innate immunity of fish (overview), Fish Shellfish Immunol. 20 (2) (2006) 137–151.
[22] J. Zou, C.J. Secombes, The function of fish cytokines, Biology (Basel) 5 (2) (2016).
[23] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (3) (1990) 403–410.
[24] W.R. Pearson, D.J. Lipman, Improved tools for biological sequence comparison, Proc. Natl. Acad. Sci. U. S. A. 85 (8) (1988) 2444–2448.
[25] R.D. Finn, T.K. Attwood, P.C. Babbitt, et al., InterPro in 2017-beyond protein family and domain annotations, Nucleic Acids Res. 45 (D1) (2017) D190–D199.
[26] A. Marchler-Bauer, Y. Bo, L. Han, et al., CDD/SPARCLE: functional classification of proteins via subfamily domain architectures, Nucleic Acids Res. 45 (D1) (2017) D200–D203.
[27] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (22) (1994) 4673–4680.
[28] J.J. Campanella, L. Bitincka, J. Smalley, MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences, BMC Bioinf. 4 (2003) 29.
[29] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method, Methods 25 (4) (2001) 402–408.
[30] K. Ikegami, Y. Katou, K. Higashi, T. Yoshimura, Localization of circadian clock protein BMAL1 in the photoperiodic signal transduction machinery in Japanese quail, J. Comp. Neurol. 517 (3) (2009) 397–404.
[31] T. Tsunoda, T. Takagi, Estimating transcription factor bindability on DNA, Bioinformatics 15 (7–8) (1999) 622–630.
[32] K. Fujisawa, T. Takami, Y. Kimoto, T. Matsumoto, N. Yamamoto, S. Terai, I. Sakaida, Circadian variations in the liver metabolites of medaka (*Oryzias latipes*), Sci. Rep. 6 (2016) 20916.
[33] D. Wu, F. Rastinejad, Structural characterization of mammalian bHLH-PAS transcription factors, Curr. Opin. Struct. Biol. 43 (2017) 1–9.
[34] J. Chaudhary, M.K. Skinner, Basic helix-loop-helix proteins can act at the E-box within the serum response element of the c-fos promoter to influence hormone-induced promoter activation in Sertoli cells, Mol. Endocrinol. 13 (5) (1999) 774–786.
[35] Z. Wang, Y. Wu, L. Li, X.D. Su, Intermolecular recognition revealed by the complex structure of human CLOCK-BMAL1 basic helix-loop-helix domains with E-box DNA, Cell Res. 23 (2) (2013) 213–224.
[36] I. Kwon, J. Lee, S.H. Chang, N.C. Jung, B.J. Lee, G.H. Son, K. Kim, K.H. Lee, BMAL1 shuttling controls transactivation and degradation of the CLOCK/BMAL1 heterodimer, Mol. Cell. Biol. 26 (19) (2006) 7318–7330.
[37] D. Wu, N. Potluri, Y. Kim, F. Rastinejad, Structure and dimerization properties of the aryl hydrocarbon receptor PAS-A domain, Mol. Cell. Biol. 33 (21) (2013) 4346–4356.
[38] C.C. Lazado, H.P. Kumaratunga, K. Nagasawa, I. Babiak, A. Giannetto, J.M. Fernandes, Daily rhythmicity of clock gene transcripts in Atlantic cod fast skeletal muscle, PLoS One 9 (6) (2014) e99172.
[39] J. Richards, M.L. Gumz, Advances in understanding the peripheral circadian clocks, FASEB J. 26 (9) (2012) 3602–3613.
[40] E.J.W. Geven, P.H.M. Klaren, The teleost head kidney: integrating thyroid and immune signalling, Dev. Comp. Immunol. 66 (2017) 73–83.
[41] I.H. Cuesta, K. Lahiri, J.F. Lopez-Olmeda, F. Loosli, N.S. Foulkes, D. Vallone, Differential maturation of rhythmic clock gene expression during early development in medaka (*Oryzias latipes*), Chronobiol. Int. 31 (4) (2014) 468–478.
[42] M.A. Patino, A. Rodriguez-Illamola, M. Conde-Sieira, J.L. Soengas, J.M. Miguez, Daily rhythmic expression patterns of clock1a, bmal1, and per1 genes in retina and hypothalamus of the rainbow trout, *Oncorhynchus mykiss*, Chronobiol. Int. 28 (5) (2011) 381–389.
[43] M. Mogi, H. Yokoi, T. Suzuki, Analyses of the cellular clock gene expression in peripheral tissue, caudal fin, in the Japanese flounder, *Paralichthys olivaceus*, Gen. Comp. Endocrinol. 248 (2017) 97–105.
[44] L.K. Fonken, M.G. Frank, M.M. Kitt, R.M. Barrientos, L.R. Watkins, S.F. Maier, Microglia inflammatory responses are controlled by an intrinsic circadian clock, Brain Behav. Immun. 45 (2015) 171–179.
[45] M. Keller, J. Mazuch, U. Abraham, G.D. Eom, E.D. Herzog, H.D. Volk, A. Kramer, B. Maier, A circadian clock in macrophages controls inflammatory immune responses, Proc. Natl. Acad. Sci. U. S. A. 106 (50) (2009) 21407–21412.
[46] A. Hida, N. Koike, M. Hirose, M. Hattori, Y. Sakaki, H. Tei, The human and mouse Period1 genes: five well-conserved E-boxes additively contribute to the enhancement of mPer1 transcription, Genomics 65 (3) (2000) 224–233.
[47] K. Lahiri, D. Vallone, S.B. Gondi, C. Santoriello, T. Dickmeis, N.S. Foulkes, Temperature regulates transcription in the zebrafish circadian clock, PLoS Biol. 3 (11) (2005) e351.
[48] M.L. Spengler, K.K. Kuropatwinski, M. Comas, et al., Core circadian protein CLOCK is a positive regulator of NF-kappaB-mediated transcription, Proc. Natl. Acad. Sci. U. S. A. 109 (37) (2012) E2457–E2465.
[49] G. Cavadini, S. Petrzilka, P. Kohler, C. Jud, I. Tobler, T. Birchler, A. Fontana, TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription, Proc. Natl. Acad. Sci. U. S. A. 104 (31) (2007) 12843–12848.
[50] G.M. Leclerc, F.R. Boockfor, Pulses of prolactin promoter activity depend on a noncanonical E-box that can bind the circadian proteins CLOCK and BMAL1, Endocrinology 146 (6) (2005) 2782–2790.
[51] A.C. Silver, A. Arjona, W.E. Walker, E. Fikrig, The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity, Immunity 36 (2) (2012) 251–261.
[52] L. Marpegan, M.J. Leone, M.E. Katz, P.M. Sobrero, T.A. Bekinstein, D.A. Golombek, Diurnal variation in endotoxin-induced mortality in mice: correlation with proinflammatory factors, Chronobiol. Int. 26 (7) (2009) 1430–1442.
[53] C.C. Lazado, M. Gesto, L. Madsen, A. Jokumsen, Interplay between daily rhythmic serum-mediated bacterial killing activity and immune defence factors in rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 72 (2018) 418–425.
[54] C. Binuramesh, R.D. Michael, Diel variations in the selected serum immune parameters in *Oreochromis mossambicus*, Fish Shellfish Immunol. 30 (3) (2011) 824–829.
[55] J.E. Long, M.T. Drayson, A.E. Taylor, K.M. Toellner, J.M. Lord, A.C. Phillips, Morning vaccination enhances antibody response over afternoon vaccination: a cluster-randomised trial, Vaccine 34 (24) (2016) 2679–2685.