Circadian rhythms of clock gene expression in Nile tilapia (*Oreochromis niloticus*) central and peripheral tissues: influence of different lighting and feeding conditions

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Abstract

The present research aimed to investigate the existence of clock gene expression rhythms in tilapia, their endogenous origin, and how light and feeding cycles synchronize these rhythms. In the first experiment, two groups of fish were kept under an LD cycle and fed at two different time points: in the middle of the light (ML) or in the middle of the dark (MD) phase. In the second experiment, fish fed at ML was fasted and kept under constant lighting (LL) conditions for 1 day. In both experiments, the samples from central (optic tectum and hypothalamus) and peripheral (liver) tissues were collected every 3 h throughout a 24 h

cycle. The expression levels of clock genes *bmal1a*, *clock1*, *per1b*, *cry2a,* and *cry5* were analyzed by quantitative PCR. All the clock genes analyzed in brain regions showed daily rhythms: *clock1*, *bmal1a,* and *cry2a* showed the acrophase approximately at the end of the light phase (ZT 8:43-11:22 h), whereas *per1b* and *cry5* did so between the end of the dark phase and the beginning of the light phase, respectively (ZT 21:16-4:00 h). These rhythms persisted under constant conditions. No effect of the feeding time was observed in the brain. In the liver, however, the rhythms of *clock1* and *cry5* were influenced by feeding, and a shift was observed in the MD fish group (ZT 3:58 h for *clock1* and 11:20 h for *cry5*). This study provides the first insights into the molecular clock of tilapia, a very important fish species for aquaculture. It also reveals the endogenous origin of clock gene rhythms and the ability of feeding time to shift the phase in some clock genes in the peripheral, but not the central, oscillator.

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Keywords

Teleost fish
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Introduction

Changes in environmental factors, such as light or food availability (e.g., prey abundance), are cyclic and can, hence, be predicted. Evolution has selected the occurrence of time-keeping systems or biological clocks, which confer animals with adaptive advantages, such as the capacity to anticipate environmental variations, and are, thus, prepared for forthcoming changes. The control of biological rhythms relies on pacemakers located in anatomical structures within the organism. The central circadian pacemaker in mammals is the suprachiasmatic nucleus (SCN), located in the hypothalamus (Reppert and Weaver 2002; Welsh et al. 2010). In mammals, the circadian system is primarily hierarchical, and

the SCN controls most overt circadian rhythms described to date (Dibner et al. 2010). The most important environmental factor that entrains the SCN is the light–dark (LD) cycle. Indeed, the SCN is also called the light entrainable oscillator (LEO) (Reppert and Weaver 2002).

At the molecular level, circadian rhythms are controlled by a self-sustainable molecular clock, the core of which is formed by both positive and negative transcriptional–translational loops (Shearman et al. 2000; Pando and Sassone-Corsi 2002). In vertebrates, the positive loop of the clock is formed mainly by two transcription factors, CLOCK and BMAL, which heterodimerize and activate the transcription of other genes by binding to a promoter region enhancer known as E-box (Gekakis et al. 1998; Reppert and Weaver 2002). Among the genes activated by CLOCK:BMAL, several are from the *Per* and *Cry* families, whose products PER and CRY translocate to the nucleus and repress CLOCK and BMAL, and close the negative feedback loop (Shearman et al. 2000; Reppert and Weaver 2002). Oscillations in the molecular clock are generally orchestrated by the SCN. However, in mammals, the molecular clock of peripheral oscillators, such as the liver, can be uncoupled from the phase of the SCN by means of restricted feeding, and also independently of lighting conditions (Damiola et al. 2000; Stokkan et al. 2001). Indeed, restricted feeding can entrain clock gene expression rhythms in the liver of SCN-ablated mice (Hara et al. 2001). These results indicate the existence of a food-entrainable oscillator (FEO) in mammals, which could work independently of the LEO (Dibner et al. 2010). In fish, however, studies on the effect of a shifted feeding time on the molecular clock of peripheral oscillators are still scarce.

After describing the existence and components of the molecular clock in mammals, several studies have searched for the presence of the molecular clock and the homologous clock genes in fish. The first fish species in which the molecular clock was described was zebrafish (*Danio rerio*) (Cahill 2002; Vatine et al. 2011), which is a major model organism used in research into genetics, development, physiology, toxicology, and biomedicine (Fishman 2001; Grunwald and Eisen 2002). In recent years, the existence of a molecular core clock has also been reported for several fish species other than zebrafish, such as medaka (*Oryzias latipes*) (Cuesta et al. 2014), goldfish (*Carassius auratus*) (Velarde et al. 2009), European sea bass (*Dicentrarchus labrax*) (Sánchez et al. 2010; Del Pozo et al.

2012), gilthead seabream (*Sparus aurata*) (Vera et al. 2013), Senegalese sole (*Solea senegalensis*) (Martín-Robles et al. 2011, 2012), Atlantic salmon (*Salmo salar*) (Davie et al. 2009), Atlantic cod (*Gadus morhua*) (Lazado et al. 2014), rainbow trout (*Oncorhynchus mykiss*) (López-Patiño et al. 2011), and two blind cavefish species (*Phreatichthys andruzzii* and *Astyanax mexicanus*) (Cavallari et al. 2011; Beale et al. 2013). Although the presence of the molecular clock and clock genes seems common in fish, describing it in other species as one of the first steps to study their circadian system is essential.

Tilapia (*Oreochromis niloticus*) is a native omnivorous fish species from Africa that belongs to the phylogenetic group of Cichlids (Eknath and Hulata 2009). This fish presents high tolerance to intensive culture, reproduction capacity throughout the year, good market acceptance, strong resistance to diseases, and relative easiness for genetic manipulation related to improved production (Ng and Romano 2013). Tilapia is bred and cultured worldwide, and is the second most cultured freshwater fish species after carp (Ng and Romano 2013). Despite the high economic and scientific relevance of tilapia, very little is known about its biological rhythms and circadian system. A very few studies have been conducted to date, focusing on daily rhythms of behavior and rhythms in the somatotropic axis (Fortes-Silva et al. 2010; Costa et al. 2016).

The objectives of this study were to test (1) the existence of daily rhythms of the expression of several clock genes from both the positive and negative loops of the molecular clock (*clock1*, *bmal1a*, *per1b*, *cry2a,* and *cry5*) on central (two brain regions: optic tectum and hypothalamus) and peripheral (liver) tilapia tissue; and (2) the endogenous origin of these rhythms under constant conditions (fasting and constant light, LL), and the synchronizing effect of light and feeding cycles.

Materials and methods

Animals and housing

Experiments were conducted on 120 fish (tilapia, *Oreochromis niloticus*) whose average body weight was 89.0 ± 36.8 g (mean ± SD). Fish were obtained from a local fish farm (Valenciana de Acuicultura, Puzol, Spain) and were housed in the Chronobiology Laboratory of the University of Murcia (east Spain). Fish were placed in 100 l tanks in a closed water

recirculation system equipped with biological and mechanical filters. Water quality parameters (pH, dissolved oxygen, ammonia, nitrate, and nitrite) were measured daily. The photoperiod was set at a 12:12 LD (light:dark) cycle, with lights on at 08:00 h (*Zeitgeber* Time 0, ZT 0 h). Water temperature was controlled at 28.0 ± 0.5 °C. Throughout acclimation and during the experimental period, fish were fed a commercial diet with 36 % of crude protein (D-4 Alterna Basic 2P, Skretting AS, Spain) at a daily rate of 1 % of fish body weight.

Experimental design

All the experimental procedures complied with the guidelines of the European Union (2010/63/UE) and Spanish legislation (RD 1201/2005 and Law 32/2007) for the use of laboratory animals. Experimental protocols were approved by the National Committee and the Committee of the University of Murcia on Ethics and Animal Welfare.

Fish were weighed and divided into 24 different tanks upon arrival (n = 5 fish per tank) and were kept under lab conditions for acclimation purposes for 2 weeks. During acclimation, fish were fed randomly in the light phase. Then, 16 groups of fish were fed once a day at a fixed time in the middle of the light phase (ML, ZT 6 h). The other 8 groups of fish were fed once a day at a fixed time in the middle of the dark phase (MD, ZT 18 h). Food was provided by automatic feeders (Eheim, Germany), adjusted to provide a daily food ratio of 1 % of fish body weight. Locomotor activity was monitored and recorded throughout the experimental period with an infrared photocell (Omron, mod E3S-AD62, Kyoto, Japan) placed inside the water and fixed to the wall of the tank. The water depth in the experimental tanks was 75 cm, and the photocell was located 15 cm below the water surface. The number of light-beam interruptions was counted and stored every 10 min by a computer connected to the photocell.

After 40 days under the experimental conditions, 8 groups of fish from each feeding condition (ML and MD feeding) were sampled every 3 h throughout a 24 h cycle. The 8 tanks from each feeding condition were sampled at different time points (one tank of fish per sampling point): the first point was collected at ZT 6 h, and then, at ZT 9, 12, 15, 18, 21, 0, and 3 h. Fish were fasted on the sampling day. The samples at ZT 0 h were collected immediately after lights went on, and those at ZT 12 h were collected immediately after lights went off. Fish were anesthetized with

eugenol (clove oil essence, Guinama, Valencia, Spain) at a concentration of 50 µl/l, and were euthanized by decapitation. Then, the hypothalamus, optic tectum, and liver tissue samples were collected and placed into sterile RNase and DNase 1.5-ml tubes. The tubes that contained the samples were immediately frozen in dry ice and stored at −80 °C until analyzed.

After the first sampling day, the other tanks (8 with fixed feeding at ML) were sampled every 3 h throughout a 24 h cycle. During this second sampling, fish were fasted, and lights were kept on throughout the 24 h (constant light, LL) to check for the presence of rhythmicity with the absence of external cues (LD and feeding). The 8 tanks were sampled at different time points (one tank of fish per sampling point): the first point was collected at CT 6 h (Circadian Time, by establishing CT 0 h as the beginning of the subjective day), and then at CT 9, 12, 15, 18, 21, 0, and 3 h. Fish manipulation, sample collection, and storage were performed as described above.

Real time RT-PCR analysis

The optic tectum, hypothalamus and liver tissue samples were transferred to sterile tubes that contained 0.5 ml of Trizol (Invitrogen, CA, USA). Tissue samples were mechanically homogenized, and total RNA extraction was performed according to the manufacturer's instructions (Invitrogen). The RNA pellet was dissolved in sterile DEPC water (Invitrogen), and the RNA concentration was determined by spectrometry (Nanodrop ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). In the next step, total RNA (1 µg) was retrotranscribed by a commercial kit (QuantiTect Reverse Transcription Kit, Qiagen, Germany), which included a genomic DNA elimination step. cDNA was subjected to quantitative PCR analyses in a light thermocycler (7500 Real-Time PCR system, Applied Biosystems, CA, USA) following this protocol: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Quantitative PCR reactions were performed using the SYBR Green PCR Master Mix (Applied Biosystems). All the samples were run in triplicate. Melting curves were run after amplification to ensure that only one DNA species was being amplified. The PCR reaction was performed in a final volume of 20 µl. The primers sequences are shown in Table 1. Primers for tilapia clock genes were designed with the Primer3 software (Untergasser et al. 2012) using the sequence obtained for each gene from the Ensembl database

(www.ensembl.org). Primer sequences for the two housekeeping genes, *ef1a* and *18-s*, were obtained from a previously published paper (Yang et al. 2013). The relative amplification efficiencies of all the genes were analyzed by cDNA dilution curves, and it was verified that they were similar for all the genes. Primer concentrations were determined by means of a primer dilution curve. The primers of *clock1*, *bmal1a*, *cry2a*, *cry5*, *ef1a,* and *18-s* were added at a final concentration in the reaction of 200 nM, and the primers of *per1b* were added at a final concentration of 400 nM. The relative expression of all genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Two housekeeping genes, *ef1a* and *18-s*, were selected after checking that the coefficient of variation (CV) for each gene within each tissue was lower than 5 %. The geometric mean of both housekeeping genes for each sample was used for the normalization, as suggested elsewhere (Vandesompele et al. 2002).

Table 1

Primer sequences used for quantitative PCR analyses

Gene	F/R	Sequence (5′-3′)	Ensembl/GenBank
clock1	F	CCAGACGCAGACAATTAGCA	ENSONIG00000002943
	R	CGAGGAGCCTGTAGGAACTG	
bmal1a	F	GATGGCTTCCTGTTTGTGGT	ENSONIG00000010970
	R	GGGGCTGTATCAGAGGATGA	
per1b	F	TCCACAGAGTCTTGGTGCAG	ENSONIG00000009248
	R	GCAAATCTGCTGAAACGTCA	
cry2a	F	GGCCCTAAATTCCATCCACT	ENSONIG00000014884
	R	CGTCCAGATCCTCCAAACAT	
cry5	F	CCGCAGACTTCTCCAAACTC	ENSONIG00000002547
	R	ACACGCTGTTACCCTCCATC	
ef1α	F	GCACGCTCTGCTGGCCTTT	ENSONIG00000017760
	R	GCGCTCAATCTTCCATCCC	
18-s	F	GGACACGGAAAGGATTGACAG	JF698683
	R	GTTCGTTATCGGAATTAACCAGAC	

Data analysis

The activity data were analyzed, and the representation of actograms and mean waveforms was performed with the El Temps chronobiology software (version 1.275, Prof. Díez-Noguera, University of Barcelona). The data of each clock gene were subjected to a Cosinor analysis, performed by El Temps. The Cosinor analysis is based on the least squares approach of time series data with a cosine function of a known period of the type Y = Mesor + Amplitude * cos ((2π(t-Acrophase)/Period). The Cosinor analysis also provides the statistical significance of the rhythm by an F test of the variance accounted for by the waveform versus a straight line of zero-amplitude (null hypothesis). The data of each gene from both the ML- and MD-fed fish were subjected to a two-way ANOVA, followed by a Tukey's post hoc test, to check for any statistically significant differences among times of day, feeding groups and both variables. The data from each gene in the group under LL were subjected to a one-way ANOVA, followed by a Tukey's post hoc test, to check for any statistically significant differences among the time points. Statistical analyses (one- and two-way ANOVAs) were performed using the SPSS software (v. 19.0, IBM, Armonk, NY, USA). In the behavioral data, the duration of food-anticipatory activity (FAA) was determined as the time which elapsed between the feeding time and increased anticipatory activity, which was defined as a 2.5-fold increase over baseline activity, and which was sustained for at least 30 min and not followed by any inflection for more than 1 h, as described elsewhere (Stephan 1997). Baseline activity was defined as the median of daily locomotor activity. Data are expressed as mean ± SEM, except the activity data in the meanwaves, which are expressed as mean ± SD. The significance threshold (α) was set at 0.05 in all the statistical tests performed.

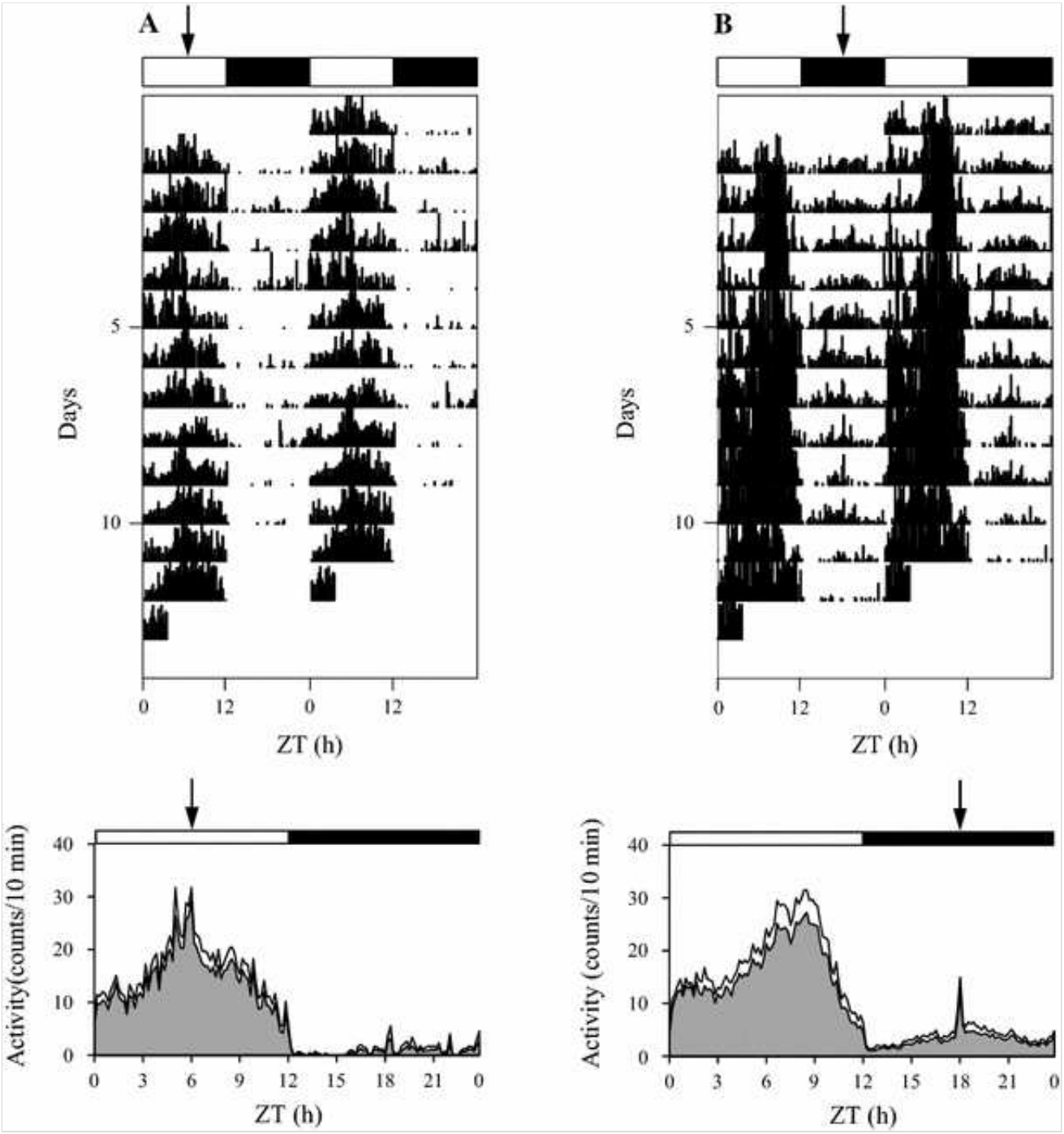
Results

During the 14 days prior to sample collection, fish displayed most of their daily activity in the light phase, although the percentage of activity displayed in this phase was significantly lower (t test, $p < 0.05$) in the animals fed at MD compared with those fed at ML (84.0 ± 3.1 % and 94.7 ± 1.8 % for MD and ML feeding, respectively) (Fig. 1). The ML groups showed food-anticipatory activity (FAA) by displaying increased locomotor activity 3.5 h before mealtime (Fig. 1a). This was followed by a gradual decrease in activity after mealtime, which lasted 4 h before

returning to the activity levels below baseline activity. The activity displayed at night by fish fed at MD was significantly higher than the activity of fish fed at ML (*t* test, $p < 0.05$), indicating some effect of feeding at night. Fish fed at MD showed a progressive increase in their activity before feeding time, followed by a peak at MD and then a gradual decrease (Fig. 1b). However, this could not be consider a significant FAA as only the sharp increase in activity for the 10 min interval immediately after feeding was above the threshold used to detect FAA (2.5-fold increase over baseline activity).

Fig. 1

Representative actograms of a group of tilapia fed at ML (**a**) or at MD (**b**). Meanwaves from each group are represented below each actogram. Mealtime is indicated by the *black arrow* at the top of the actograms and meanwaves. In the actograms, for convenient visualization, data were *double-plotted* (48 h) with activity binned every 10 min. The height of each point represents the number of interruptions of the infrared light beam. The *bars* above each actogram and meanwave represent the light regime; *open and black bars* represent light and dark, respectively. Each point in the mean waveform was calculated as mean ± SD from the 10 min binned data over the last 14 days prior to sample collection ($n = 14$), as represented in the actogram. The *gray area* represents the mean values and the continuous line SD



In the optic tectum, all the clock genes analyzed in the fish under LD cycles displayed significant daily rhythms (Cosinor, $p < 0.05$) (Fig. 2; Table 2). The genes from the positive loop (*clock1* and *bmal1a*) showed the acrophase at the end of the light phase (ZT 9:42–10:51 h) (Fig. 2a, c; Table 2). Among the genes from the negative loop of the clock, *cry2a* presented the acrophase around ZT 8–9 h (Fig. 2g), *per1b* presented it at the end of the dark phase (ZT 21:27 and 22:44 h in the fish fed at ML and MD, respectively) (Fig. 2e), and *cry5* did so at the beginning of the light phase (ZT 1:35 and 1:51 h in the fish fed at ML and MD, respectively) (Fig. 2i). Besides the significance of sinusoidal rhythmicity, the five clock genes showed statistically significant differences depending on the time of

the day (two-way ANOVA, $p < 0.05$), but no significant differences were observed between the ML- and MD-fed fish in any of the analyzed clock genes (two-way ANOVA, $p > 0.05$). Under the LL conditions, all the analyzed clock genes displayed circadian rhythmicity (Cosinor, $p < 0.05$) (Fig. 2; Table 2). The acrophase calculated by the Cosinor analysis under LL showed a delay compared with the acrophase calculated under the LD cycles in *clock1*, *bmal1a*, *per1b,* and *cry2a* (Table 2). The acrophase of *cry5* under LL showed an advance (CT 23:35 h) compared with its acrophase at LD in the ML-fed fish (ZT 1:35 h) (Table 2). Statistically significant differences depending on the time of the day were observed for all clock genes analyzed (one-way ANOVA, $p < 0.05$) (Fig. 2b, d, f, h, j). In addition, statistically significant differences between ML- and MD-fed fish were observed for *cry5* expression (two-way ANOVA, $p < 0.05$).

Fig. 2

Daily variations of the relative expression of clock genes in the optic tectum of tilapia under the LD (**a**, **c**, **e**, **g**, and **i**) and LL (**b**, **d**, **f**, **h**, and **j**) conditions. The analyzed clock genes were *clock1* (**a**, **b**), *bmal1a* (**c**, **d**), *per1b* (**e**, **f**), *cry2a* (**g**, **h**), and *cry5* (**i**, **j**). The tilapia maintained under LD cycles was divided into two groups, and fed once a day at different times: in the middle of the light phase (ML) (*white circles*) or in the middle of the dark phase (MD) (*black circles*). Another group of fish were maintained under LD and were fed once a day at ML, then lights remained constant (LL), and fish were fasted on the sampling day (*gray circles*). The *white and black bars* above the graphs represent the light and dark periods, respectively. The *white and black arrows* indicate the feeding times at ML and MD, respectively. Data are expressed as mean ± SEM ($n = 5$ for each time point). The data from the groups sampled at LD were subjected to a two-way ANOVA ($p < 0.05$), followed by a Tukey's post hoc test. The data from the group sampled at LL were subjected to a one-way ANOVA ($p < 0.05$), followed by a Tukey's post hoc test. *Different letters* indicate significant differences among the time points in the same graph

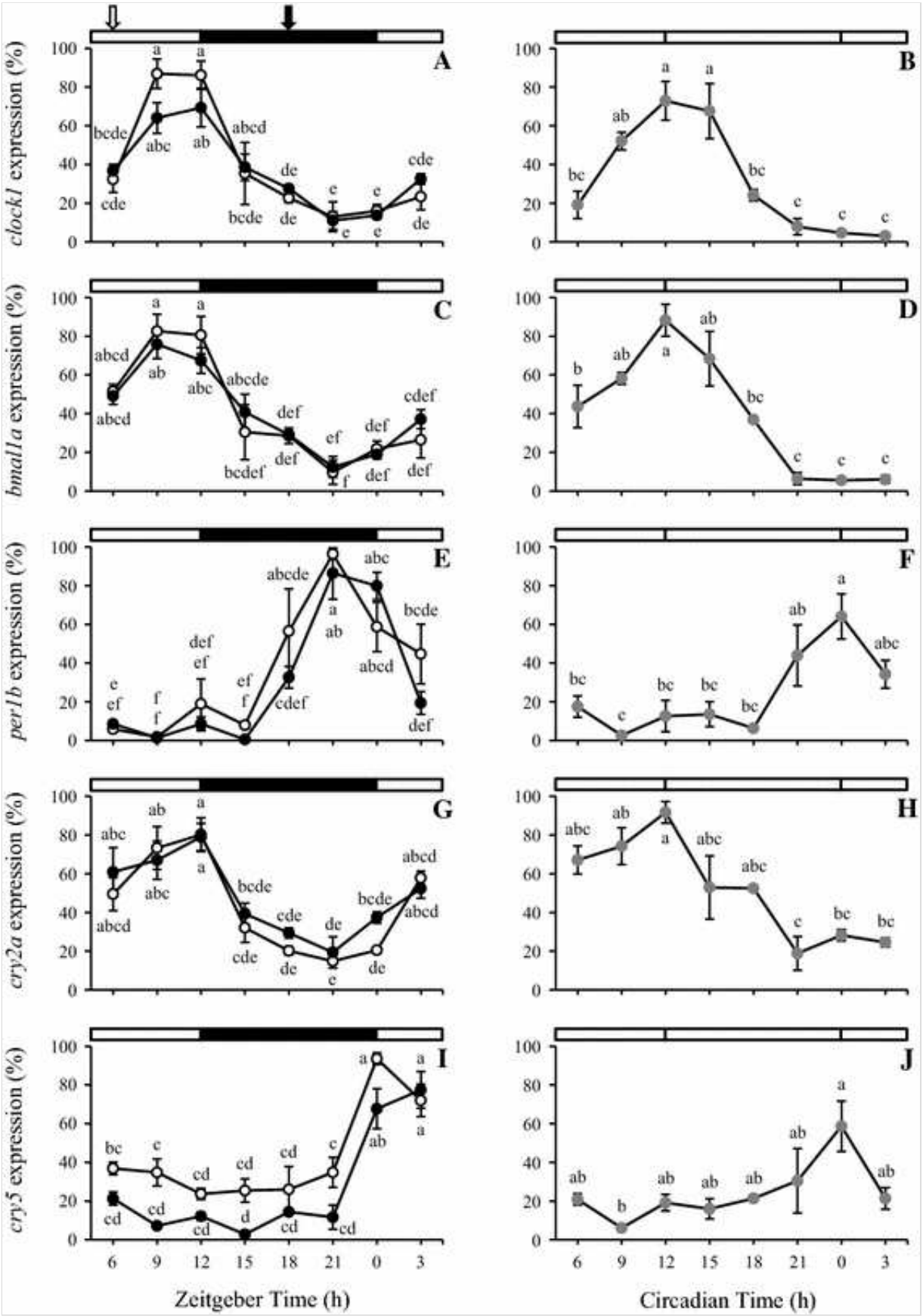


Table 2

Acrophase and statistical significance values of the variables subjected to the Cosinor

	Optic Tectum		Hypothalamus		Liver
	Acrophase (ZT)	Sign.	Acrophase (ZT)	Sign.	Acrophase (ZT)

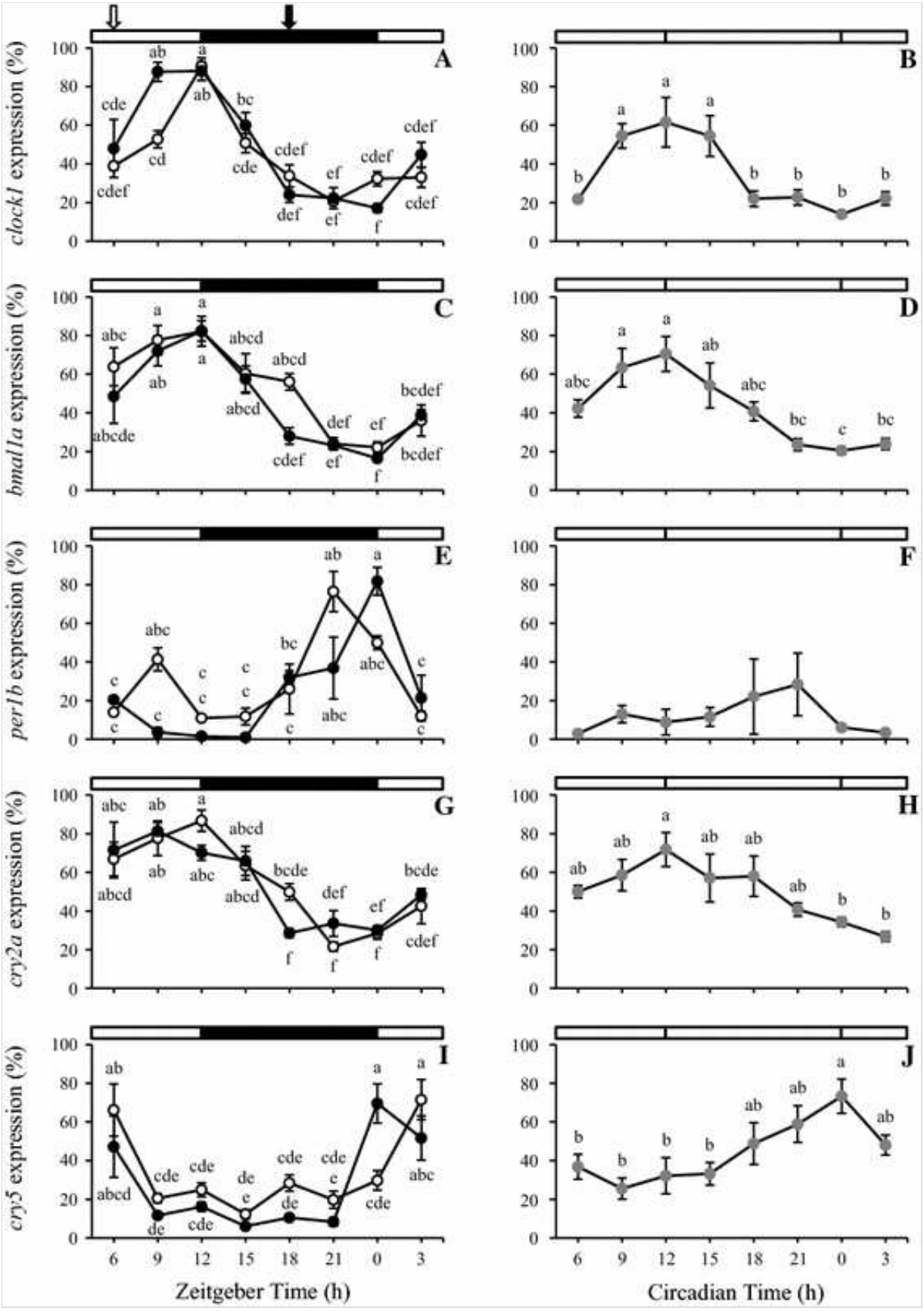
clock1					
LD/ML	10:51 ± 1:12	***	11:22 ± 1:22	***	9:31 ± 1:20
LD/MD	10:18 ± 1:09	***	10:20 ± 1:04	***	3:58 ± 3:56
LL	12:27 ± 1:01	***	12:04 ± 1:31	***	14:13 ± 1:41
bmal1a					
LD/ML	10:19 ± 1.12	***	11:04 ± 1:11	***	9:07 ± 1:18
LD/MD	9:42 ± 1:03	***	10:39 ± 1:09	***	–
LL	11:54 ± 0:51	***	11:40 ± 1:23	***	14:31 ± 1:26
per1b					
LD/ML	21:27 ± 1:53	***	21:16 ± 3:59	*	20:53 ± 4:50
LD/MD	22:44 ± 1:09	***	23:14 ± 1:57	***	–
LL	23:53 ± 1:56	***	–	0.17	7:46 ± 3:11
cry2a					
LD/ML	8:57 ± 1:24	***	10:38 ± 1:04	***	–
LD/MD	8:43 ± 1:34	***	9:36 ± 1:19	***	–
LL	10:34 ± 1:44	***	12:50 ± 2:11	***	–
cry5					
LD/ML	1:35 ± 1:45	***	4:00 ± 1:55	***	4:24 ± 2:55
LD/MD	1:51 ± 1:24	***	2:50 ± 1:43	***	11:20 ± 4:22
LL	23:35 ± 3:13	*	22:47 ± 1:59	***	20:19 ± 4:16
Data are expressed as value ± fiducial limits (set at 95 %). The acrophase is indica *Zeigeber* time hours (ZT) for the samples under LD or in circadian time hours (CT the samples under LL					
* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$					

In the hypothalamus, all the analyzed genes displayed significant daily rhythms under LD cycles in both the ML- and MD-fed fish (Cosinor, $p < 0.05$) (Fig. 3; Table 2). Under these conditions, the acrophases were similar to those observed in the optic tectum (Table 2): *clock1*, *bmal1a,* and *cry2a* presented the acrophase at the end of the light phase (ZT 9:36–11:22 h) (Fig. 3a, c, g), *per1b* presented it at the end of the dark phase (ZT

21:16 and 23:14 h in the fish fed at ML and MD, respectively) (Fig. 3e), and *cry5* did so at the beginning of the light phase (ZT 4:00 and 2:50 h in the fish fed at ML and MD, respectively) (Fig. 3i). All the genes showed statistically significant differences depending on the time of the day under the LD cycles (two-way ANOVA, $p < 0.05$), but no significant differences were observed between the ML- and MD-fed fish (two-way ANOVA, $p > 0.05$). Under the LL conditions, *clock1*, *bmal1a*, *cry2a,* and *cry5* displayed circadian rhythmicity (Cosinor, $p < 0.05$), but not *per1b* (Cosinor, $p = 0.17$) (Table 2). As observed in the optic tectum, the acrophase of *clock1*, *bmal1a,* and *cry2a* under LL showed a delay compared with the acrophase calculated under LD, whereas that of *cry5* under LL showed an advance (Table 2). In addition, *clock1*, *bmal1a*, *cry2a,* and *cry5* showed statistically significant differences depending on the time of the day (one-way ANOVA, $p < 0.05$) (Fig. 3b, d, h, j), but not *per1b* (one-way ANOVA, $p = 0.56$) (Fig. 3f).

Fig. 3

Daily variations of the relative expression of the clock genes in the hypothalamus of the tilapia under the LD (**a**, **c**, **e**, **g**, and **i**) and LL (**b**, **d**, **f**, **h**, and **j**) conditions. The analyzed clock genes were *clock1* (**a**, **b**), *bmal1a* (**c**, **d**), *per1b* (**e**, **f**), *cry2a* (**g**, **h**), and *cry5* (**i**, **j**). The tilapia maintained under LD cycles was divided into two groups, and fed once a day at different times: in the middle of the light phase (ML) (*white circles*) or in the middle of the dark phase (MD) (*black circles*). Another group of fish were maintained under LD and were fed once a day at ML, then lights remained constant (LL), and fish were fasted on the sampling day (*gray circles*). The *white and black bars* above the graphs represent the light and dark periods, respectively. The *white and black arrows* indicate the feeding times at ML and MD, respectively. Data are expressed as mean ± SEM ($n = 5$ for each time point). The data from the groups sampled at LD were subjected to a two-way ANOVA ($p < 0.05$), followed by a Tukey's post hoc test. The data from the group sampled at LL were subjected to a one-way ANOVA ($p < 0.05$), followed by a Tukey's post hoc test. *Different letters* indicate significant differences among the time points in the same graph



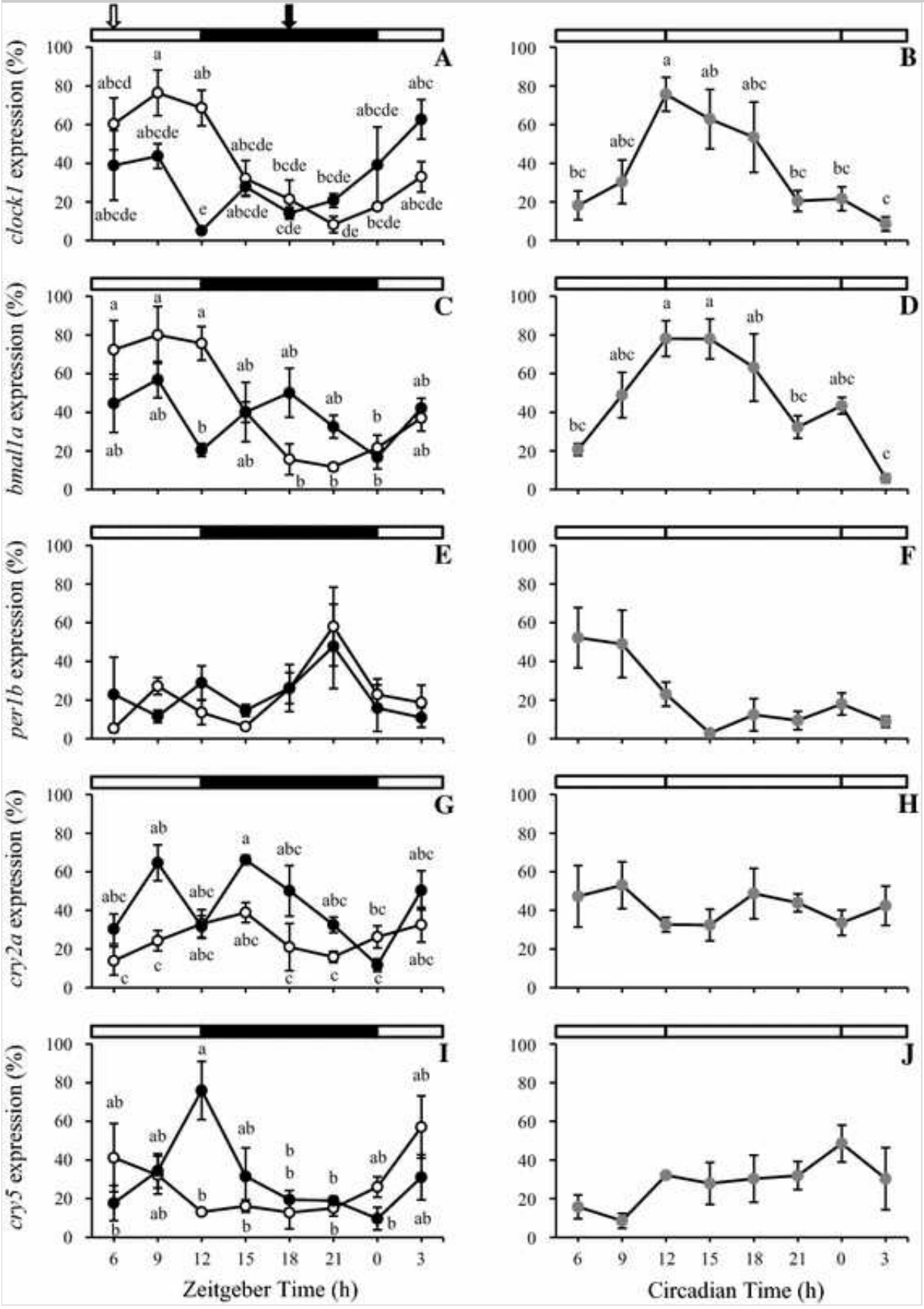
In the liver, unlike that observed in brain regions, the clock gene expression results differed in the ML compared with the MD-fed fish (Fig. 4; Table 2). In the ML-fed group, *clock1*, *bmal1a*, *per1b,* and *cry5* displayed statistically significant daily rhythms (Cosinor, $p < 0.05$) (Table 2) with acrophases located at similar times to those observed in the

optic tectum and the hypothalamus: *clock1* and *bmal1a* toward the end of the light phase (ZT 9:31 and 9:07 h, respectively) (Fig. 4a, c), *per1b* at the end of the dark phase (ZT 20:53 h) (Fig. 4e), and *cry5* in the first half of the light phase (ZT 4:24 h) (Fig. 4i). On the other hand, fish fed at MD only showed significant rhythms in *clock1* and *cry5* (Fig. 4a, i). Interestingly, the acrophases of both genes (ZT 3:58 h for *clock1* and 11:20 h for *cry5*) shifted compared with the liver of the ML-fed fish (ZT 9:31 for *clock1* and 4:24 h for *cry5*) and the rhythms observed in brain regions (Table 2). The shift observed in the liver of MD-fed fish compared with the liver of the ML group was of 5:33 h for *clock1* and 6:56 h for *cry5* (Table 2). In addition, *clock1*, *bmal1a*, *cry2a,* and *cry5* showed statistically significant differences depending on the time of the day (two-way ANOVA, $p < 0.05$) (Fig. 4c, e, i), whereas *cry2a* and *cry5* also showed significant differences between groups (ML *vs.* MD feeding) (two-way ANOVA, $p < 0.05$). Under LL, *clock1*, *bmal1a*, *per1b,* and *cry5* displayed circadian rhythmicity (Cosinor, $p < 0.05$) (Fig. 4b, d, f, j; Table 2), but not *cry2a* (Cosinor, $p = 0.75$) (Fig. 4h; Table 2). The acrophase of *clock1* and *bmal1a* showed a delay compared with the acrophase under LD: 4:43 h later for *clock1* and 5:24 h later for *bmal1a* (Table 2). A shift of more hours was noted for *per1b* and *cry5* between the acrophase under LL and that under LD: 10:53 h later for *per1b* and 8:05 h earlier for *cry5* (Table 2). Finally, only *clock1* and *bmal1a* gave statistically significant differences depending on the time of the day in the liver of fish under LL (one-way ANOVA, $p < 0.05$) (Fig. 4b).

Fig. 4

Daily variations of the relative expression of clock genes in the liver of the tilapia under the LD (**a**, **c**, **e**, **g**, and **i**) and LL (**b**, **d**, **f**, **h**, and **j**) conditions. The analyzed clock genes were *clock1* (**a**, **b**), *bmal1a* (**c**, **d**), *per1b* (**e**, **f**), *cry2a* (**g**, **h**), and *cry5* (**i**, **j**). The tilapia maintained under LD cycles was divided into two groups, and fed once a day at different times: in the middle of the light phase (ML) (*white circles*) or in the middle of the dark phase (MD) (*black circles*). Another group of fish were maintained under LD and were fed once a day at ML, lights remained constant (LL), and fish were fasted on the sampling day (*gray circles*). The *white and black bars* above the graphs represent the light and dark periods, respectively. The *white and black arrows* indicate the feeding times at ML and MD, respectively. Data are expressed as mean ± SEM ($n = 5$ for each time point). The data from the groups sampled at LD were subjected to a two-way ANOVA ($p < 0.05$),

followed by a Tukey's post hoc test. The data from the group sampled at LL were subjected to a one-way ANOVA ($p < 0.05$), followed by a Tukey's post hoc test. *Different letters* indicate significant differences among the time points in the same graph



Discussion

In this study, all the clock genes displayed daily rhythms in the analyzed brain regions (optic tectum and hypothalamus). These rhythms persisted under constant conditions, which indicate that tilapia possesses an endogenous molecular clock with similar characteristics to those described in other fish species. In the liver, clock genes in the fish fed at ML showed similar circadian rhythms to those observed in the brain regions. On the other hand, in the liver of fish fed at MD, only *clock1* and *cry5* displayed significant rhythmicity. Interestingly, the daily rhythms of *clock1* and *cry5* in the liver were shifted by MD feeding compared with ML, suggesting a strong influence of food on this peripheral oscillator.

When food is periodically restricted, feeding can act as a potent synchronizer of rhythms in animals by eliciting an increase in locomotor activity several hours before mealtimes, which is known as food-anticipatory activity (FAA), and is driven by the FEO (Mistlberger 2009). FAA confers an adaptive advantage as it allows animals to anticipate and prepare for forthcoming meals (Mistlberger 2009). The present experiment is, as far as we know, the first to submit tilapia to a scheduled feeding time. The fish fed at ML showed FAA, which has been observed in other fish species (López-Olmeda and Sánchez-Vázquez 2010; López-Olmeda et al. 2010). The fish fed in the dark phase was predominantly diurnal and failed to show FAA. Although the Nile tilapia was first described as a diurnal animal, recent studies have also reported nocturnal patterns under some conditions, e.g., isolated individuals or tilapia groups allowed to self-feed (Vera et al. 2009; Fortes-Silva et al. 2010). This ability to display either diurnal or nocturnal behavior in the same species is known as dualism, which, in fish, is related to a flexible circadian system (López-Olmeda et al. 2012). In some fish species, a switch from diurnal to nocturnal behavior can be achieved by feeding fish at a fixed time in the dark phase (Sánchez-Vázquez et al. 1997; Montoya et al. 2010), but this does not seem to be the case in tilapia. Instead nocturnal behavior in this species seems to occur spontaneously; e.g., when tilapia is allowed to self-feed (Fortes-Silva et al. 2010).

Generation of all circadian rhythms relies on the molecular clock present in cells, whose core is formed by the so-called clock genes. Fish have undergone a genome duplication event during their evolution, which is why they present a larger number of clock gene copies compared with

mammals (Postlethwait et al. 1998). Some groups of clock genes present different acrophases depending on the isoform, which is especially relevant for *per* and *cry* genes, and suggest the possibility of different roles in the organism (Vatine et al. 2011). In tilapia brain regions, the analyzed clock genes showed similar daily rhythmic patterns to other fish species that have been studied to date. Positive elements *clock1* and *bmal1a* presented a peak at the end of the light phase and the beginning of the dark phase, which has been observed in zebrafish, medaka, gilthead seabream, and rainbow trout (Cahill 2002; López-Patiño et al. 2011; Vera et al. 2013; Cuesta et al. 2014). Regarding the negative molecular clock elements, *per1b* presented the acrophase at the end of the dark phase, which also occurs in zebrafish, medaka, and European sea bass (Cahill 2002; Sánchez et al. 2010; Cuesta et al. 2014), whereas *cry2a* in tilapia peaked toward the end of the light phase, as reported for zebrafish, goldfish, and European sea bass (Velarde et al. 2009; Vatine et al. 2011; Del Pozo et al. 2012). Finally, the acrophase of *cry5* in tilapia was located during the first hours after lights went on as described in zebrafish and medaka (Cavallari et al. 2011; Cuesta et al. 2014).

One characteristic of circadian pacemakers is their ability to continue the oscillations under constant environmental conditions, which is known as free-running. When the synchronizer is removed, the biological rhythm free-runs from a phase determined previously by the synchronizer (Moore-Ede et al. 1982). The phase in free-running is similar to, but not the same as, the synchronizer. In free-running, the period of the biological rhythm matches the oscillator's endogenous period (τ), which comes close to 24 h (circadian) (Moore-Ede et al. 1982). In the pacemaker, the molecular clock is formed by a self-sustainable transcriptional–translational feedback loop that also free-runs under constant conditions (Reppert and Weaver 2002). In this study, almost all the clock genes analyzed in tilapia under LL, except *per1b* in the hypothalamus and *cry2a* in the liver, displayed free-running under constant conditions. The phase of the clock genes under LL was similar to the phase observed under LD, which shows that the molecular clock in this species forms part of a circadian pacemaker entrained by the LD cycle.

In mammals, a shift in feeding time is able to shift the phase of peripheral oscillators, e.g., the liver, regardless of lighting conditions (Damiola et al. 2000; Stokkan et al. 2001). In fish, the effects of conflicting *zeitgebers*

(light *vs.* food) have barely been explored to date. In tilapia, no effect of food was observed in central brain tissues, which has also been reported in other fish species, such as zebrafish and gilthead seabream (López-Olmeda et al. 2010; Vera et al. 2013), which have shown that light is the most important synchronizer in these areas. Conversely, in the liver of tilapia, the phase of *clock1* and *cry5* was shifted 5.5 and 7 h, respectively, by MD feeding compared with the phase of these genes in the liver of the ML-fed fish and in the brain. This partial shift is similar to that observed in previous studies conducted with fish under shifted feeding schedules (ML *vs.* MD) and LD cycles, which showed a shift of between 6 and 8 h in the phase of several clock genes in the liver (López-Olmeda et al. 2010; Vera et al. 2013). This is a different situation to that observed in mammals, in which a 12 h shift is observed when they are submitted to restrict feeding schedules with a 12 h difference (Damiola et al. 2000). Indeed, the mammalian FEO seems independent of the LEO as the SCN is not required for feeding to entrain rhythms in the livers of SCN-ablated mice (Hara et al. 2001). Conversely, in fish, data from the clock gene expression in peripheral tissues and behavior have suggested that both the LEO and FEO display some degree of coupling, and two hypothesis have been put forward: (1) both oscillators are anatomically separated, but tightly coupled, through some signaling (nervous, endocrine, etc.); and (2) a single oscillator entrainable by both light and food is present, and one synchronizer is stronger than the other (Sánchez-Vázquez et al. 1997; Aranda et al. 2001; López-Olmeda and Sánchez-Vázquez 2010).

In the liver, some genes were affected by feeding time (*clock1* and *cry5*), whereas other genes seemed to be more affected by light (*bmal1a*, *per1b,* and *cry2a*). These different responses to the two *zeitgebers* could be due to differences in the promoter region. In fish, two main regulatory elements of clock gene expression have been described: E-boxes and D-boxes. E-boxes are essential in circadian regulation, and they are the binding site for a multitude of basic helix-loop-helix transcription factors, among them CLOCK:BMAL heterodimers (Vallone et al. 2004), whereas D-boxes are implicated in light-regulated phase shifting of the clock and in clock output pathways (Vatine et al. 2009). In addition, other elements can play an important role in the regulation of clock gene expression. For instance, glucocorticoids have been suggested to act as an input used by the central pacemaker to set the phase of peripheral oscillators, such as the liver, an effect that seems to be mediated, at least in part, through glucocorticoid

response elements (GRE) present in the promoter regions of the clock genes (Sánchez-Bretaño et al. 2016). Moreover, zebrafish cells and tissues are photoreceptive, and their clock gene expression can be directly entrained by light (Whitmore et al. 2000); hence, a direct light response in peripheral tissues of tilapia, such as the liver, could not be discarded. Nevertheless, further research is required to elucidate this hypothesis, especially sequencing and identification of the elements present in the promoter regions of clock genes in tilapia.

In summary, this is the first paper to describe the circadian rhythms of the clock gene expression in tilapia, a fish that is most important for worldwide aquaculture. This study revealed the presence of a functional molecular clock in this species with similar characteristics to other fish species, in which feeding time had different synchronizing effects on central and peripheral oscillators. Since the molecular clock is the core of the generation of circadian rhythms, its characterization is crucial for further studies into biological rhythms in tilapia.

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