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ORIGINAL PAPER

Cloning, tissue expression pattern and daily rhythms of *Period1*, *Period2*, and *Clock* transcripts in the flatfish Senegalese sole, *Solea senegalensis*

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Abstract An extensive network of endogenous oscillators governs vertebrate circadian rhythmicity. At the molecular level, they are composed of a set of clock genes that participate in transcriptional–translational feedback loops to control their own expression and that of downstream output genes. These clocks are synchronized with the environment, although entrainment by external periodic cues remains little explored in fish. In this work, partial cDNA sequences of clock genes representing both positive (*Clock*) and negative (*Period1*, *Period2*) elements of the molecular feedback loops were obtained from the nocturnal flatfish Senegalese sole, a relevant species for aquaculture and chronobiology. All of the above genes exhibited high identities with their respective teleost clock genes, and Per–Arnt–Sim or basic helix–loop–helix binding domains were recognized in their primary structure. They showed a widespread distribution through the animal body and some of them displayed daily mRNA rhythms in central (retina, optic tectum, diencephalon, and cerebellum) and peripheral (liver) tissues. These rhythms were most robust in retina and liver, exhibiting marked *Period1* and *Clock* daily oscillations in transcript levels as revealed by ANOVA and cosinor analysis. Interestingly, expression profiles were inverted in retina and optic tectum compared to liver. Such differences suggest the existence of tissue-dependent zeitgebers for clock gene expression in this species (i.e., light for retina and optic tectum and feeding time for liver). This study provides novel insight into the location of the molecular clocks (central vs. peripheral) and their different phasing and synchronization pathways, which contributes to better understand the teleost circadian systems and its plasticity.

Keywords Clock genes · Fish · Central nervous system · Liver · Light · Feeding

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Introduction

It is now well established that interlocked feedback loops operate to sustain circadian rhythms in very different model systems such as Cyanobacteria, *Neurospora*, *Drosophila*, mouse and zebrafish. At the core of the mammalian circadian clockwork model, *Clock* and *Bmal1* act as positive regulators, while three *Period* (*Per1, Per2* and *Per3*) and two *Cryptochrome* (*Cry1* and *Cry2*) genes form the negative regulators. The CLOCK–BMAL1 heterodimer activates the

transcription of PER and CRY proteins, which interact in the cytoplasm and, after nuclear entry, negatively regulate their own transcription by inhibiting CLOCK and BMAL1 transcriptional activators (Reppert and Weaver 2002). Post-translational modifications, including phosphorylation of clock proteins, may impact upon the workings of the molecular clock (Lee et al. 2001). This autoregulatory mechanism results in a cyclic, self-sustained expression of clock genes with an approximately 24-h period.

In mammals, outside the suprachiasmatic nucleus (SCN), the master circadian pacemaker, a number of brain areas as well as peripheral (non-neural) tissues show circadian rhythms in core clock genes (Balsalobre 2002; Guilding and Piggins 2007). These molecular oscillators are differentially reset by periodic external and internal cues. The light–dark (LD) cycle is the most reliable of these time cues and represents a powerful synchronizer of the central clock, whereas peripheral oscillators can be strongly affected by daily feeding cycles (Damiola et al. 2000; Stokkan et al. 2001). Recently, it has been shown that food-related cues might also influence the expression of clock genes in the SCN and other multiple brain oscillators (reviewed in Challet and Mendoza 2010). In fish, however, little is known about the role of light and feeding as synchronizers of the molecular clock, although recent research revealed the unique model that fish offers to tackle this issue (Cavallari et al. 2011).

Teleost fish are the largest and most diverse group of vertebrates, showing high divergence in physiology regarding biological rhythms in terms of feeding behavior, locomotor activity and reproductive strategies, which reflects the evolution of complex and diverse timekeeping mechanisms (Falcon et al. 2010; Migaud et al. 2007). Therefore, fish represent very attractive models to study circadian clocks (Lahiri and Foulkes 2010). As in mammals, rhythmic clock gene expression has been described in fish in central and peripheral tissues, although a larger number of clock genes have been identified derived from a teleost-specific genome duplication (Meyer and Van de Peer 2005). Moreover, in zebrafish (*Danio rerio*), peripheral tissues and cells lines have been shown to be light responsive (Tamai et al. 2005; Whitmore et al. 2000), which allows direct entrainment of peripheral circadian clocks independently from the central oscillator. Interestingly, in most recent works, entrainment of central (brain) and peripheral (liver) tissues by both light and feeding cycles has also been demonstrated in fish (Cavallari et al. 2011; Feliciano et al. 2011; Lopez-Olmeda et al. 2010).

The *Solea senegalensis* is a flatfish with nocturnal locomotor and feeding rhythms (Bayarri et al. 2004; Navarro et al. 2009), marked daily and seasonal plasma melatonin rhythms (Oliveira et al. 2010; Vera et al. 2007), reproduction and development (Blanco-Vives et al. 2011a; Confente et al. 2010; Isorna et al. 2009, 2011; Oliveira et al. 2009). This flatfish has a great commercial value and is of particular interest to chronobiologists as sole experiences a light-dependent switch (from diurnal to nocturnal) of its daily activity rhythms during metamorphosis (Blanco-Vives et al. 2012) and exposure of larvae to daily thermocycles alters the timing of gonad development and sex ratio (Blanco-Vives et al. 2011b). However, the molecular bases underlying sole circadian rhythms remain to be deciphered. In a preliminary study, we cloned a full-length coding cDNA sequence of Senegalese sole *Per3*, which showed a significant daily oscillation in visually related structures such as the retina and the optic tectum (Martin-Robles et al. 2011). The aim of this research was to extend our knowledge about the molecular clock components and its regulation in this species, by investigating positive (*Clock*) and negative (*Per1* and *Per2*) elements of the transcription–translation feedback loops, their tissue distribution and their daily rhythms in central versus peripheral tissues.

Materials and methods

Animals and sampling

Adult vitellogenic female sole specimens from 200 to 300 g in body mass were housed in the "Laboratorio de Cultivos Marinos" (University of Cadiz, Puerto Real, Spain). Animals were kept in running seawater at a constant temperature and salinity of 19 ± 1°C and 39 ppt, respectively, in indoor facilities receiving natural environmental light (12 h light:12 h darkness), with lights on at ZT0. Fish were fed by automatic feeders three times a day between ZT5–ZT8, ZT11–ZT14 and ZT17–ZT20 with commercial 2-mm dry pellets (Skretting España S.A, Burgos, Spain) and a daily ration of about 1% body weight. Fish were fasted for 24 h before sampling. They were anesthetized in MS-222 (Sigma, St Louis, MO; 100–200 mg/l of water) before killing. This study was approved by the Animal Experimentation and Ethics Committee of the University of Cádiz (Spain) and was conducted according to international ethical standards.

For the partial cloning and the tissue distribution study, fish were killed during daytime between Zeitgeber Time (ZT) 3 and ZT5 for sole *Per1* and *Per2*, and during the night between ZT14 and ZT15 for *Clock*, and neural (olfactory bulbs, telencephalon, optic tectum, diencephalon, cerebellum, medulla, retina) and peripheral (pituitary, gills, heart, liver, kidney, intestine, and ovary) tissues were collected.

To analyze daily transcript levels of *Per1*, *Per2*, and *Clock*, 36 sole specimens from the same broodstock were

Table 1 Sequences of primers used for the *Per1*, *Per2*, and *Clock* partial cloning, RT-PCR and qPCR expression analysis. The different PCR product sizes are also indicated

Primer name	Sequence (5′–3′)	PCR product size (bp)
Molecular cloning		
PER1F1	GGAGAGCAACAAGAGCTCMAAC	715
PER1R2	GCGATGAGCAGGCAGCAGGGCT	
DRPER2F283	CAGTGTGTTCTACAGCTTCACCAC	499
DRPER2R284	AAGCTGGACCAGCTGGTGTC	
CLOCKF1	CAGTTCAATGTSCTCATCAAGGA	613
CLOCKR1	GTGCACATYTCCTTGATAAACTG	
Tissue expression pattern		
SSPER1F5	GGAGCTGAAGCTGCGTCTGCC	216
SSPER1R7	TTGAGGGTGTATTCGGAGGTGA	
SSPER2F8	AACAGTTCTGCTGCCTCCTGCTG	219
SSPER2R3	GCATTAACGGTCGGTCACTTGGG	
SSCLOCKF1E	AAGGAAATCGCTGCTCAGTCGG	385
SSCLOCKR1E	CATATTCGTACACAGGAGGCTCT	
SSACTINF	GACATGGAGAAGATCTGGCATCA	488
SSACTINR	GGCAGCTCATAGCTCTTCTCC	
Real-time qPCR		
SSPER1qPCRF1	ACATCACCTCCGAATACACC	141
SSPER1qPCRR1	ACACAGACCCCTGAAGACAC	
SSPER2qPCRF1	TGAGCTCGCTGAAGAACAG	108
SSPER2qPCRR1	TGTGTGGTGGTGAAGATGC	
SSCLOCKqPCRF1	TATCATGGAGGGAGAGACA	173
SSCLOCKqPCRR1	TTCGGGTACAGTTAGGCAC	
SSACTB2F	AATCGTGACCTCTGCTTCCCCCTGT	92
SSACTB2R	TCTGGCACCCCATGTTACCCCATC	
SSRPS4F	GTGAAGAAGCTCCTTGTCGGCACCA	83
SSRPS4R	AGGGGGTCGGGGTAGCGGATG	

sampled every 4 h ($n = 6$ at each point) over a 24-h LD cycle corresponding to ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20 (sunrise 07:28; sunset 19:20). Neural (retina, optic tectum, diencephalon, cerebellum) and peripheral (liver) tissues were rapidly removed as previously reported (Martin-Robles et al. 2011). At night, sampling was performed under a dim red light. All samples were frozen immediately in liquid nitrogen and stored at −80°C until use.

Partial molecular cloning of sole *Per1*, *Per2*, and *Clock* cDNAs

Total RNA was extracted from a pool of sole retinas by using the TRIsure Reagent® (Bioline, London, UK) according to the manufacturer's instructions. An aliquot of 2 µg was DNase I-treated (USB, Cerdanyola, Spain) and reverse transcribed to cDNA in a 20-µl reaction volume using oligo-dT_{20}, random hexamers and the SuperScrip® III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Partial cDNA fragments were amplified by using touch-down polymerase chain reactions (PCR) using the Advantage® 2 PCR kit (BD Bioscience, Clontech, Palo Alto, CA, USA) or the GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA). PCR were performed using optimized degenerated primers designed by means of ClustalW2 algorithm (http://www.ebi.ac.uk/Tools/clustalw2/) from highly conserved regions among the available vertebrate clock genes sequences (Table 1). PCR conditions for *Per1* and *Per2* were set as follows: 95°C for 2 min, 5 cycles of 95°C for 5 s, 68°C for 3 min, 5 cycles of 95°C for 5 s, 66°C for 3 min, and 30 cycles of 95°C for 5 s, 64°C for 3 min. A final extension step was added at 66°C for 5 min. For *Clock*, similar amplification conditions were used, but annealing-extension temperatures were set at 70, 68 and 66°C.

Amplified products were gel purified with Eppendorf Perfectprep® Gel Cleanup Kit (Eppendorf, Hamburg, Germany) and subcloned into pGEM®-T Easy vector system (Promega) following commercially available protocols. Five positive clones per fragment were sequenced using the

BigDye® Terminator v3.0 Ready Reaction Cycle Sequencing Kit in the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) with vector specific primers (T7 and SP6).

Sequence analysis

The identity of the partial sole cDNA fragments was confirmed by BLAST (http://blast.ncbi.nlm.nih.gov/) and deduced amino acid sequences were obtained using the ExPASy Proteomics Server (http://www.expasy.ch/). Conserved domains were determined using the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/). Phylogenetic analysis was accomplished by comparing sole sequences with complete sequences of PER and CLOCK proteins available in the GenBank and Ensembl databases. The tree was constructed by using the neighbor-joining method with the Phylo_win 2.0 software (Galtier et al. 1996), and bootstrap analysis with 1,000 replications was used to test the strictness of the tree.

Tissue expression analysis of sole clock genes by RT-PCR

Total RNA from a pool of sole neural and peripheral tissues was extracted as described above. Aliquots of 100 ng were digested with DNase I (USB) to eliminate genomic DNA and retro-transcribed with the iScript cDNA synthesis kit (Bio-Rad, Alcobendas, Spain). Specific primers were designed using the Oligo Explorer v 1.1.0 software [T. Kuulasma, University of Kuopio, Kuopio, Finland (http://www.uku.fi/~kuulasma/OligoSoftware)] from the sequences previously obtained (Table 1), and PCR were performed with the BIOTAQ™ DNA polymerase (Bioline). PCR conditions were set at 94°C for 2 min followed by 30 cycles of denaturation at 94°C (15 s), annealing at 58, 62 and 55°C (30 s) for *Per1*, *Per2*, and *Clock*, respectively, extension at 72°C (30 s) and completed at 72°C for 5 min. Amplified products were visualized in 1.2% agarose gels with ethidium bromide, and then sequenced to verify their identities. Sole *β-actin* (Genbank accession number DQ485686) was used as the internal control gene. Signal intensity of amplified products was estimated using the Quantity one® 4.2.1 software (Bio-Rad) and represented referring to the *β-actin* signal.

Real-time quantitative PCR expression analysis

Total RNA was extracted from retina, optic tectum, diencephalon, cerebellum, and liver using TRIsure Reagent® (Bioline) and 100 ng was reverse transcribed with the Quantitec® Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time quantitative PCR (RT-qPCR) reactions were developed in a Chromo 4™ Four-Color Real-Time System (Bio-Rad) as previously described (Martin-Robles et al. 2011). Specific primers for sole *Per1*, *Per2*, and *Clock* were designed using the Primer3 v 0.4.0 software (Whitehead Institute for Biomedical Research, Cambridge, MA, Table 1). PCR conditions were set as follows: initial denaturation and enzyme activation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 10 s, and annealing-extension at 58.3°C for 35 s. Sole *β-actin* and ribosomal protein S4 *Rps4* (GeneBank accession number AB291557) were selected as housekeeping genes, using the PCR conditions detailed in Martin-Robles et al. (2011). Standard curves were generated for each gene with tenfold serial dilutions of cDNA and all calibration curves exhibited slopes close to −3.32 and efficiencies around 100%. Melting curves were generated for each sample to confirm that a single product was amplified. Non-template controls and non-retro-transcribed total RNA samples were used as negative controls. The ΔΔCt method (Livak and Schmittgen 2001) was used to determine the relative mRNA expression.

Data analysis

Statistical variations in sole clock genes mRNA levels among different daily time points were analyzed by one-way ANOVA followed by post hoc LSD multiple comparisons test. When necessary, values were transformed to get normal distribution and homogeneity of variances. In all cases, statistical significance was accepted at $p < 0.05$. All statistical tests were made using Statgraphics Plus 5.1 software (Statpoint Technologies, Warrenton, VA, USA). Rhythm analyses were performed by the cosinor method (Nelson et al. 1979), using the software developed by Prof. A. Díez Noguera ("El Temps", University of Barcelona) and they were considered significant when $p < 0.05$.

Results

Molecular cloning of Senegalese sole clock genes

Partial *Per1*, *Per2*, and *Clock* cDNA fragments were 715, 499, and 613 base pairs (bp), respectively (GenBank accession numbers FM180505, FM200425 and FM179317), from which 238, 166, and 204 partial amino acid (aa) sequences were deduced. Conserved Per–Arnt–Sim (PAS) binding domains were identified in sole partial PER1 and PER2 predicted proteins and a basic helix–loop–helix (bHLH) DNA binding domain was found in sole partial CLOCK (data not shown). These sole PER1, PER2, and CLOCK fragments exhibited identities between 78–90, 79–88, and 83–95%, respectively, when compared with their respective teleost clock proteins. The identity was lower with other non-fish vertebrate sequences and fluctuated

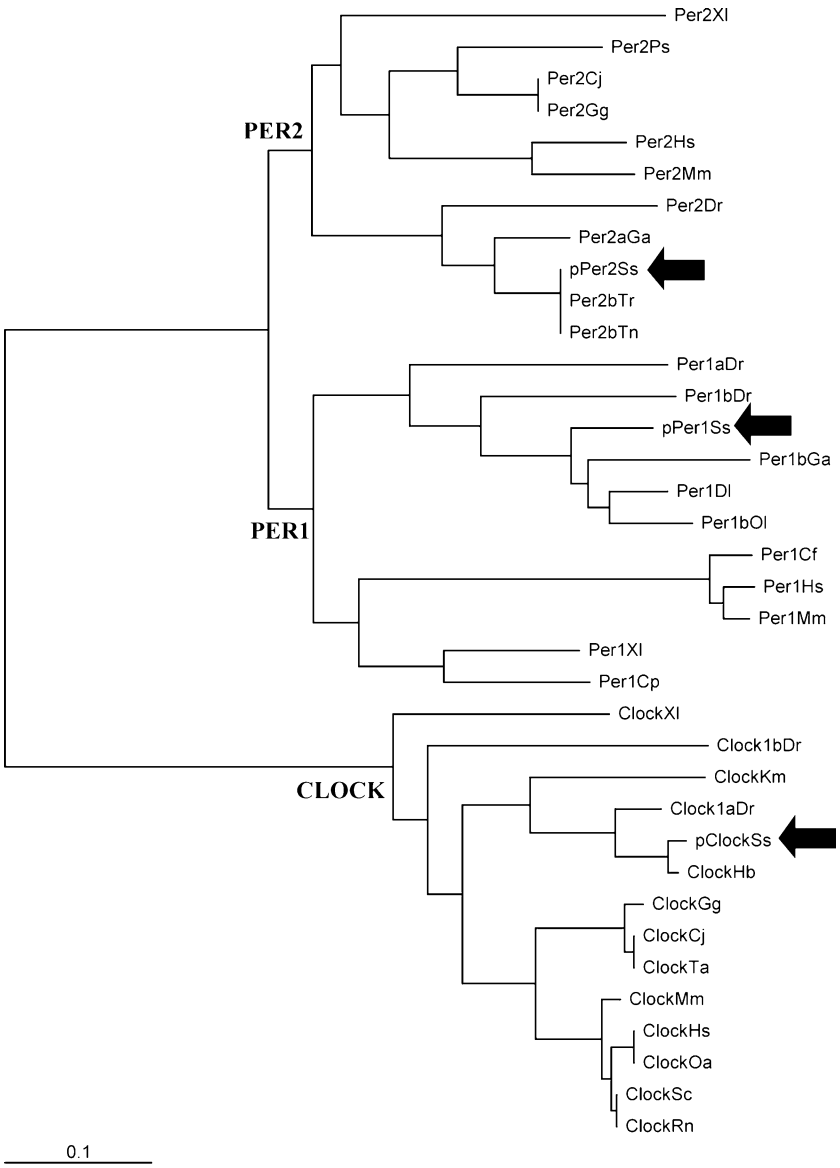

Fig. 1 Phylogenetic tree of PER and CLOCK proteins created by the neighbor-joining method. Only complete protein sequences were used for the analysis. The length of the branches is proportional to the phylogenetic distance. *Solea senegalensis* circadian proteins positions are indicated by *black arrows*. Cf, *Canis familiaris*; Cj, *Coturnix japonica*; Cp, *Cynops pyrrhogaster*; Dl, *Dicentrarchus labrax*; Dr, *Danio rerio*; Ga, *Gasterosteus aculeatus*; Gg, *Gallus gallus*; Hb, *Haplochromis burtoni*; Hs, *Homo sapiens*; Km, *Kryptolebias marmoratus*; Mm, *Mus musculus*; Oa, *Ovis aries*; Ol, *Oryzias latipes*; Ps, *Podarcis sicula*; Sc, *Spalax carmeli*; Rn, *Rattus norvegicus*; Ss, *Solea senegalensis*; Ta, *Tyto alba*; Tn, *Tetraodon nigroviridis*; Tr, *Takifugu rubripes*; Xl, *Xenopus laevis*. Protein accession numbers and Ensembl protein IDs used for alignment and phylogenetic analysis were the following: ENSCAFP00000025009 (*Canis familiaris* PER1); BAB03455, BAB03454, (*Coturnix japonica* PER2, CLOCK); BAC98490 (*Cynops pyrrhogaster* PER1); GQ353293 (*Dicentrarchus labrax* PER1); NP_001025354,NP_997604, NP_878277, NP_571032, NP_840080 (*Danio rerio* PER1a, PER1b, PER2, CLOCK1a, CLOCK1b); ENSGACP00000025523, ENSGACP00000017830 (*Gasterosteus aculeatus* PER1b, PER2a); NP_989593, NP_989505 (*Gallus gallus* PER2, CLOCK); ABP97104 (*Haplochromis burtoni* CLOCK); NP_002607, NP_073728, NP_004889 (HsPER1, PER2, CLOCK); ACL00861 (*Kryptolebias marmoratus* CLOCK); NP_001152839, NP_035196; NP_031741 (*Mus musculus* PER1, PER2, CLOCK); NP_001124404 (*Ovis aries* CLOCK); NP_001129992 (*Oryzias latipes* PER1b); CAI43981 (*Podarcis sicula* PER2); Q91YB2 (*Spalax carmeli* CLOCK); NP_068628 (*Rattus norvegicus* CLOCK); FM180505, FM200425, FM179317 (present work, *Solea senegalensis* pPER1, pPER2, pCLOCK); Q6YGZ4 (*Tyto alba* CLOCK); GSTENP00026769001(*Tetraodon nigroviridis*, PER2b); SINFRUP00000174412, (*Takifugu rubripes* PER2b); NP_001079172, NP_001081098, NP_001083854 (*Xenopus laevis* PER1, PER2, CLOCK)

between 60 and 64% for sole PER1, 61–69% for PER2, and 72–77% for CLOCK. This result was confirmed by the phylogenetic analysis that clearly located sole PER1, PER2, and CLOCK within the corresponding fish branches, showing higher divergence in relation to amphibian, avian, and mammalian sequences (Fig. 1).

Tissue distribution of *Per1*, *Per2,* and *Clock* genes

RT-PCR analysis using sole specific primers revealed clock genes expression in almost all central and peripheral tissues examined (Fig. 2a). In central tissues, a conspicuous band was observed in retina, cerebellum and, to a lesser extent, in diencephalon, olfactory bulbs, pituitary, optic tectum, telencephalon, and medulla oblongata (Fig. 2a–d). In the case of *Per2*, cDNA amplification was not detected in telencephalon and medulla oblongata (Fig. 2a, c). In peripheral tissues, intense signal was observed in ovary for the three genes analyzed, as well as in liver and gill for *Per1*. A moderate band was present for *Per1* in the remaining tissues, whereas moderate to faint signals were found for *Per2* and *Clock* in most peripheral tissues analyzed (Fig. 2a–d).

Daily expression of clock genes in central and peripheral tissues

We analyzed the daily rhythms of *Per1*, *Per2*, and *Clock* transcripts in central tissues such as retina, optic tectum, diencephalon, and cerebellum (Figs. 3, 4, 5, 6; Table 2). These tissues were selected because they have been implicated in the processing of visual/light information and contain melatonin binding sites and express melatonin receptors in sole (Oliveira et al. 2008; Confente et al. 2010). The liver was also selected (Fig. 7; Table 2), because it exhibited an important expression of clock genes in the RT-PCR study and feeding-entrained liver oscillators have been reported in fish (Cavallari et al. 2011; Feliciano et al. 2011; Lopez-Olmeda et al. 2010). In all tissues examined, *Per2* expression was significantly lower than those of *Per1* and *Clock* (compare the scales of *y* axis in Figs. 3, 4, 5, 6, 7). In retina, cerebellum and especially in liver, the highest expression was found for *Per1*, while in diencephalon it was observed for *Clock.* In optic tectum, both genes showed similar expression levels.

Statistical analysis including cosinor and ANOVA indicated differences in phase depending on the tissue analyzed (Figs. 3, 4, 5, 6, 7; Table 2). Retina and liver were the most rhythmic tissues. In retina, *Per1* and *Clock* but not *Per2* transcript levels displayed significant cyclic oscillations over the 24-h cycle (Fig. 3a–c; Table 2). *Per1* expression diminished during the day and significantly increased during the dark phase, showing its acrophase during midnight, at ZT 18.12 (Fig. 3a; Table 2). In contrast, *Clock* mRNA levels were higher during the light phase and decreased in the nighttime, with the acrophase positioned at midday (ZT 6.38, Fig. 3c; Table 2). In optic tectum, only *Per1* transcripts exhibited significant daily rhythms by both ANOVA and cosinor analysis (Fig. 4a–c; Table 2). Its expression decreased during the light phase and the early night, but it increased in the second half of the night and peaked near

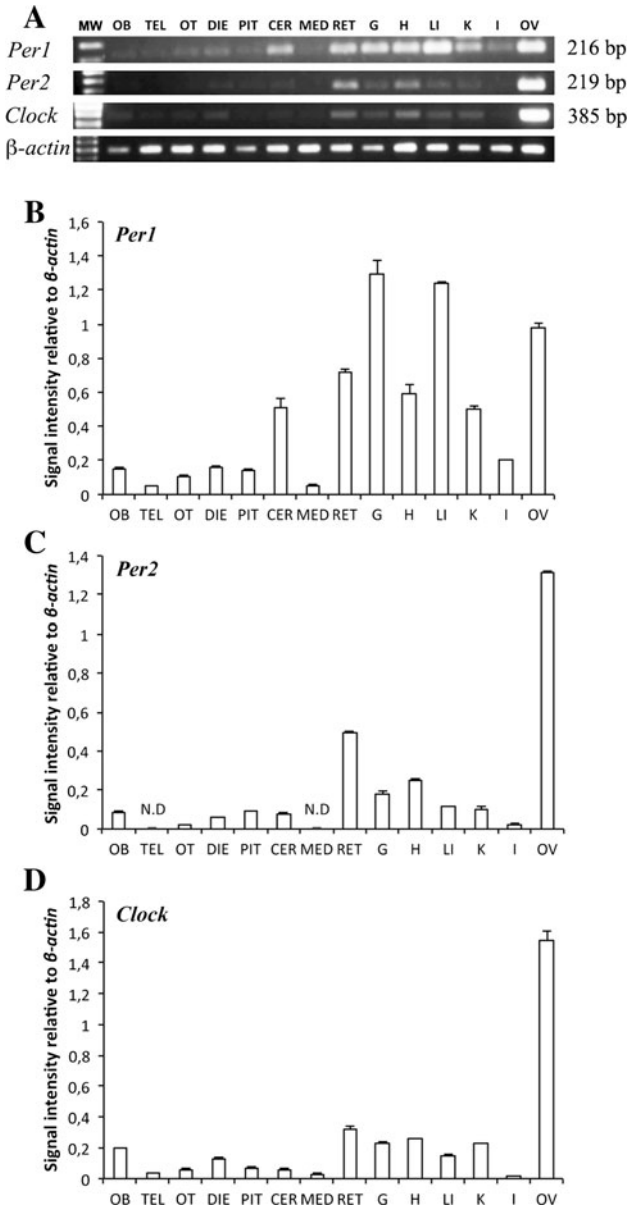

Fig. 2 Tissue distribution of Senegalese sole clock genes in central and peripheral tissues. **a** Specific *Per1*, *Per2*, and *Clock* products of 216, 219, and 385 bp, respectively, were amplified following the RT-PCR conditions described in "Materials and methods". Sole *β-actin* was used as internal control gene (*lower panel*). **b–d** Signal intensity of above amplified products relative to *β-actin* for *Per1* (**b**), *Per2* (**c**), and *Clock* (**d**) was calculated by using the Quantity one® software (Bio-Rad). *Columns* represent the mean ± SEM of three different measurements. *Per2* signal was not detected in telencephalon and medulla. *MW* molecular weight marker (100-bp Dominion, Córdoba, Spain), *OB* olfactory bulbs, *TEL* telencephalon, *OT* optic tectum, *DIE* diencephalon, *PIT* pituitary, *CER* cerebellum, *MED* medulla, *RET* retina, *G* gills, *H* heart, *LI* liver, *K* kidney, *I* intestine, *OV* ovary, *N.D.* nondetected

dawn (ZT 22.27, Fig. 4a; Table 2). *Per2* mRNA levels showed significant daily variations only by ANOVA, its expression being higher at ZT4, during the daytime (Fig. 4b). In the diencephalon, only *Per2* transcript levels

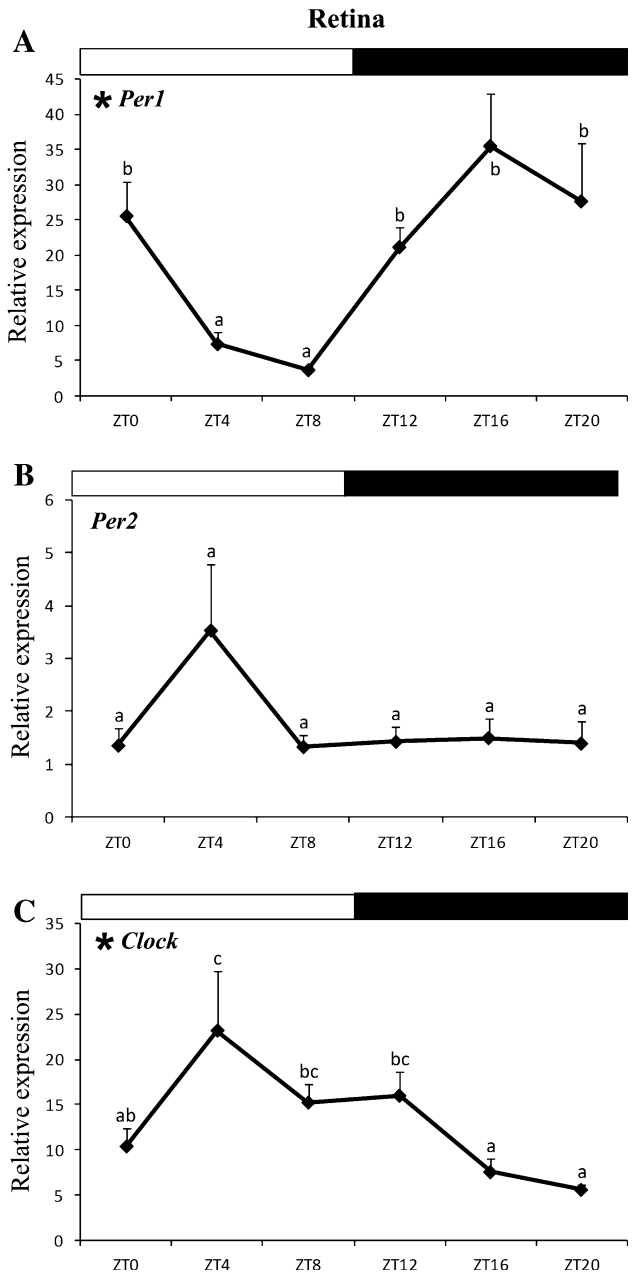

Fig. 3 Relative expression of *Per1* (**a**), *Per2* (**b**), and *Clock* (**c**) in Senegalese sole retina during the daily light–dark (LD) cycle. Samples were taken every 4 h and *each value* represents the mean ± SEM of six different specimens ($n = 6$). The *bars above each graph* indicate the daily photoperiod conditions. *White bars* represent the light phase (sampling points ZT0, ZT4 and ZT8) and *black bars* represent phases of darkness (sampling points ZT12, ZT16 and ZT20). *Different letters* indicate statistically significant differences between mean values ($p < 0.05$). The *asterisk* indicates a significant rhythm by cosinor analysis (see Table 2 for details)

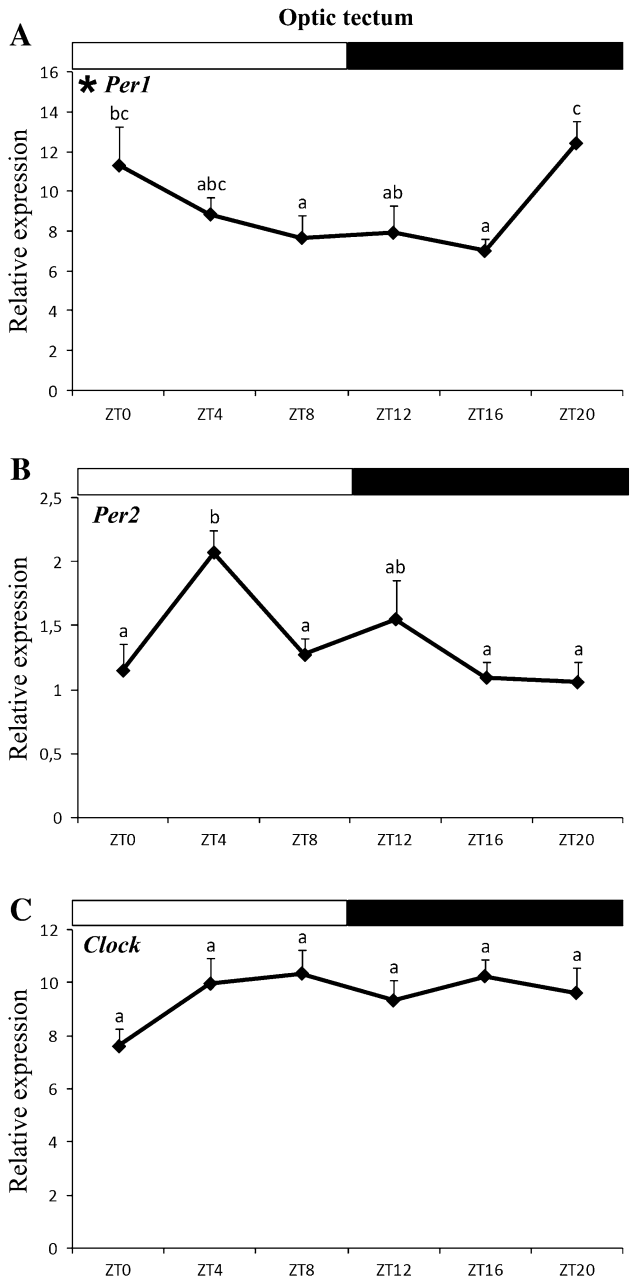

Fig. 4 Relative expression of *Per1* (**a**), *Per2* (**b**), and *Clock* (**c**) in Senegalese sole optic tectum during the daily LD cycle. For further details, see the legend of Fig. 3

were revealed as rhythmic by ANOVA and cosinor analysis (Fig. 5b; Table 2). Its expression peaked near the mid of the light phase at ZT 5.27, and decreased toward the end of the night. In cerebellum, none of the clock genes investigated was found to be rhythmic by both ANOVA and cosinor analysis, although *Clock* expression values support the cosinor analysis ($p < 0.05$), showing its acrophase in the early night at ZT 15.42 (Fig. 6c; Table 2).

Finally in liver, significant daily rhythms were observed for *Per1* and *Clock* but not for *Per2* (Fig. 7a–c; Table 2). Opposite to the retina and the optic tectum, *Per1* expression increased during daytime, peaked at ZT 9.59, and started to decline in the day–night transition, showing its minimum values near midnight (Fig. 7a; Table 2). *Clock* mRNA

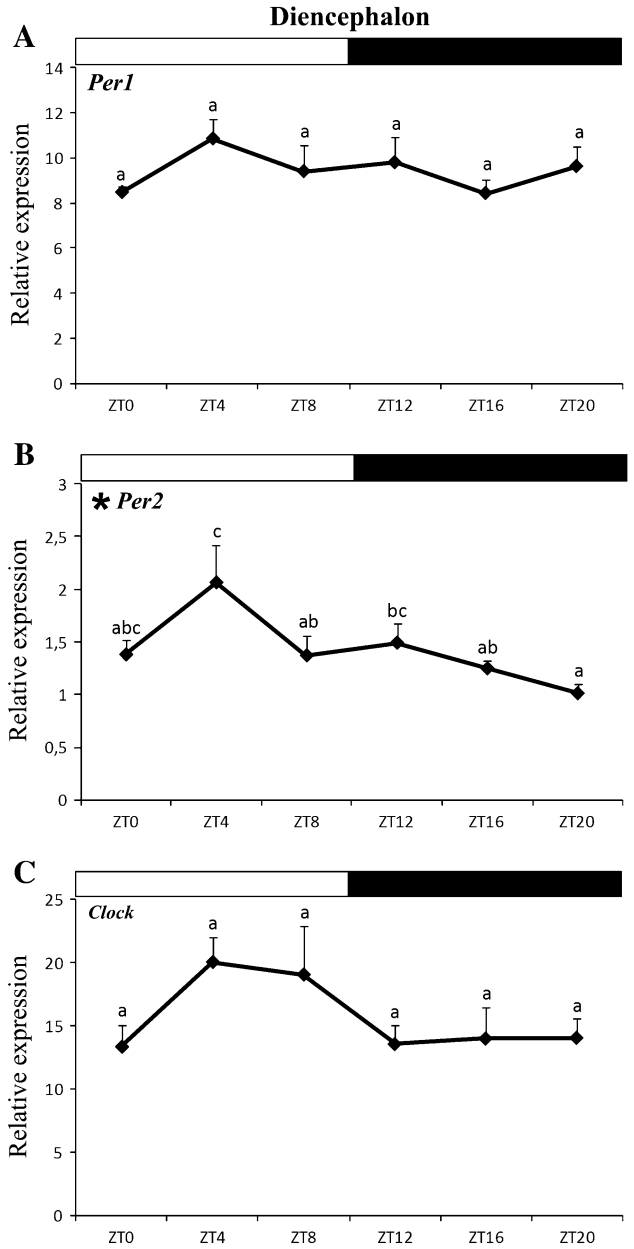

Fig. 5 Relative expression of *Per1* (**a**), *Per2* (**b**), and *Clock* (**c**) in the diencephalon of Senegalese sole during the daily LD cycle. For further details, see the legend of Fig. 3

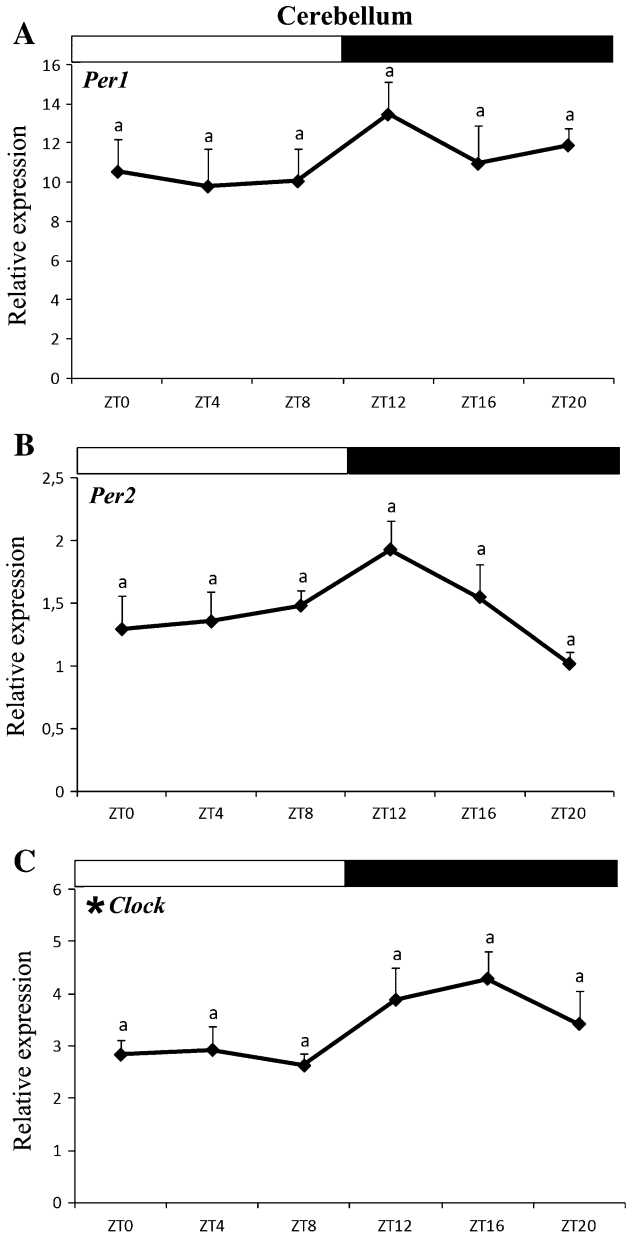

Fig. 6 Relative expression of *Per1* (**a**), *Per2* (**b**), and *Clock* (**c**) in the cerebellum of Senegalese sole during the daily LD cycle. For further details, see the legend of Fig. 3

levels also exhibited an inverse daily pattern in liver in relation to the rhythmic central tissues. Its expression remained low during daytime and the early night, increasing during the rest of the dark phase (Fig. 7c). *Clock* acrophase was observed around the mid of the night at ZT 18.56 (Table 2).

Discussion

In the present study performed in Senegalese sole, we have cloned cDNA sequences corresponding to partial coding regions of *Per1*, *Per2*, and *Clock*, three of the main clock genes in vertebrates. Expression of these genes shows a broad tissue distribution in this species, some of them exhibiting significant daily rhythms in retina, optic tectum, diencephalon, and liver. In all tissues analyzed, *Per1* always precedes the *Per3* acrophase (Martin-Robles et al. 2011) and both genes are able to anticipate dawn in retina and brain, as their expression is activated before sunrise. Furthermore, *Per* genes oscillate in anti-phase to the oscillations displayed by *Clock*, which is consistent with their putative roles as negative and positive elements of the clock mechanism in mammals and fish (Pando and Sassone-Corsi 2002).

Table 2 Parameters defining clock gene expression rhythms in central and peripheral tissues of sole

	Mesor (r.e.)	Amplitude (r.e.)	Acrophase (ZT)	Significance (*p*-value)
Retina				
Per1	20.12	14.94	18.12	0.0002
Per2	1.68	0.50	4.01	N.S. $p > 0.2$
Clock	12.71	6.81	6.38	0.001
Optic tectum				
Per1	9.26	2.37	22.27	0.009
Per2	1.35	2.37	6.13	N.S. $p > 0.05$
Clock	9.48	0.74	11.12	$p > 0.3$
Diencephalon				
Per1	9.45	0.63	5.39	N.S. $p > 0.4$
Per2	1.42	0.35	5.27	0.01
Clock	15.66	3.19	5.52	N.S. $p > 0.07$
Cerebellum				
Per1	11.08	1.22	14.56	N.S. $p > 0.4$
Per2	1.44	0.32	11.12	N.S. $p > 0.05$
Clock	3.33	0.77	15.42	0.02
Liver				
Per1	66.06	33.97	9.59	0.002
Per2	2.45	0.71	20.22	N.S. $p > 0.2$
Clock	10.82	9.82	18.56	0.001

Numeric values of mesor, amplitude, and acrophase, as well as the significance of the rhythms (*p*-value) reported by the cosinor analysis are presented for all genes and tissues studied. Mesor and amplitude are given as relative expression values (r.e.) and acrophases as Zeitgeber Time (ZT) hours. Rhythms were considered significant when $p < 0.05$

N.S. nonsignificant

In silico structural analysis of the partial amino acid sequences indicated the presence of PAS and bHLH domains. Both domains are highly conserved among PER and CLOCK transcription factors from different species, and are required for the proper functioning of the circadian clock (Hirayama and Sassone-Corsi 2005). The phylogenetic tree grouped the sole PER1, PER2, and CLOCK sequences in clearly separate branches together with their corresponding teleost clock proteins. This phylogenetic analysis confirms the high identity of sole amino acid sequences with their teleost and mammalian homologs and highlights their function in sole circadian system. Further studies are required to corroborate that these genes are the only *Per1*, *Per2*, and *Clock* existing in sole, as divergent resolution after the fish-specific genome duplication resulted in retention of different duplicates in different fish species (Wang 2008a, b).

We have shown that *Per1, Per2*, and *Clock* are widely expressed in *Solea senegalensis*. This is consistent with all other species in which they have been examined, including mouse (King et al. 1997; Shearman et al. 1997), zebrafish (Whitmore et al. 1998), goldfish (Velarde et al. 2009), sea bass (Sanchez et al. 2010), and *Drosophila* (Hall 1995). Similar ubiquitous spatial expression pattern have been reported in many species for other clock genes as *Per3*, *Bmal*, or *Cry* (Cermakian et al. 2000; Kobayashi et al. 2000; Miyamoto and Sancar 1999; Zylka et al. 1998), and there are now several examples of peripheral (non-neural) tissues that contain endogenous circadian clocks (Plautz et al. 1997; Whitmore et al. 1998; Yamazaki et al. 2000). The presence of *Per1*, *Per2*, and *Clock*, together with *Per3* (Martin-Robles et al. 2011) mRNAs in central and peripheral tissues reinforce that sole may also have multiple clocks dispersed through the body.

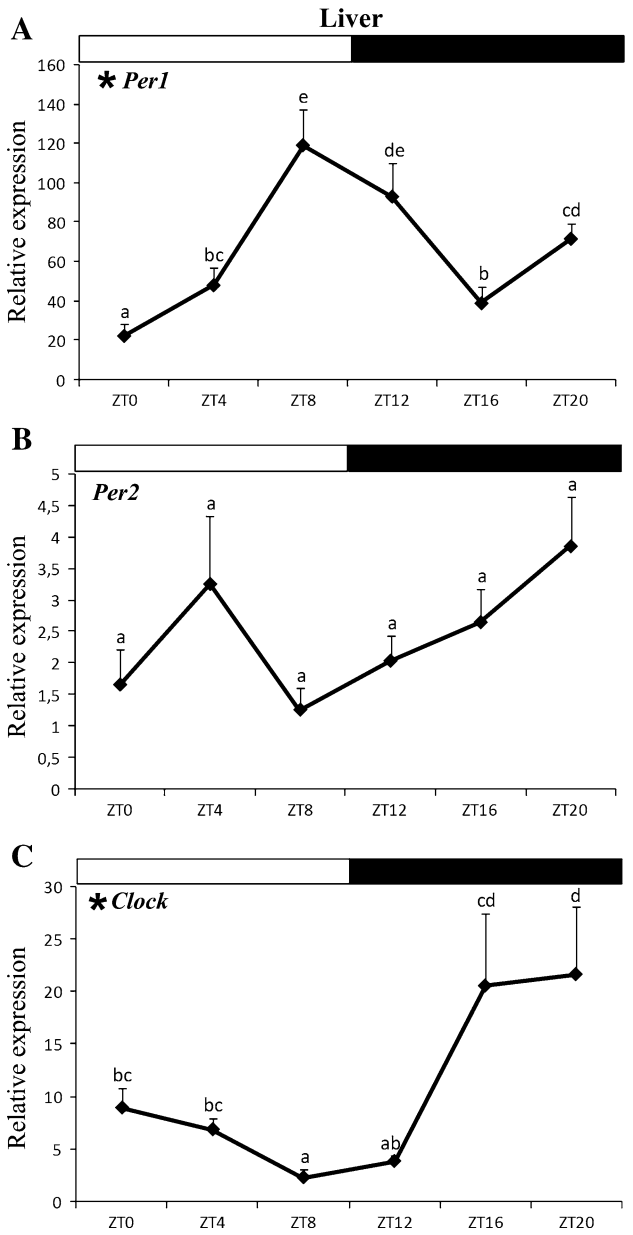

Fig. 7 Relative expression of *Per1* (**a**), *Per2* (**b**), and *Clock* (**c**) in the liver of Senegalese sole during the daily LD cycle. For further details, see the legend of Fig. 3

In sole retina, *Per1* and *Clock* transcripts were rhythmic but *Per2* did not show significant daily variations. *Per1* expression was highest at midnight (ZT 18.12) and minimum mRNA levels were observed during daytime (ZT 4-ZT 8). This profile is quite similar to that described for the retina in other diurnal fish species as the golden rabbitfish (*Siganus gutatus*), goldfish (*Carassius auratus*), and rainbow trout (*Oncorhynchus mykiss*) (Park et al. 2007; Patiño et al. 2011; Velarde et al. 2009) and 6 h-advanced to that found in *Xenopus laevis* retina, where *Per1* peak expression occurs during the time of light onset, at ZT 0 (Zhuang et al. 2000). However, it differs considerably from that previously reported for mammals, where *Per1* peak expression in retina arises later during daytime (Peirson et al. 2006; Tosini et al. 2007; Zylka et al. 1998). In the retina of sole, *Clock* peaked during midday at ZT 6.38, while minimum expression values were reached at night. It is interesting to note that this pattern of rhythmicity differs slightly from that reported in fish species as rainbow trout and zebrafish (Patiño et al. 2011; Whitmore et al. 1998; Zhdanova et al. 2008), where retinal *Clock* showed a major peak of expression at the end of the light phase (ZT9 and ZT11 for rainbow trout and zebrafish, respectively) and the onset of the night (ZT12 and ZT15 for rainbow trout and zebrafish, respectively). In addition, *Clock* is constitutively expressed in the retina of quail (*Coturnix japonica)* and *Xenopus* (Yoshimura et al. 2000; Zhu et al. 2000), but contradictory results have been obtained in both avian and mammalian species depending on the techniques used and the species studied (Helfer et al. 2006; Iuvone et al. 2005). The regulatory mechanisms responsible for these differences in *Clock* mRNA levels in the retina and their significance on the circadian clock function are unknown. Surprisingly, *Per2* expression was arrhythmic in sole retina, although a daily rhythm of this gene has been described in the retina of several species (Chaurasia et al. 2006; Kamphuis et al. 2005; Velarde et al. 2009; Yoshimura et al. 2000; Zhuang et al. 2000). Transcript levels of *Per2* change rapidly in response to variations in the light conditions, and a role for this clock gene in phase resetting by light in *Xenopus*, zebrafish, quail and mammals has been suggested (Besharse et al. 2004; Namihira et al. 2001; Pando et al. 2001; Shearman et al. 1997; Yoshimura et al. 2000; Zylka et al. 1998). The lack of rhythmicity in sole retina could be related to shifted phases of *Per2* expression in different retinal cell types (Tosini et al. 2008). Alternatively, it could be associated with seasonal variations in *Per2* retinal rhythms and/or to the benthonic habits of sole, which determines differences in light exposition (i.e., light intensity and/or spectrum) in relation to pelagic species. Taken together, our results pointed to interspecies differences in circadian organization of the vertebrate retina, as has been suggested before (Iuvone et al. 2010).

Daily expression analysis of clock genes in discrete brain areas showed that *Per1* was rhythmically expressed in the optic tectum and *Per2* in the diencephalon. Temporal expression of *Clock* mRNA levels fits a cosinor function also in sole cerebellum, although these data did not show statistically significant variations over time by ANOVA. In contrast, in the optic tectum, *Per2* mRNA levels showed significant daily variations only by ANOVA. Rhythmic expression of *Per1* in sole optic tectum resembled the *Per1* daily profile observed in retina, although it exhibited a phase delay and the amplitude was higher in the photoreceptive organ, as occurred for *Per3* (Martin-Robles et al. 2011). Tectal *Per1* acrophase was placed at the end of the night (ZT 22.27), in agreement with *Per1* rhythms recently reported in rabbitfish, sea bass, or zebrafish brain (Lopez-Olmeda et al. 2010; Park et al. 2007; Sanchez et al. 2010; Sanchez and Sanchez-Vazquez 2009). In the diencephalon, only *Per2* transcript exhibited daily oscillations by both ANOVA and cosinor analyses, its expression being higher during daytime. To the best of our knowledge, precise data of *Per2* daily expression are not available in fish diencephalon, but *Per1* transcripts also peaked in the rainbow trout hypothalamus during daytime at ZT3 (Patiño et al. 2011). The diencephalon and the optic tectum receive or integrate information from sensory organs in teleost fish (e.g., retinal and pineal projections) and contain high concentration of melatonin receptors in most species including sole (Confente et al. 2010; Herrera-Perez et al. 2010; Mazurais et al. 1999; Oliveira et al. 2008). In addition, as mentioned before, *Per2* is light-inducible in *Xenopus*, zebrafish, quail, and mammals, and it has been shown that some diencephalic areas are photosensitive in different non-mammalian vertebrates (Falcon et al. 2010; Kojima et al. 2000; Menaker et al. 1997). It should be noted that no central master pacemaker has been identified in fish yet. Therefore, a more anatomically precise approach appears necessary to interpret the presence or the lack of rhythmicity of some clock genes in particular neural regions. Furthermore, whether diencephalic expression of *Per2* is present in putative deep brain photoreceptors or in cells receiving direct retinal/pineal projections remains to be investigated in sole.

Together with the retina, the liver is one of the tissues that exhibited robust rhythms in sole, showing significant daily variations for *Per1* and *Clock*. In this case, both genes displayed an 8–12 h phase shift compared to retina and optic tectum, with their acrophases at ZT 9.59 and ZT 18.56, respectively. This result could suggest an uncoupling from the sole neural tissues and entrainment of the liver oscillator to cues different from light, highlighting a possible role of feeding time. Actually, entrainment of peripheral oscillators by feeding has been demonstrated in fish (Cavallari et al. 2011; Feliciano et al. 2011; Lopez-Olmeda et al. 2010) and mammals (Damiola et al. 2000; Stokkan et al.

2001). In addition to the phase shift, the higher mesor and amplitude of *Per1* in sole liver, compared to the retina and the optic tectum and also to *Per2* and *Clock*, further suggest that this gene could play an important role in feeding entrainment, being more susceptible to feeding cues. This result is consistent with that reported in goldfish where the negative elements *Per1* and *Cry3* also showed higher amplitude rhythms in liver compared to the brain, pointing to the liver as more sensitive to feeding entrainment (Feliciano et al. 2011). However, further studies are needed to clarify how different light (LD vs. constant conditions) and feeding schedules (e.g., daytime, nighttime, or random restricted feeding) are able to entrain the brain and liver oscillators in sole.

In summary, we have developed new and valuable tools to explore the molecular basis of the Senegalese sole circadian system, providing original information on clock genes from the positive (*Clock*) and negative (*Per1, Per2*) molecular loops in this species. Such clock genes were expressed in all analyzed tissues of sole and displayed significant daily oscillations, which were particularly evident in the retina, optic tectum, and liver. Neural (brain and retina) and peripheral (liver) tissues showed marked differences in phase, suggesting the existence of different entraining cues (light vs. feeding time) and emphasizing the great flexibility of the fish circadian system. These results could be useful to deepen the understanding of how reproductive, metabolic, developmental, and behavioral rhythms are controlled in this species, and might provide valuable information to improve the practices in sole aquaculture.

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