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Circadian clock genes of goldfish, *Carassius auratus*: cDNA cloning and rhythmic expression of *Period* and *Cryptochrome* transcripts in retina, liver, and gut

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Abstract

Clock genes are known to be the molecular core of biological clocks of vertebrates. They are expressed not only in those tissues considered central pacemakers, but also in peripheral tissues. In the present study, partial cDNAs for six of the principal clock genes (*Period 1-3* and *Cryptochrome 1-3*) were cloned from a teleost fish, the goldfish (*Carassius auratus*). These genes showed high homology (approximately 90%) with the respective cDNAs of zebrafish (*Danio rerio*), the only other teleost from which clock genes have been cloned. The daily expression pattern of each gene in retina, gut and liver of goldfish was investigated using quantitative RT-PCR and cosinor analysis. All clock genes analyzed in the retina showed circadian rhythmicity; however, only *Per 2-3* and *Cry 2-3* were rhythmic in goldfish liver and gut. The amplitude and phase of the expression in liver and gut were different from those found in goldfish retina. Such differences suggest that other cues, such as feeding time, may contribute to the entrainment of oscillators in goldfish liver and gut. Our results support the use of goldfish as a teleost model to investigate the location and functioning of the circadian oscillators.

Keywords

goldfish; *Period* gene; *Cryptochrome* gene; circadian oscillators; retina; nonphotic entrainment

INTRODUCTION

Living organisms exhibit daily rhythms in physiology, behaviour, and gene expression due to the existence of endogenous circadian clocks. These clocks synchronize biological processes to the 24-h light/dark cycle, allowing the organisms to anticipate changes in their environment. In metazoans, the generation of circadian rhythmicity appears to be consequence of specialised tissues known as “master clocks”, which have different locations among species, i.e. lateral neurons in *Drosophila* (Stanewsky, 2002), the pineal gland and retina in fish (Falcon, 1999), pineal gland, retina and hypothalamus in birds (Underwood *et al*., 2001), and the hypothalamic suprachiasmatic nuclei (SCN) in mammals (Klein *et al.*, 1991).

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The core oscillator of the circadian clock consists of transcriptional-translational feedback loops that involve a highly conserved set of "clock genes". The mechanism of the endogenous clocks is known to be conserved along the phylogenetic scale, from unicellular organisms and plants to insects and mammals (Panda *et al.,* 2002). In mammals, these loops are formed by positive elements, CLOCK and BMAL1, which heterodimerize and enhance the transcription of the negative components *Period* (*Per1*, *Per2* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. The PER and CRY proteins form complexes that inhibit their own transcription by binding to the CLOCK:BMAL1 complex and blocking its function. This negative loop allows a daily rhythm in expression of *Per* and *Cry* transcripts and protein products (Iuvone *et al.,* 2005; Okamura *et al.,* 2002). The molecular basis of circadian clocks in teleost fish has been studied in zebrafish, where the homologues of the mammalian clock genes have been cloned (Cahill, 2002). The main differences with respect to the mammalian system may be the regulation of *Per2* and the number of *Cry* genes; up to six *Cry* genes have been found, all of them rhythmically expressed (Kobayashi *et al.,* 2000).

However, the expression of clock genes is not restricted to the master clocks. The mRNA products of these genes have been found in peripheral tissues such as liver, heart, muscle, kidney, pancreas, adipose tissue and lung from mammals (Balsalobre, 2002; Muhlbauer *et al*., 2004; Peirson *et al*., 2006; Zvonic *et al.* 2006), heart, spleen and gall bladder in zebrafish (Kaneko *et al.,* 2006), and lung, heart, liver, muscle and testis in lizard (Della Ragione *et al.,* 2005; Vallone *et al.,* 2007). Although these circadian oscillators may need an external messenger to sustain their rhythmicity, they can be entrained to other cues besides the light/dark cycle, such as temperature, the reproductive cycle, or feeding time (Brown *et al*., 2002; Mendoza, 2007; Vallone *et al.,* 2007). Regarding feeding time, some studies have shown it to be a potent zeitgeber that is able to entrain the master circadian clock in the absence of photic cues in mammals (Castillo *et al.,* 2004) and in fish (Boujard and Leatherland, 1992), where feeding time also influences the daily changes of metabolic parameters such as cortisol, glucose and triglyceride plasma levels (Polakof *et al.,* 2007). These facts, together with the well-studied increase in locomotor activity prior to food intake (food anticipatory activity, FAA), have led investigators to propose the existence of a food-entrainable oscillator (FEO), whose location is still unknown (Mendoza, 2007).

The goldfish is a well studied species, with a robust circadian system and a demonstrated capacity of entrainment by feeding cues, as shown by its pattern of FAA (Aranda *et al*., 2001; Vera *et al.,* 2007). However, there is no data available on clock gene expression in this species.

The aim of the present study was to investigate some of the main components of the molecular clock in the goldfish (*Carassius auratus*). First, six cDNA transcripts from the *Period* and *Cryptochrome* genes were cloned in this teleost species. Second, their expression pattern in central and peripheral tissues was characterized. Lastly, possible daily rhythms of expression of their mRNA were analyzed.

MATERIALS AND METHODS

1. Animals and tissue collection

All animal experiments were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals and complied with the Spanish legal requirements. For the cloning and tissue expression of the genes, goldfish (*Carassius auratus*) with a body weight of 9–12 g were used. Fish were kept in 100 l tanks with filtered and aerated water, and were fed once a day at zeitgeber time (ZT) 2 on a commercial pellet diet (1% bw, Sera Biogran). Animals were maintained under 12L:12D photoperiod, with lights on at ZT0. For the tissue distribution study, fish were killed at ZT2. Brain, neural retina, heart, liver and gut were removed and immediately

placed in RNA*later* (Sigma, Saint Louis, MO, USA) for tissue preservation. For the study of daily variations in gene expression, we used 30 goldfish of 10–15 g, kept under the same housing conditions. Fish were sacrificed at ZT2, ZT8, ZT14, ZT20 and ZT2 of the following day. Neural retina, liver and gut were excised and rapidly immersed in RNA*later*.

2. RNA isolation and first-strand cDNA synthesis

Tissues removed from RNA*later* were homogenized in RLT buffer (Qiagen Inc., Valencia, CA, USA) by a rotor homogenizer (Polytron, Brinkmann Instruments, Westbury, NY). Total RNA was extracted by a silica-based filter-binding RNeasy mini kit (Qiagen Inc.) and treated with RNase-free DNase I (1 U) according to the manufacturer's instructions (Qiagen Inc.). The yield and quality of RNA were quantified by the 260/280 nm absorbance ratio (SmartSpec3000, Bio-Rad Laboratories, Hercules, CA, USA) and by electrophoresis under non-denaturing conditions on 1.5% agarose gel.

An aliquot of 200 ng of total RNA from tissues was reverse transcribed (RT) in a 20 μl reaction volume using oligo-dT or random primers, RNase inhibitor and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The reaction was carried out for 50 min at 55° C, followed by 15 min at 70°C to inactivate the reverse transcriptase. In the case of random primers, there was a 5-min step at 25°C prior to the inactivation of the enzyme.

3. RT-PCR cloning of goldfish Period and Cryptochrome cDNAs

To amplify the cDNAs of the clock genes, PCR was performed by using specific primers (Table 1) for the homologous *Per1, Cry1, Cry2* and *Cry3* of zebrafish (*Danio rerio*) transcripts. For *Per2* and *Per3*, degenerate primers were designed using the CLUSTALW algorithm from the sequences of zebrafish, *Siganus guttatus* and *Xenopus tropicalis*.

The reaction mixture contained cDNA, 10 pmol each of forward and reverse primers (see Table 1), 1 U Taq DNA polymerase (Invitrogen), 5 μl of 10× PCR buffer, 1 μl of 10 mM dNTPs (200nM final), and 2 μl of 50 mM $MgCl_2$ (2 mM final). PCRs were performed in a total volume of 50 μl. PCR conditions were set at 95 °C for 2 min, followed by 40 cycles of denaturation, annealing and extension, each one at 95 °C, 54 °C and 72 °C for 30 s. The amplified products were gel purified using QIAE's gel extraction kit (Qiagen). Purified PCR products (426–849 bp) were ligated into pGEM® T-vector (Promega, Madison, WI, USA). Ligated products were transformed into *Escherichia coli* JM109 cells. Positive clones were obtained and plasmid DNA extraction was performed using the QIAprep Spin miniprep kit (Qiagen). Nucleotide sequences were determined by sequencing with Agencourt Bioscience Corporation (Beverly, MA) with two T-vector-specific primers (T7 and SP6).

4. Quantitative Real-time RT-PCR (qRT-PCR)

Primers (Table 1) for qRT-PCR were designed based on the sequences of the cloned cDNAs and on the available sequence for goldfish *18s rRNA*. Amplification reactions were performed in an iCycler (Bio-Rad) using cDNA (2μl from each sample), 1x SYBR Green PCR Master mix (Biorad) and 400nM gene-specific forward and reverse primers, up to a volume of 25μl. Samples were incubated at 95°C for 3 min, followed by 45 cycles of denaturation, annealing and extension, each one at temperatures of 95°C, 54°C and 72°C for 30 seconds. All samples were assayed in duplicates. PCR products were checked by agarose electrophoresis, and quantification was carried out by comparing the threshold cycle for amplification of the unknown product with those of six concentrations of standard cDNA for each gene (standard curves were generated using from 1 fg to 100 pg of cDNA). The gene expression levels were calculated and normalized by dividing the calculated values for the mRNA samples by that of 18S rRNA.

5. Statistical analysis

To test for variation in mRNA levels among time points, statistical analyses were carried out with one-way ANOVA. To evaluate rhythmicity in gene expression, cosinor analysis was performed by fitting periodic sinusoidal functions to the expression values for the genes across the five time points using the formula $f(t)=M+A\cos(t\pi/12-\phi)$, where $f(t)$ was the gene expression level in a given time, the mesor (M) is the mean value, A is the sinusoidal amplitude of oscillation, t is time in hours and ϕ is the acrophase (time of peak expression). Non-linear regression allows the estimation of M, A, and ϕ, and their standard error (SE) (Delgado et al, 1993). Significance of cosinor analysis was defined by the noise/signal of amplitude calculated from the ratio $SE(A)/A$. Expression was considered to display a daily rhythm if it had both $P<0.05$ by ANOVA and $SE(A)/A<0.3$ by cosinor analysis.

RESULTS

Cloning of goldfish *Period* and *Cryptochrome* genes

PCR-generated partial cDNAs of *Per1, 2*, and *3* and *Cry1, 2*, and *3* of goldfish were cloned into a pGEM®-T easy vector (Promega, Madison, WI) and sequenced. The sequences were compared to the homologous zebrafish sequences. The size of the PCR products, their homology with corresponding zebrafish genes, and the GenBank accession numbers of the cloned goldfish clock genes are presented in Table 2. More than 80% and 90% sequence identity was observed between goldfish *Periods* and *Cryptochromes* cDNA products and their corresponding zebrafish sequences, respectively (Table 2).

Expression of *Period* and *Cryptochrome* genes in goldfish

The mRNA expression pattern of *Per1, Per2, Per3, Cry1, Cry2* and *Cry3* genes was examined in goldfish retina, brain, heart, liver, and gut, dissected at ZT2 (Figure 1). The expression of *Cryptochromes* was higher than that of *Periods* in all the tissues examined. *Per 1* and *Per3* were more highly expressed in the retina and liver compared to the other tissues. *Per2* expression appeared to be relatively low in all tissues. The relative tissue distribution of *Periods* and *Cryptochromes* is presented in Table 3.

Rhythmic expression of circadian clock genes

The daily rhythms of *Per1, Per2, Per3* and *Cry1, Cry2*, and *Cry3* transcript levels were examined by qRT-PCR in the goldfish retina, liver, and gut during a 12: 12h light/dark cycle (Figs. 2–4). Statistical analysis, including cosinor and ANOVA, showed that mRNAs for the six clock genes in retina displayed significant cyclic oscillations as a function of a 24-h cycle. The data presented in Table 4 show that the mean expression (mesor) of *Cryptochrome* genes in goldfish retina was higher than that of *Periods*, and that their peak expression (acrophase) occurred at the day-night transition (*Cry1* and *2*) or mid-night (*Cry3*). Peak expression of *Per1* also occurred at night. In contrast, peak expression of *Per2 and Per3* transcripts occurred during the daytime.

In gut, *Per2* and *Per3*, but not *Per1*, transcript levels showed a significant daily variation by ANOVA and cosinor analysis (Figure 3; Table 5). Similarly, *Cry2* and *Cry3* showed significant daily rhythms, but *Cry1* did not. The rhythms in *Per2*, *Per3*, *Cry2* and *Cry3* showed differences in amplitude and phase (Fig. 3, Table 5). Thus, *Per2* and *Cry2* peaked in the early and mid-daytime, respectively, while *Per3* and *Cry3* had their acrophase at near midnight. *Per1* and *Cry1* did not show significant rhythms.

Significant daily rhythms of expression of *Cry2*, and *Cry3* and *Per3* transcripts were observed in the liver, as assessed by both ANOVA and cosinor analysis, although with different temporal profiles of expression (Fig. 4; Table 5). Peak expression *Cry3* mRNA occurred near the day-

to-night transition, while *Cry2* peaked in the middle of the daytime. *Per3* mRNA levels were highest in the middle of the night. The liver *Per1*, *Per2*, and *Cry1* transcript levels did not display significant rhythms.

Comparing the significant rhythms in the three tissues, *Cry3* peaked earlier in the liver (~ ZT14) than in gut and retina (~ ZT18). The levels of mRNA were similar for both peripheral tissues, but approximately 10 times more abundant in retina. In liver and gut, *Cry2* had a similar acrophase at the middle of the day, while the peak in retina was delayed until the beginning of the dark phase. As occurs with *Cry3* expression, mRNA expression for *Cry2* was approximately an order of magnitude higher in retina than in gut and liver. The maximum values for *Per3* expression in liver and gut were acquired late in the scotophase (ZT21 for gut and ZT19 for liver), whereas it occurred at the beginning of the subjective day in retina. The amount of *Per3* mRNA gene was an order of magnitude lower in retina than in peripheral tissues.

DISCUSSION

In the present study, we have cloned, for the first time in the goldfish, partial cDNAs of some of the principal clock genes, which exhibit robust oscillations of mRNA expression through the light-dark cycle in retina as well as in liver and gut.

The partial cDNAs of the goldfish clock genes have a high degree of homology with zebrafish clock genes. Although alignment is not shown, goldfish *Per1, Per2*, and *Per3* cDNA sequences are 84–88% identical with those of zebrafish, which are true homologs of the three mammalian *Period* genes (Cahill, 2002; Delaunay *et al.*, 2003), supporting the role played by these genes in the molecular clock machinery of the goldfish. Regarding to *Cryptochrome* transcripts, the cDNA sequences are 91–93% identical with those of zebrafish. While there are only two *Cryptochrome* genes and proteins characterized in mammals (CRY1 and CRY2) (Kobayashi *et al.*, 1998), six rhythmically expressed *Crytochrome* genes have been reported in zebrafish (Kobayashi *et al.*, 2000). Both being teleosts, zebrafish and goldfish are expected to have two paralogs of most mammalian genes due to genome duplication (Postlethwait *et al.*, 1998), but this fact is not sufficient to explain the six zebrafish *Cryptochromes*. In the present study we obtained the goldfish cDNAs from primers designed upon sequences of zebrafish *Cry1a* and *Cry2a* (both most similar to *mCry1*) and zebrafish *Cry3*, whose product does not inhibit the transcription mediated by the CLOCK:BMAL1 dimer (Kobayashi *et al.*, 2000). The high homology of the goldfish cDNA fragments with their zebrafish homologs, and therefore with mammals, highlights their function in goldfish circadian system. Moreover, the presence of a probable *zCry3* homolog in goldfish is a first approach to delve into the existence and role of *Cryptochrome* genes in the teleost lineage.

Analysis of goldfish clock gene expression in retina reveals that, under LD conditions, all transcripts are rhythmic. Regarding the *Period* genes, *Per3* expression is highest at dawn, as occurs in the retina of zebrafish or Japanese quail (Delaunay *et al*., 2000; Yoshimura *et al.*, 2000). Moreover, *Per1* and *Per2* also exhibit rhythms but with opposite phase relationships: while *Per1* peaks during midnight, *Per2* shows its maximum at midday. These patterns of rhythmicity are the inverse from that previously reported for *Xenopus* photoreceptors (Zhuang *et al*., 2000). However, goldfish *Per2* parallels the rhythm described for quail, chicken and mammalian retina, with higher levels during the daytime (Chaurasia *et al*., 2006; Kamphuis *et al.*, 2005; Yoshimura *et al.*, 2000). The high nocturnal expression of cryptochrome transcripts in goldfish retina is similar to the expression profile of *Cry2* in zebrafish. In contrast, *zCry1a* and *zCry3* are expressed during daytime (Kobayashi *et al.*, 2000) as occurs with *Cry1* in the quail (Fu *et al*., 2002) and chicken retina (Chaurasia *et al*., 2006; Haque *et al*., 2002). These findings support the key role of *Period* and *Cryptochrome* genes in the molecular clock of

goldfish retina, although further experiments may be necessary to unravel the implication of each component.

Our results demonstrate the expression of all three *Cryptochrome* and *Period* genes in peripheral organs of the goldfish, such as liver or intestine. Rhythmic clock gene expression in such tissues is already reported in organisms like zebrafish (Kaneko *et al.*, 2006; Whitmore *et al.*, 1998), lizard (Della Ragione *et al.*, 2005), chicken (Chong *et al*., 2003), Japanese quail (Fu *et al*., 2002), sheep (Andersson *et al*., 2005), mice (Peirson *et al.*, 2006) and humans (Pardini *et al.*, 2005). This rhythmic expression of clock genes in mammalian peripheral tissues is self-sustained (Balsalobre, 2002; Yoo *et al.*, 2004), temperature compensated (Reyes *et al.*, 2008), and can synchronize to cues different from the light/dark cycle, such as temperature (Brown *et al.*, 2002) or feeding (Damiola *et al.*, 2000; Stokkan *et al*., 2001). Characteristics of peripheral clock gene expression in fish have been studied most in zebrafish, where photo-entrainable peripheral oscillators have been found in tissues such as heart, kidney or spleen, suggesting a circadian system made of a number of widely distributed pacemakers synchronized by light, perhaps due to its semi-transparent body (Kaneko *et al.*, 2006; Whitmore *et al.*, 1998). Thus, our results of rhythmic circadian clock gene expression in goldfish peripheral tissues, together with their robust FAA both along the light/dark cycle and with restricted feeding (Aranda *et al*., 2001; Vera *et al*., 2007), support the use of goldfish as a model to study the existence of a food entrainable oscillator (FEO) in fish.

The clock genes studied have been implicated in entrainment by feeding cues, and therefore affecting food anticipatory activity (FAA), principal output of the so-called FEO (Stephan *et al.*, 1979). Studies with *Clock* mutant mice showed the FAA persisted even in constant darkness, suggesting that *Clock* gene is not part of the FEO clockwork (Pitts *et al*., 2003), although it might be necessary in maintaining rhythmicity in peripheral oscillators (DeBruyne *et al*., 2007). The genes considered a part of the FEO due to its involvement in food anticipatory behaviours are the *Cryptochromes*, which appear to be necessary for the development of the FAA (Iijima *et al*., 2005), and *Period* genes. Specifically, *Per2* mutant mice have significant impairment of the FEO output (Feillet *et al*., 2006; Mendoza *et al*. 2005).

Clock gene expression in goldfish liver and gut has revealed rhythms of both *Period* and *Cryptochrome* genes under LD conditions. *Per2* is rhythmic only in goldfish gut, with a maximum at ZT2, preceding the peak in retina and occurring just prior to the feeding time. To our knowledge, circadian expression of clock genes in the gastrointestinal tract has been studied only in mice, where *Per2* exhibits its maximum expression at the beginning of the night with *ad libitum* feeding; this peak shifts to midday when food availability is restricted (Hoogerwerf *et al*., 2007). Rhythmic expression of *Per3* occurs in goldfish gut and liver with similar acrophase late in the night, different from the peak in retina. In the case of mice, *Per3* in the gastrointestinal tract shows an expression pattern parallel to *Per2*, peaking early in the night when fed *ad libitum* and shifting to early day in timed feeding (Hoogerwerf *et al*., 2007). Moreover, *Per3* expression in mouse liver reaches its maximum at the beginning of the dark phase with *ad libitum* food access (Peirson *et al*., 2006), but shifts to midnight when food is restricted to their activity period (Damiola *et al.*, 2000).

Concerning *Cryptochrome* genes, our study shows that *Cry3* is rhythmically expressed in liver and gut of goldfish with a maximum during midnight in gut (similar to retina) and early in the night in liver. There is no data available to compare these results, as this is the first time that the expression rhythm of this gene is characterized in a teleost species, and *Cry3* is not expressed in mammals. In both goldfish peripheral tissues, *Cry2* exhibits a maximum in the middle of the light phase, while in retina it occurs early in the night. This could be due to an entrainment of the peripheral oscillator of the goldfish to restricted feeding (feeding time in our experiment was at ZT2), resulting in an uncoupling from the central pacemaker. Our results

agree with studies in the colon of mice subjected to restricted feeding (Hoogerwerf *et al*., 2007). In liver, *ad libitum*-fed mice present *Cry2* maximums at the beginning of the dark phase (Peirson *et al.*, 2006) but early in the light phase with restricted feeding (Damiola *et al*., 2000). Thus, feeding time for the goldfish may be essential to synchronize its peripheral oscillators, as occurs in mammals, supporting the existence of a FEO in this species.

In summary, *Period* and *Cryptochrome* genes are expressed in a circadian manner in retina, liver and gut of the goldfish, with differences in phase and amplitude, suggesting entrainment of goldfish peripheral tissues to cues different from light and, therefore, highlighting a probable role of feeding. Thus, the goldfish can be considered a model to delve into the mechanisms of peripheral oscillators, as well as to unravel the location and physiology of the food entrainable clock.

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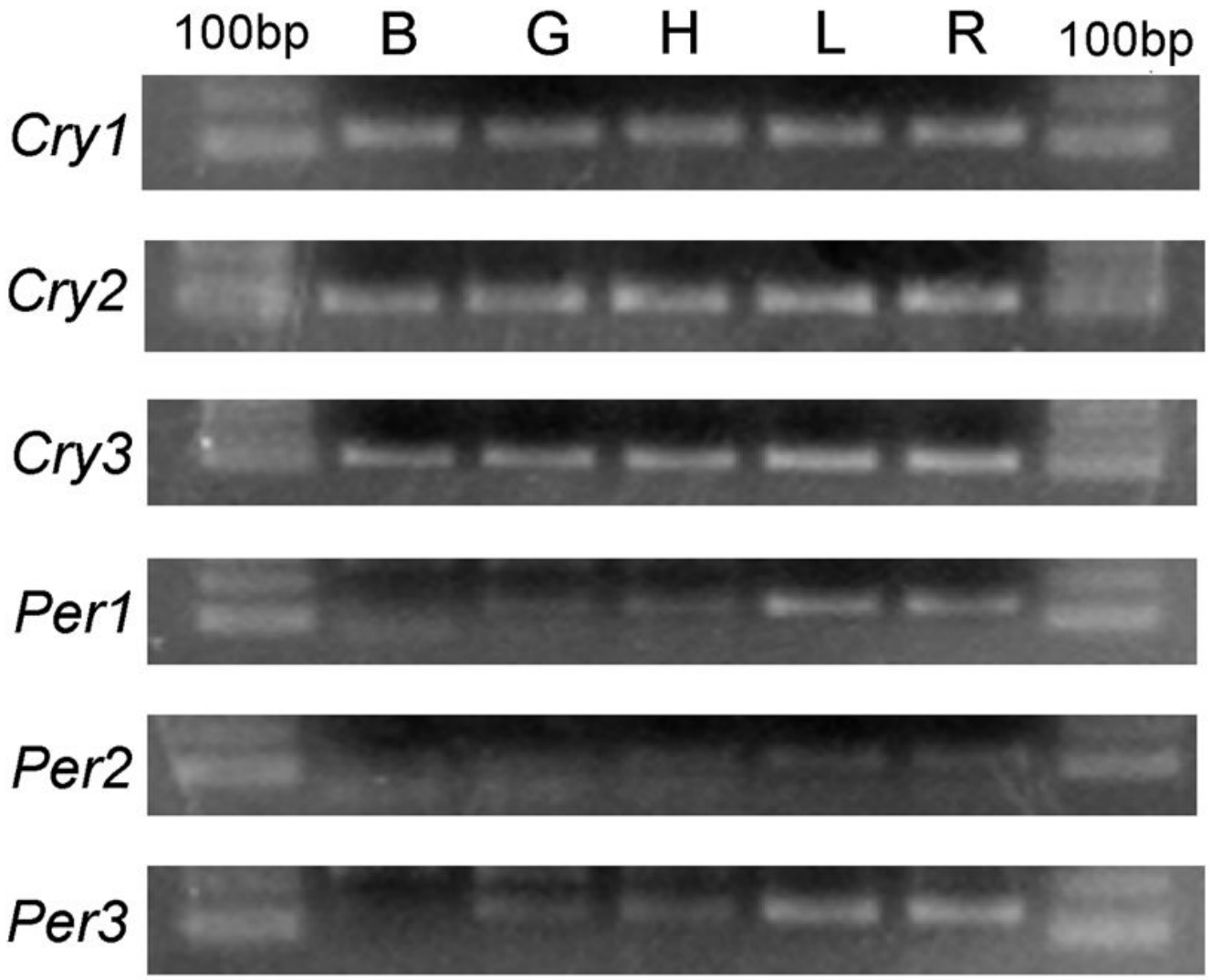

Fig 1. Tissue distribution of goldfish clock genes

RT-PCR analysis of *Cry1-3* and *Per1-3* transcripts in different tissues collected at ZT2, as shown in a 1.5% agarose electrophoresis gel with ethidium bromide and 100bp molecular marker. Abbreviations: B, brain; G, gut; H, heart; L, liver; R, retina.

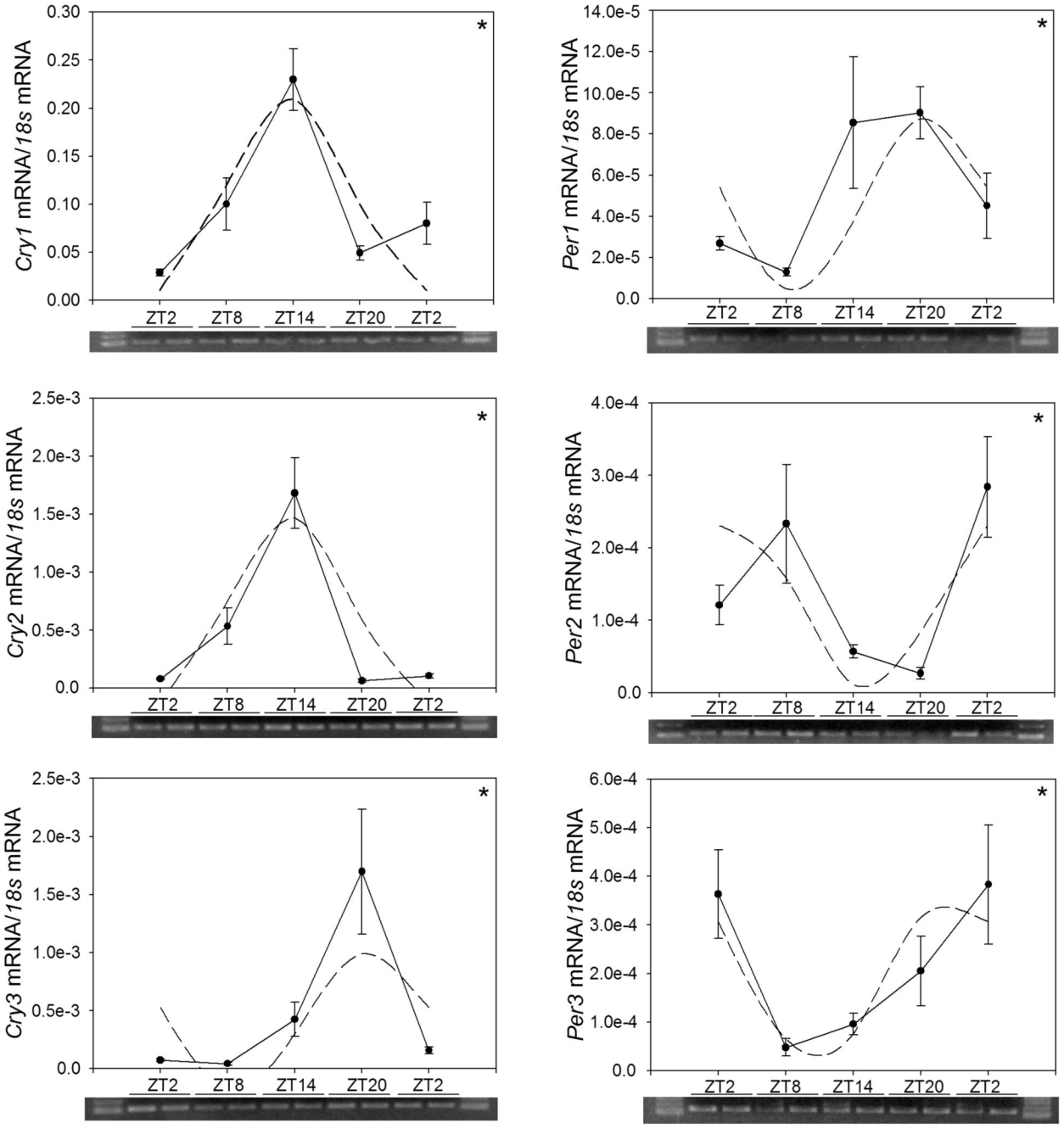

Fig 2.

Expression of clock genes in the retina of goldfish (*Carassius auratus*) during the daily light-dark cycle. Relative mRNA levels were quantified by qRT-PCR. Each data point represents the mRNA amount of the corresponding clock gene normalized to *18s* rRNA, expressed as mean ± S.E.M. (n=6 retinas per time point). The dashed lines in the graphs represent the periodic sinusoidal functions determined by the cosinor analysis. The asterisks indicate a significant rhythm (SE(A)/A<0.3). ANOVA: *Per1* P=0.01; *Per2* P=0.004; *Per3* P=0.02; *Cry1* P=0.03; *Cry2* P<0.001; *Cry3* P=0.008.

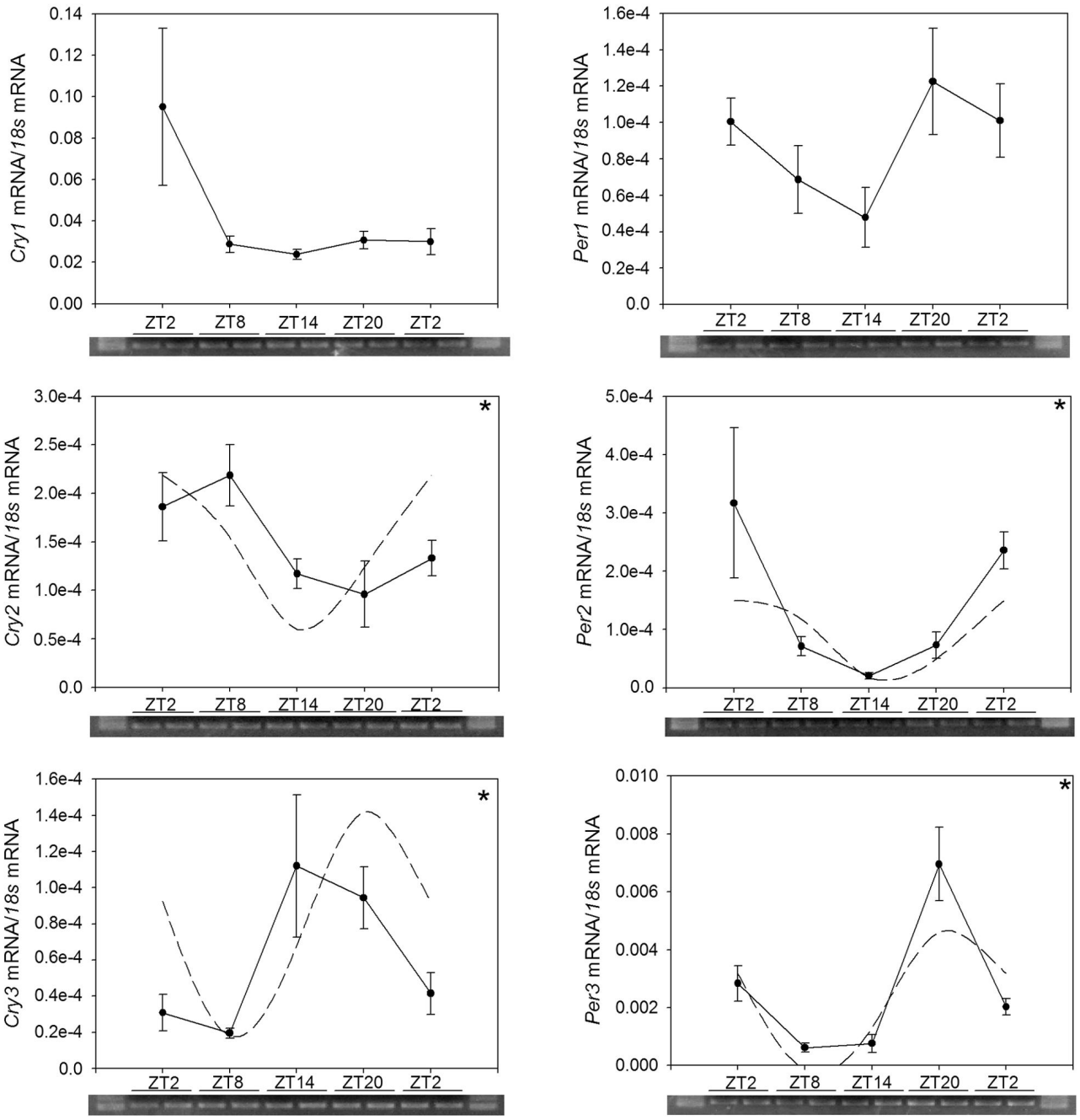

Fig 3.

Expression levels of clock genes in the gut of goldfish (*Carassius auratus*) during the daily light-dark cycle. Relative mRNA levels were quantified by qPCR. Each data point represents the mRNA amount of the correspondent clock gene normalized to *18s* rRNA, and are expressed as mean ± S.E.M. (n=6 intestines per time point). The dashed line in the graphs represents the periodic sinusoidal functions determined by the cosinor analysis. The asterisks indicate a significant rhythm (SE(A)/A<0.3). ANOVA: *Per1* P=0.138; *Per2* P=0.005; *Per3* P=0.006; *Cry1* P<0.001; *Cry2* P=0.033; *Cry3* P=0.018.

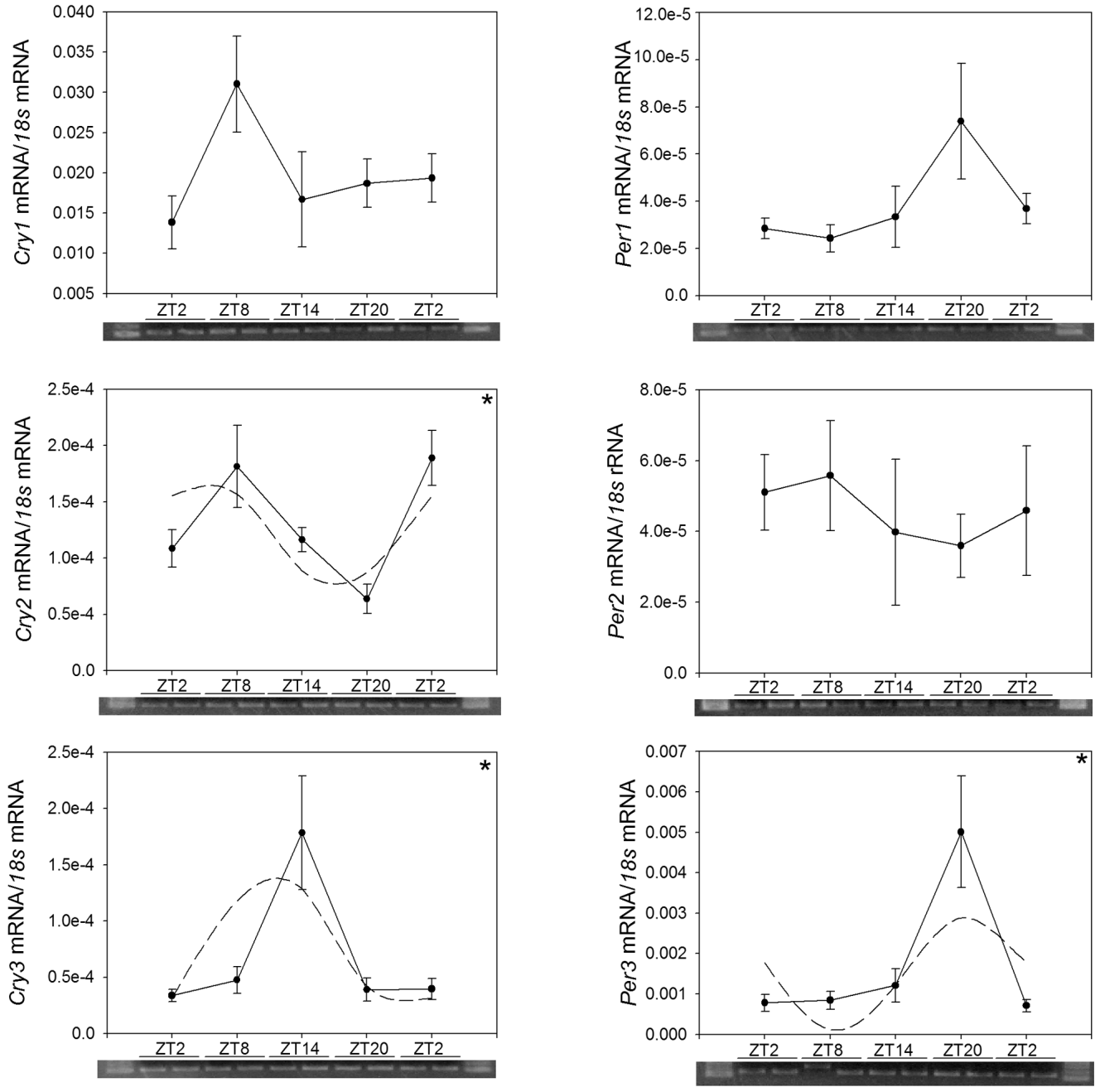

Fig 4.

Expression levels of clock genes in the liver of goldfish (*Carassius auratus*) during the daily light-dark cycle. Relative mRNA levels were quantified by qPCR. Each data point represents the mRNA amount of the correspondent clock gene normalized to *18s* rRNA, and are expressed as mean ± S.E.M. (n=6 livers per time point). The dashed line in the graphs represents the periodic sinusoidal functions determined by the cosinor analysis. The asterisks indicate a significant rhythm (SE(A)/A<0.3). ANOVA: *Per1* P=0.103; *Per2* P=0.211; *Per3* P=0.001; *Cry1* P=0.365; *Cry2* P=0.002; *Cry3* P<0.001.

Table 1

Sequences of primers used for real-time PCR.

PRIMER NAME		TARGE T GENE	SEQUENCE (from 5′ to 3′)	PCR product (bp)	ACCESSION NUMBER	SPECIES
For gene cloning:						
zP1F-1377	Forward	*Per1*	5′-TGTACCGCAGCTGAATGAACTGA-3′	531	NM001030183	*Danio rerio*
zP1R-1974	Reverse		5′-TCAAAGGTCGTTCCTGCAGCAC-3′			
zCry1F831	Forward	*Cry1a*	5′-AAAGAACAGCTCACCTCCTCTG-3′	396	NM131789	*Danio rerio*
zCry1R1311	Reverse		5′-GCGATATCTGCCAGTCTTAAGG-3′			
zCry2F830	Forward	*Cry2a*	5′-GAAAACCAGCACTCCTCCGCT-3′	492	NM131791	*Danio rerio*
zCry2R1322	Reverse		5′-CTATTCTCCGAGGTTTCCCTGC-3′			
zCry3F711	Forward	*Cry3*	5′-AGGACTCAGGGTGACAGCTTACA-3′	712	NM131786	*Danio rerio*
zCry3R1423	Reverse		5′-GAGGTACATCCCAAAGTTAAAGG-3′			
g18SF571	Forward	*18s rRNA*	5′-ATGATTAAGAGGGACGGCCGG-3′	509	EF189737	*Carassius auratus*
g18SR1080	Reverse		5′-AATAGTTACGCGGCCCCGTG-3′			
Degenerate primers for gene cloning:						
dPer2F-2	Forward	*Per2*	5′-AGCTTYRTCAATCCCTGGAGY-3′	849	NM182857	*Danio rerio*
dPer2R-2	Reverse		5′-CAGYTACAGCAGCACMATTGT-3′		EF208027	*Siganus guttatus*
					AF199499	*Xenopus laevis*
dPer3F	Forward	*Per3*	5′-GCCTGTTCATAATAATGGATCCAG-3′	460	NM131584	*Danio rerio*
dPer3R	Reverse		5′-CAGCCTGAGTCMGAAGTCACT-3′		NM001079228	*Xenopus tropicalis*
For qRT-PCR:						
gP1F56	Forward	*Per1*	5′-AGCGCCACTTCCTCCTCTGA-3′	130	EF690698	*Carassius auratus*
gP1R186	Reverse		5′-CCAACGGACAGCAGGTCTTCA-3′			
gP2F4	Forward	*Per2*	5′-TTTGTCAATCCCTGGAGCCGC-3′	116	EF690697	*Carassius auratus*
gP2R119	Reverse		5′-CGTGGCTGAGGGCAAATCCTT-3′			
gP3F26	Forward	*Per3*	5′-GGCTATGGCAGTCTGGCTAGTAA-3′	130	EF690699	*Carassius auratus*
gP3R155	Reverse		5′-GACATTGCAGCGGTTTTGTGCTG-3′			
gC1F11	Forward	*Cry1*	5′-TACCGGCTGCCACCAACAAC-3′	106	EF690700	*Carassius auratus*
gC1R117	Reverse		5′-TTTGGCCAAGTGGGCTGAGG-3′			
gC2F16	Forward	*Cry2*	5′-CGCTCTCCCTGTATGGTCAAC-3′	102	EF 690701	*Carassius auratus*
gC2R118	Reverse		5′-AAGGCAACCCGATCTGTGTGC-3′			
gC3F38	Forward	*Cry3*	5′-GGTGAGACAGAAGCCCTGGAA-3′	102	EF690702	*Carassius auratus*

PRIMER NAME		TARGE T GENE	SEQUENCE (from 5′ to 3′)	PCR product (bp)	ACCESSION NUMBER	SPECIES
gC3R140	Reverse		5′-CGCTCAATCACTGTTCGCAAGC-3′			
g18SF571	Forward	*18s rRNA*	5′-ATGATTAAGAGGGACGGCCGG-3′	143		
g18SR714	Reverse		5′-GTCGGAGGTTCGAAGACGATCA-3′			

Table 2

Characteristics of the partial cDNA sequences for each gene.

GENE NAME	FRAGMENT SIZE (nt)	*Danio rerio* IDENTITY (%)	GenBank ACCESSION NUMBER
Per1	531	88	EF690698
Per2	849	88	EF690697
Per3	460	84	EF690699
Cry1	396	92	EF690700
Cry2	492	93	EF690701
Cry3	712	91	EF690702

Table 3

Relative tissue expression pattern of *Period* and *Cryptochrome* transcripts in the goldfish.

GENE	TISSUE EXPRESSION PATTERN	
	High expression	**Low expression**
Per1	Liver, retina	Gut, brain, heart
Per2		Retina, gut, liver, heart, brain
Per3	Liver, retina	Gut, brain, liver, heart
Cry1	Brain, gut, heart, liver, retina	
Cry2	Gut, brain, retina, heart, liver	
Cry3	Gut, heart, brain, retina, liver	

Table 4

Parameters defining the gene expression rhythms in the retina of goldfish (*Carassius auratus*).

	Mesor ± SE (mRNA/*18s* mRNA)	**Amplitude ± SE (mRNA/*18s* mRNA)**	**Acrophase ± SE (hours)**
Per1	$(0.46 \pm 0.04)\cdot10^{-4}$	$(0.42 \pm 0.06)\cdot10^{-4}$	18.55 ± 0.50
Per2	$(1.21 \pm 0.21)\cdot10^{-4}$	$(1.16 \pm 0.31)\cdot10^{-4}$	6.15 ± 0.92
Per3	$(1.91 \pm 0.37)\cdot10^{-4}$	$(1.70 \pm 0.49)\cdot10^{-4}$	0.30 ± 1.25
Cry1	0.13 ± 0.01	0.11 ± 0.01	12.14 ± 0.43
Cry2	$(0.67 \pm 0.09)\cdot10^{-3}$	$(0.80 \pm 0.12)\cdot10^{-3}$	12.87 ± 0.61
Cry3	$(0.41 \pm 0.06)\cdot10^{-3}$	$(0.59 \pm 0.09)\cdot10^{-3}$	18.67 ± 0.47

The cosinor analysis was used to obtain the rhythmic parameters as defined by a sinusoidal function. Statistical significance was assumed when the ratio *SE(A)*/*A* was below 0.3

Table 5

Parameters defining the statistically significant gene expression rhythms of peripheral tissues of the goldfish (*Carassius auratus*).

	Mesor ± SE (mRNA/*18s* mRNA)	**Amplitude± SE(mRNA/*18s* mRNA)**	**Acrophase± SE(hours)**
LIVER			
Per3	$(1.50 \pm 0.19)\cdot10^{-3}$	$(1.41 \pm 0.29)\cdot10^{-3}$	18.88 ± 0.65
Cry2	$(1.34 \pm 0.09)\cdot10^{-4}$	$(0.79 \pm 0.14)\cdot10^{-4}$	7.49 ± 0.59
Cry3	$(8.00 \pm 1.24)\cdot10^{-5}$	$(6.19 \pm 1.60)\cdot10^{-5}$	13.73 ±1.17
GUT			
Per2	$(8.38 \pm 1.35)\cdot10^{-5}$	$(7.44 \pm 1.76)\cdot10^{-5}$	1.94 ± 1.05
Per3	$(2.23 \pm 0.24)\cdot10^{-3}$	$(2.55 \pm 0.37)\cdot10^{-3}$	20.94 ± 0.46
Cry2	$(1.39 \pm 0.12)\cdot10^{-4}$	$(0.81 \pm 0.18)\cdot10^{-4}$	6.99 ± 0.72
Cry3	$(1.30 \pm 0.14)\cdot10^{-4}$	$(1.13 \pm 0.20)\cdot10^{-4}$	18.34 ± 0.63

The cosinor analysis was used to obtain the rhythmic parameters as defined by a sinusoidal function. Statistical significance was assumed when the ratio *SE(A)/A* was below 0.3. Parameters are shown only for transcripts displaying significant rhythms.