Daily expression of a clock gene in the brain and pituitary of the Malabar grouper (*Epinephelus malabaricus*)

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Abstract

Recent studies have revealed that, in addition to regulating the circadian system, clock genes such as *cryptochrome* (*Cry*) genes are involved in seasonal and lunar rhythmicity in fish. This study clarified the transcriptional characteristics of a *Cry* subtype (*mgCry2*) in the brain of the Malabar grouper, *Epinephelus malabaricus*, which is an important aquaculture species that spawns around the new moon. The cDNA sequence of *mgCry2* showed high identity (97–99%) with fish *Cry2* and had an open reading frame encoding a protein with 170 amino acids. Phylogenetic analyses revealed that mgCRY2 had high identity with CRY in other fish species. Real-time quantitative polymerase chain reaction (qPCR) showed the widespread distribution of *mgCry2* in neural (brain, pituitary, and retina) and peripheral (heart, liver, kidney, spleen, intestine, and ovary) tissues. When immature Malabar groupers were reared under a light-dark cycle (LD = 12:12) and the amounts of *mgCry2* mRNA in the telencephalon and diencephalon were measured at 4-h intervals, the levels increased during photophase and decreased during scotophase. Day–night variation in *mgCry2* mRNA abundance was also observed in the pituitary. These daily profiles suggest that *mgCry2* is a light-responsive gene in neural tissues. *In situ* hybridization analyses showed that *mgCry2* was strongly transcribed in the nucleus lateralis tuberis of the ventral hypothalamus, peripheral area of the proximal pars distalis, and the pars intermedia of the pituitary. We conclude that clock genes expressed in the pituitary and diencephalon play a role in entraining the endocrine network of the Malabar grouper to periodic changes in external cues.

Keywords:

Circadian, Clock gene, Cryptochrome, Daily rhythm, Grouper, Lunar cycle, Pituitary

1. Introduction

In vertebrates, most physiological and behavioral events are rhythmic and controlled endogenously by biological clock systems (Pittendrigh, 1993). The survival and success of each species are ensured by clock-related predictions of and adaptive entrainment to environmental changes in habitats. It is generally accepted that the circadian rhythm oscillates over an approximately 24-h cycle and is regulated by a core feedback loop, which consists of positive [Circadian locomotor output cycles kaput (CLOCK) and Brain and muscle Arnt-like protein 1 (BMAL1)] and negative [PERIOD and CRYPTOCROME (CRY)] transcription factors. CLOCK and BMAL1 drive the rhythmic transcription of *period* (*Per*) and *cryptochrome* (*Cry*); PERIOD and CRY interact negatively with CLOCK and BMAL1 (Gachon et al., 2004). This molecular mechanism of the circadian system is conserved in vertebrates (Duston and Bromage, 1987; Norberg et al., 2004).

Recently, it was reported that clock genes are involved in the seasonal reproduction of fish (Herrero and Lepesant, 2014; Takeuchi et al., 2015). In the tropical damselfish (the sapphire devil, *Chrysiptera cyanea*), which is a long-day spawner (Bapary et al., 2009; Bapary and Takemura, 2010) and has a photoinducible phase for ovarian development (Takeuchi et al., 2015), manipulation of the photoperiod influenced the expression of *Per2*, *Cry1*, and *Cry2*, but not *Per1*, in the brain (Takeuchi et al., 2015). This implies that variation in clock gene expression according to the change in photoperiod contributes to seasonal reproduction. Similar insight was obtained into the lunar-related reproduction of the goldlined spinefoot, *Siganus guttatus*, which showed repeated lunar-dependent rhythmicity of *Cry1* and *Cry3* in its midbrain during the spawning season (Fukushiro et al., 2011). A more recent study revealed that the expression profiles of *Cry3* and *Per4* were moon phase-dependent under moonlight-disrupting conditions in the diencephalon of the goldlined spinefoot, suggesting that they act as circalunar-like clocks (Toda et al., 2014). These findings led to the hypothesis that the oscillation of clock

genes in the brain is involved in the reproductive cycle occurring on monthly and annual bases. However, little is known about how clock genes are involved in the phase shift and entrainment of the reproductive cycle in fish (Migaud et al., 2010), although this knowledge is important for efficient artificial control of breeding processes in aquaculture.

The Malabar grouper *Epinephelus malabaricus* (order Perciformes, family Serranidae) is distributed widely in the tropical waters of the Indo-West Pacific and is an important aquaculture species with high commercial value. Although it is a new moon spawner in nature, it tends to spawn sporadically from the full moon to the new moon under culture conditions. It is important to develop scheduled breeding methods by manipulating light and temperature. The present study examined the transcriptional characteristics of a *Cry* gene (*mgCry2*) in the brain of the Malabar grouper because moonlight-dependent fluctuations in *Cry2* were reported in the reef-building coral *Acropora millepora* (Levy et al., 2007). We focused mainly on the diencephalon and pituitary, where the neural center of the endocrine network for reproduction is located (Zohar et al., 2010). We cloned Malabar grouper *mgCry2* cDNA from the brain and examined its day–night variation under a programmed light-dark cycle. *In situ* hybridization (ISH) was used to determine its localization in the diencephalon and pituitary of this species.

2. Materials and Methods

2.1. Animals and experimental regimes

The Malabar grouper (body mass 69.0–72.4 g) used in this study were obtained from Okinawa Prefectural Sea Farming Center, Motobu, Okinawa, Japan, and transferred to Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan. They were reared in fiber-reinforced plastic stock tanks (3 metric ton capacity) with aerated running seawater under a natural photoperiod and natural water temperatures (ranged from 19.4 to 30.6 ^{o}C), and fed commercial pellets (Himesakura, Higashimaru, Kagoshima, Japan) daily at 1000

h. All experiments were conducted in compliance with the Animal Care and Use Committee guidelines of the University of the Ryukyus and regulations for the care and use of laboratory animals in Japan.

The fish used for molecular cloning (n = 4) and to examine the tissue distribution (n = 3 – 6) of *mgCry2* were taken from the stock tanks at 1200 h, anesthetized on ice, and then sacrificed by decapitation. The whole brain was taken from the skull for molecular cloning. In addition to the whole brain, the retina and other peripheral tissues (heart, liver, spleen, gill, intestine, and ovary) were sampled to examine the tissue distribution of *mgCry2*. The brain was further separated into the diencephalon, telencephalon, optic tectum, pituitary, cerebellum, and medulla oblongata. The tissues were immediately frozen in liquid nitrogen, and then stored at –80°C until use. Total RNA was extracted from the tissues using TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions. Following pretreatment with gDNA Eraser at 37℃ for 15 min to avoid contamination with genomic DNA, first-strand cDNA was synthesized from 1 μg of total RNA using Prime Script RT reagent with the gDNA Eraser kit (Takara Bio, Kusatsu, Japan), according to the manufacturer's protocol.

To examine day–night variation in *mgCry2* mRNA abundance, fish were taken from the stock tanks and housed in seven aquariums (six individuals per aquarium) with running seawater and aeration under a programmable photoperiod with 12 h of light and 12 h of darkness (LD = 12:12, lights on at 0600 h and off at 1800 h). Fluorescent lamps were set on the aquariums; the light intensity was 440 lx at the water surface. After acclimatization for 1 week, fish (were taken from each tank at Zeitgeber time (ZT) 5, 9, 13, 17, 21, 25, and 29 (for the telencephalon and diencephalon, n = 6 per each sampling point) and ZT9 and 21 (for the pituitary, n = 6 and 4, respectively), according to the results of the preliminary experiments (Yamashina, 2016). After anesthetizing the fish on ice, the brain was removed from the skull.

The telencephalon, diencephalon, and pituitary were separated and immediately frozen in liquid nitrogen, and then stored at –80°C until use. Samples were collected during scotophase (ZT13, ZT17, and ZT21) under dim lighting. RNA extraction from the tissues and reverse transcription were carried out using the abovementioned methods.

For ISH, fish (n = 4) were taken from the tanks at 1200 h, anesthetized on ice, and then decapitated. The brain was removed from the skull and fixed in 4% paraformaldehyde at 4°C for 24 h. Following dehydration in an ethanol series and clearance with xylene, the samples were embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA), sectioned serially every 5 μm, and stored at 4°C until use.

2.2. Cloning

The primers used in this study were designed from the sequences of zebrafish (*Danio rerio*) *Cry1a* and *Cry1b* (GenBank accession no. **NM_131790**), goldlined spinefoot *Cry1* (**AB643455**), threespot wrasse (*Halichoeres trimaculatus*) *Cry1a* (**HQ893881**), and Atlantic salmon (*Salmo salar*) *Cry1* (**BT058825**) (Table 1). The cDNA fragments encoding *mgCry2* were amplified by polymerase chain reaction (PCR) in 10 μL containing 50% Go Taq DNA Polymerase Mixture (Promega, Madison, WI, USA), 0.3 μM forward and reverse primers, and 1.6% cDNA under the following cycling conditions: 95°C for 3 min; 40 cycles at 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s; and a final extension period for 5 min at 72°C. The products were fractioned by 2% agarose gel electrophoresis, subcloned into pGEM-T easy vector (Promega), and sequenced using a 3730xl DNA Analyzer (Applied Biosystems, Waltham, MA, USA).

2.3. Sequence analysis

149 The amino acid sequence of the Malabar grouper CRY2 cDNA was deduced using the program
150 ORF Finder (NCBI; http://www.ncbi.nlm.nih.gov/projects/gorf/). Its identities were verified by
151 searching the NCBI database using blast (https://blast.ncbi.nlm.nih.gov/blast.cgi). ClustalX2
152 was used to generate a neighbor-joining phylogenetic tree with bootstrap confidence values
153 based on 1000 replicates (Felsenstein, 1985).
154
155 2.4. Real-time quantitative PCR (qPCR)
156 The tissue distribution of and daily variation in *mgCry2* mRNA abundance were examined
157 using a CFX96 Real Time System (Bio-Rad, Hercules, CA, USA) and Go Taq qPCR Master
158 Mix (Promega). Table 1 lists the primer pairs used for the amplification of *mgCry2* and
159 elongation factor 1 alpha (*mgEf1α*). Each PCR was carried out in a final volume of 10 μL
160 containing 2× GoTaq qPCR Master Mix, the forward and reverse primers (0.3 μM each), cDNA
161 template (20 ng), and nuclease-free water. The qPCR conditions were 2 min at 95°C followed
162 by 40 cycles of 95°C for 15 s and 60°C for 1 min. Plasmid DNA or pooled cDNA from the
163 brain at 10-fold dilutions were subjected to qPCR to construct the standard curve. The *mgCry2*
164 and *mgEf1α* levels in all tissues were measured in duplicate. The relative mRNA expression of
165 *mgCry2* to *mgEf1α* was calculated using the ΔΔCt method (Figs. S1 and S2).
166 Verification of *mgEf1α* as an internal control gene was preliminary checked by comparing
167 transcript levels among candidate genes including *mgß-Actin*, *mgEf1α*, and *mgRpl8* (Fig. S3,
168 Table S1).
169
170 2.5. ISH
171 The hybridization probes were prepared from the primer sets (Table 1) by labeling with
172 digoxigenin (DIG) using a DIG RNA Labeling Kit (Sp6/T7; Roche, Indianapolis, IN, USA),
173 according to the manufacturer's instructions. ISH was performed according to the DIG

application manual for nonradioactive ISH (Roche), with minor modifications (Takeuchi et al., 2015). Every section was washed with phosphate-buffered saline and treated with 10 μg/mL of proteinase K (Sigma-Aldrich) for 15 min at 37°C. Hybridization was performed with 500 ng/mL of DIG-labeled sense and antisense RNA probes at 55°C for 12h. A sense RNA probe was used as a negative control. After hybridization, the slides were washed three times with 2× saline sodium citrate containing 50% formamide and 1× saline sodium citrate, blocked with 1.5% blocking reagent (Roche, Basel, Switzerland) for 1 h at room temperature, and incubated with an alkaline phosphatase-conjugated anti-DIG antibody (Roche) for overnight at 4°C. The hybridization signal was detected using nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphatase *p*-toluidine salt solution. Sections were rinsed in Tris-EDTA (TE) buffer, mounted using 87% glycerol, and observed under a light microscope.

2.6. Statistics

All data are expressed as the mean ± standard error of the mean (SEM). Values were compared using Student's *t*-test (day–night difference in *mgCry2* in the pituitary) and a one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparison test (tissue distribution of and daily variation in *mgCry2* in the telencephalon and diencephalon). Values of $P < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Cloning and characterization of Malabar grouper *Cry2*

The partial *mgCry2* cDNA sequence (**LC468787**) consisted of 512 bases with an open reading frame (ORF) encoding a protein with 170 amino acids. The amino acid sequence shared 97% identity with the zebrafish sequence, 98% with the European seabass (*Dicentrarchus labrax*)

199 sequence, and 99% with both the Nile tilapia (*Oreochromis niloticus*) and sapphire devil
200 sequences. The phylogenetic tree shows that mgCRY2 clustered within a clade composed of
201 teleost CRY2s (Fig. 1A).
202
203 3.2. Distribution of Malabar grouper *Cry2* mRNA
204 The tissue distribution of *mgCry2* mRNA at 1200 h was assessed using qPCR (Fig. 1B). The
205 expressoin of *mgCry2* could be detected in all tissues tested. Strong expression was observed
206 in neural tissues (brain and retina). In the separated parts of the brain, strong *mgCry2* expression
207 was seen in the cerebellum and retina. No amplified products were observed in the negative
208 control (data not shown).
209
210 3.3. Day–night variation in Malabar grouper *Cry2*
211 The mRNA abundance of *mgCry2* was measured in the telencephalon (Fig. 2A) and
212 diencephalon (Fig. 2B) at 4-h intervals. Its abundance showed daily variation, with a decrease
213 during scotophase (ZT21 for the telencephalon and ZT17–ZT21 for the diencephalon). When
214 day–night variation in *mgCry2* mRNA in the pituitary was compared between ZT9
215 (photophase) and ZT21 (scotophase), the level was significantly higher ($P < 0.001$) at ZT9 than
216 at ZT21 (Fig. 3).
217
218 3.4. ISH
219 The distribution of *mgCry2* transcripts in the diencephalon and pituitary was examined using
220 ISH (Fig. 4). Strong signals were noted in the nucleus lateralis tuberis of the ventral
221 hypothalamus and the peripheral area of the proximal pars distalis and pars intermedia in the
222 pituitary. No labeled cells were detected in the control sections when the *mgCry2* sense probe
223 was used (Fig. 6S).

4. Discussion

The cDNA of *mgCry2* was successfully cloned and its transcription was seen widely in neural and peripheral tissues. This tissue distribution is in agreement with that in various fish (del Pozo et al., 2012b; Hoskins and Volkoff, 2012; Martín-Robles et al., 2012; Sánchez et al., 2010; Velarde et al., 2009), suggesting that, in addition to the master clocks in the brain, peripheral circadian clocks exist in fish, as reported in zebrafish cell lines and embryos (Whitmore and Foulkes, 2000). High-level *mgCry2* expression was confirmed in neural tissues, including the retina and separated brain parts (e.g., the cerebellum) of the Malabar grouper. The abundance of this gene in tissues may differ among species because relatively strong expression of *Cry2* was seen in the peripheral tissues (heart, muscle, spleen, and intestine) of the European seabass *Dicentrarchus labrax* (del Pozo et al., 2012b), whereas its expression levels were relatively low in the Malabar grouper.

The present study shows that under LD conditions, *mgCry2* fluctuated daily with an increase during photophase and decrease during scotophase in the telencephalon and diencephalon, suggesting that it is a light-inducible gene. In the European seabass, the acrophase of *Cry1* and *Cry2* expression in the brain occurred at the beginning and end of the light phase when fish were reared under LD conditions (del Pozo et al., 2012b). The daily transcript profile of *Cry1*, but not of *Cry2*, in the brain matched that in the liver. In the European seabass, it was also reported that the expression of a clock gene (*Per1*) in these two tissues increased around the onset of the light phase (Sánchez et al., 2010), and that feeding time influenced *Per1* transcription in the liver (feeding-entrained clock), but not in the brain (light-entrained clock) (del Pozo et al., 2012a). Therefore, the synchronous oscillation of *Cry1* and *Per1* suggests the existence of a negative feedback loop in the circadian system (del Pozo et al., 2012b). The asynchronous oscillation of *Cry2* in the brain suggests a light-responsive function.

There is also seasonal variation in clock gene expression patterns in the Atlantic salmon brain, in which *Clock*, *Bmal1*, and *Per2* showed significant variation under short-day, but not long-day, conditions, while the expression of *Cry2* varied under both short- and long-day conditions (Davie et al., 2009). Seasonal variation in clock genes was also found in the pituitary of the European seabass, suggesting that changes in melatonin and temperature both mediate the photoperiodic effect of clock gene expression (Herrero and Lepesant, 2014). The present study indicates significant day-high and night-low variation in *mgCry2* in the pituitary, although the *mgCry2* transcripts in this tissue were determined only at time two points (ZT10 and ZT22). However, clock genes in the pituitary exhibit an oscillating pattern similar to those in the diencephalon and telencephalon because the peak and basal levels of *mgCry2* transcripts were determined in the present study.

Immunohistochemical studies of the pituitary of the Malabar grouper (30–360 days after hatching) detected immunoreactivity against the β-subunit of follicle-stimulating hormone (FSH) in cells in the center of the proximal pars distalis area, and immunoreactivity against the β-subunit of luteinizing hormone (LH) in cells in the center of the proximal pars distalis area and in the peripheral pars intermedia area (Murata et al., 2012). Our ISH analyses showed that *mgCry2* was transcribed in the inferior part of the proximal pars distalis and the peripheral area of the pars intermedia of the pituitary of the Malabar grouper. A comparison of the results of the two studies indicates that clock genes are expressed in gonadotrophs (mainly in LH-producing cells) or that cells expressing clock genes are located near gonadotrophs. In the pituitary of the Nile tilapia, in addition to cells containing FSH and LH, those containing ACTH and α-MSH were stained immunohistochemically in the pars intermedia. Therefore, clock genes may function as timekeepers for the daily/seasonal secretion of these hormones in the pituitary. Our ISH study also revealed the transcription of *mgCry2* in the nucleus lateralis tuberis of the ventral hypothalamus. In this regard, the existence of immunoreactivities against

LH-RH and ß-endorphin was evident in this area of the southern platyfish *Xiphophorus maculatus* (Schreibman et al., 1979) and the mrigal carp *Cirrhinus mrigala* (Sakharkar et al., 2006), respectively. This may be an indirect evidence suggesting a possible linkage between clock genes and reproduction. Alternatively, clock genes in this area may be related to vision-related behavior because a real-time imaging technique showed the abolishment of prey-capture behavior on ablation of the pretectum (Muto et al., 2017).

In conclusion, clock genes (e.g., *mgCry2*) expressed in the pituitary and diencephalon can convey external cues in relation to natural lights to endocrine networks and behavioral mechanisms in the brain of the Malabar grouper. Special attention may be paid to lunar light because the Malabar grouper is a typical new moon spawner. A recent qPCR analyses revealed that the transcript levels of *fshß* and *lhß* increased towards the first quarter moon in the pituitary of the honeycomb grouper *E. merra*, suggesting that these genes exhibit the lunar-related transcription (Fukunaga, 2018). Additional studies are needed to clarify the involvement of moonlight in transcription of clock genes in the Malabar grouper and in the lunar related reproduction.

Conflict of interest

The authors have declared no conflict of interest.

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Authors' contributions

FY designed and performed all the experiments and analyzed all of the data obtained in the present study. YT, KF, SU, EST, CY, and JB were contributors in preparing samples and performing the experiments (*in situ* hybridization and molecular cloning/characterization, respectively). They participated in preparing the manuscript. AT was a collaborator and supervisor in analyzing the data and writing the manuscript. All authors have read and approved the final manuscript.

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Table 1. Primes used in the present study.*

Primer	Sequence
Cloning	
dgCryF	5'-CHGTGTGGCCHGGDGGAG-3'
dgCryR	5'-AYRCCYTCYTCCCAGCTGAT-3'
Real-time PCR	
mgCry2-realF	5'-ATAGAGCGCCATCTGGAGAG-3'
mgCry2-realR	5'-CAAGTGCTTCGGGATTTTTG-3'
mgEf1α-realF	5'-ACGTGTCCGTCAAGGAAATC-3'
mgEf1α-realR	5'-GGGTGGTTCAGGATGATGAC-3'
In situ hybridization	
Forward	5'-ATAGAGCGCCATCTGGAGAG-3'
Reverse	5'-CAAGTGCTTCGGGATTTTTG-3'

*The part of the sequence amplified by the primer pairs (using base pair numbering of the sequence submission).

Figure legends

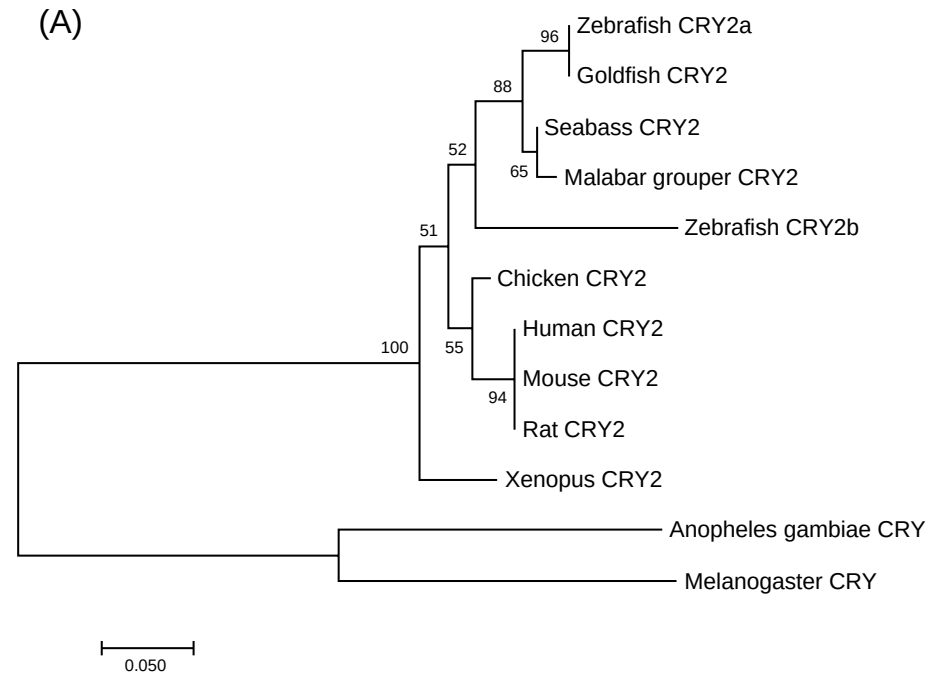
Fig. 1. Phylogenetic analysis of mgCRY2 (A) and tissue distribution of *mgCry2* (B). For phylogenetic analyses, one thousand bootstrap repetitions were performed, and values are shown at the in inner nodes. The scale bar is calibrated in substitutions per site. Drosophila CRY and Anopheles CRY were used as the outgroup. The following amino acid sequences were used for alignment and phylogenetic analysis; Malabar grouper CRY2 (*Epinephelus malabaricus* **LC468787**), Anopheles gambiae CRY (*Anopheles gambiae* **Q7PYI7**), Melanogaster CRY (*Drosophila melanogaster* **NP_732407.1**), Zebrafish CRY2a (*Danio rerio* **CAQ13306.1**), Zebrafish CRY2b (*Danio rerio* **NP_571867.1**), Human CRY2 (*Homo sapiens* **NP_066940.2**), Mouse CRY2 (*Mus musculus* **NP_034093.1**), Chicken CRY2 (*Gallus gallus* **NP_989575**), Xenopus CRY2 (*Xenopus laevis* **AAH77381**), Rat (*Rattus norvegicus* **NP_596896.1**), Seabass (*Dicentrarchus labrax* **AFP33463**), Goldfish (*Carassius auratus* **ABU93791.1**). For tissue distribution, neural and peripheral tissues were collected from the Malabar grouper (n = 3 - 6). Expression levels of *mgCry2* were measured using real-time quantitative PCR. The data were normalized by determining the amount of *mgEf1α* mRNA. Different letters indicate statistically significant differences ($P < 0.05$; Tukey-Kramer test). Each value was expressed as mean ± SEM. Re; Retina, Tel; Telencephalon, Op; Optic tectum, Di; Diencephalon, Pt; Pituitary, Ce; Cerebellum, Md; Medulla oblongata, H; Heart, L; Liver, K; Kidney, S; Spleen, G; Gut, In; Intestine, O; Ovary.

Fig. 2 Daily changes in *mgCry2* in the telencephalon (A) and diencephalon (B) of the Malabar grouper under light-dark conditions (LD12:12). The brain was collected from the fish (n = 6) and the telencephalon and diencephalon were separated. Expression levels of *mgCry2* in these two parts of the brain were measured using real-time quantitative PCR. The data were normalized by determining the amount of *mgEf1α.* Mean values with

different letters in the figure show significant differences ($P < 0.05$; Tukey-Kramer test). Horizontal bars with white and black colors in the figures indicate photophase and scotophase, respectively.

Fig. 3. Day-night variation in *mgCry2* mRNA levels in the pituitary. The pituitary was collected from the fish at ZT9 (n = 6) and ZT21 (n = 4). Expression levels of *mgCry2* were measured using qPCR. The data were normalized by determining the amount of *mgEf1α*. Line across and length of each box indicate median and interquartile range of the sample, respectively. Maximum and minimum of sample are shown by whisker. An asterisk in the figure shows significant difference ($P < 0.001$; Student's t-test).

Fig. 4. Detection of *mgCry2* signals in the brain of the Malabar grouper. The whole brain was fixed in 4% paraformaldehyde and sectioned at 5 μm. Transcription of *mgCry2* was localized by *in situ* hybridization. Arrow heads indicate *mgCry2* signals. NLT; Nucleus lateralis tuberis, PI; Pars intermedia, PPD, Proximal pars distalis. Inserted bar shows 200 μm.


(A)
Zebrafish CRY2a
Goldfish CRY2
Seabass CRY2
Malabar grouper CRY2
Zebrafish CRY2b
Chicken CRY2
Human CRY2
Mouse CRY2
Rat CRY2
Xenopus CRY2
Anopheles gambiae CRY
Melanogaster CRY
96
88
52
65
51
100
55
94
0.050

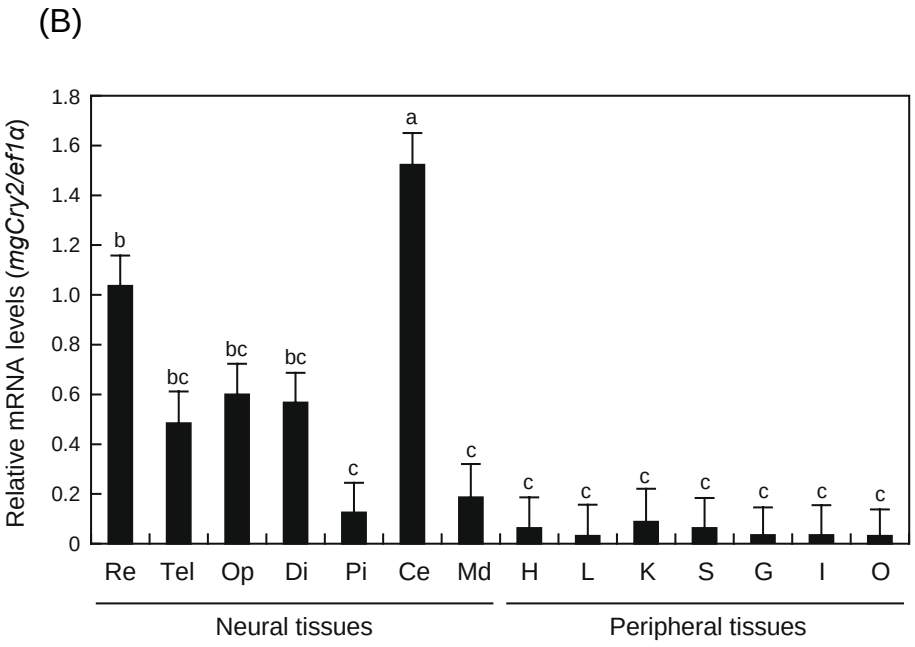

(B)
Relative mRNA levels (mgCry2/ef1α)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
b
bc
bc
bc
c
a
c
c
c
c
c
c
c
c
Re
Tel
Op
Di
Pi
Ce
Md
H
L
K
S
G
I
O
Neural tissues
Peripheral tissues

Figure 1

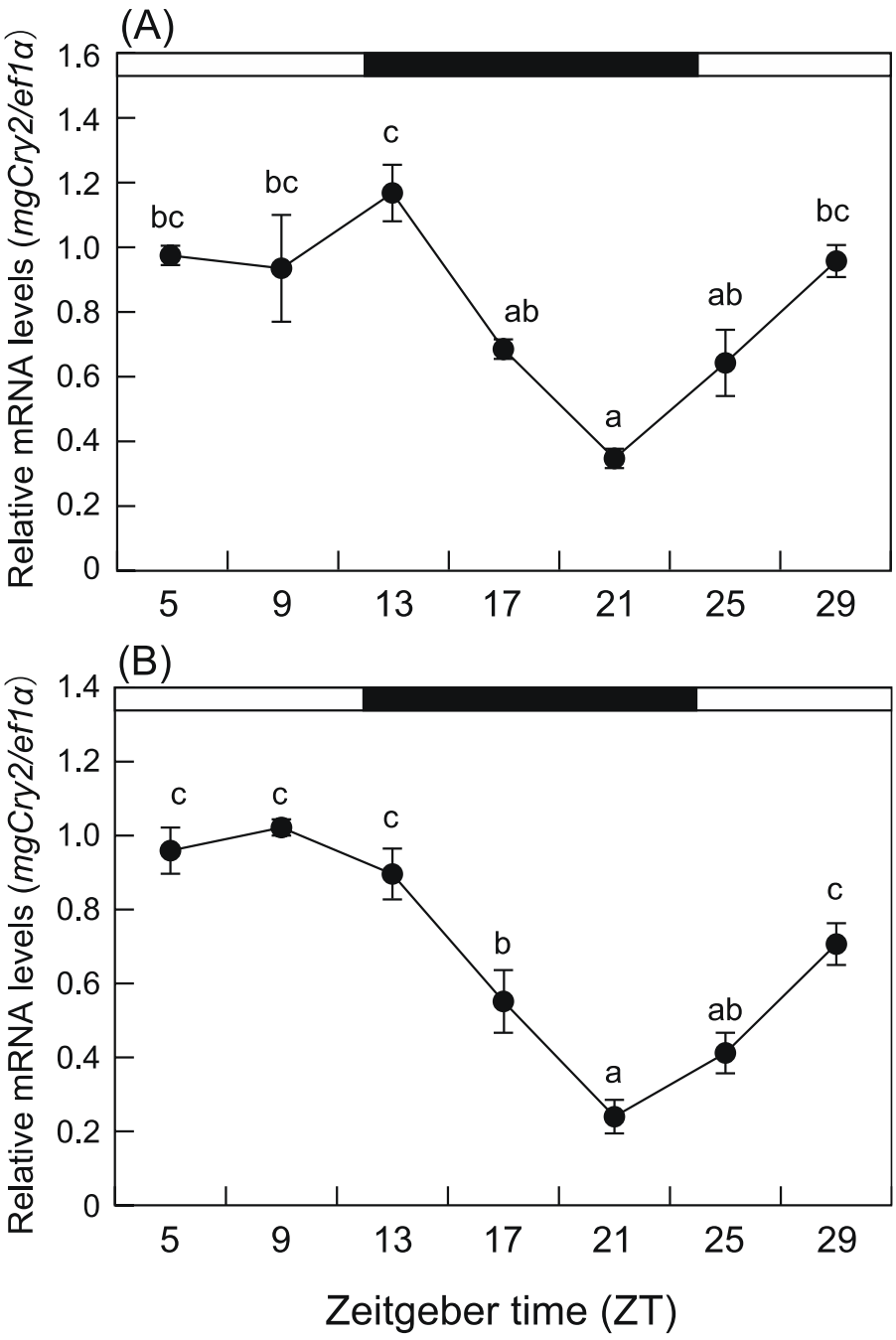

(A)
Relative mRNA levels (mgCry2/ef1α)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
bc
bc
c
ab
a
ab
bc
5
9
13
17
21
25
29
(B)
Relative mRNA levels (mgCry2/ef1α)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
c
c
c
b
a
ab
c
5
9
13
17
21
25
29
Zeitgeber time (ZT)

Figure 2

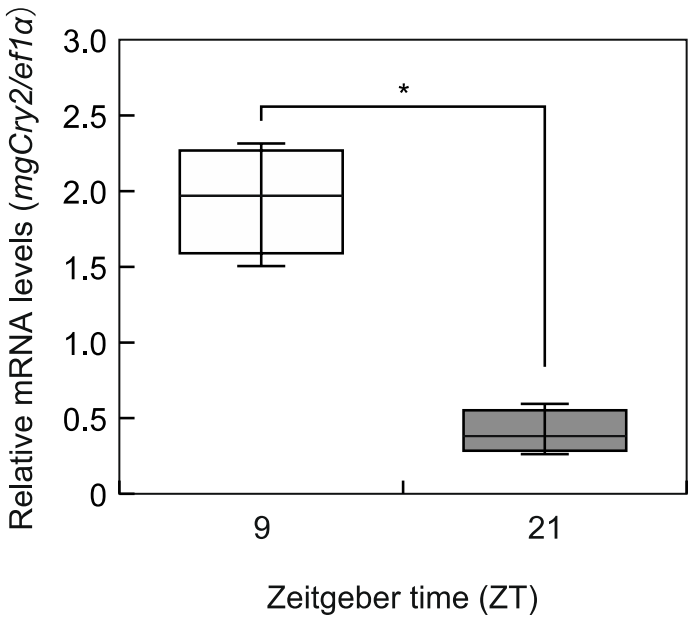

Relative mRNA levels (mgCry2/ef1α)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
9
21
Zeitgeber time (ZT)

Figure 3

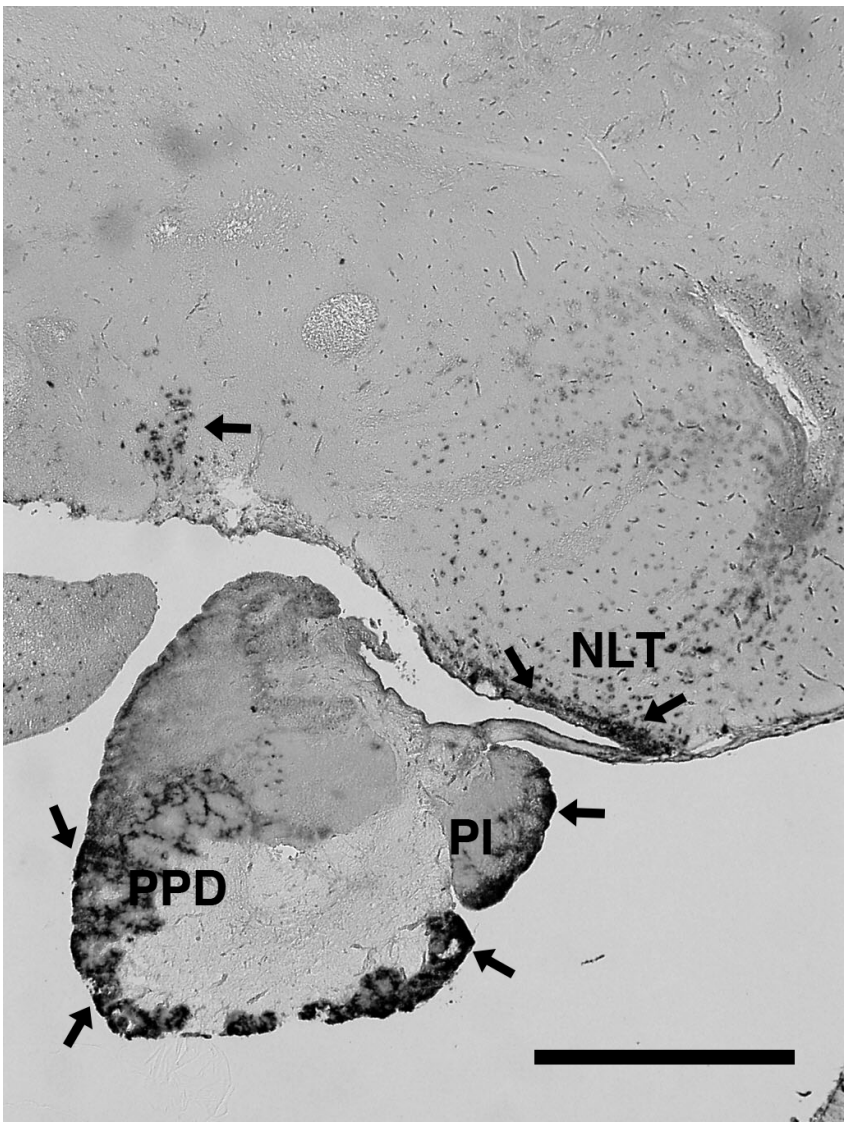


Figure 4