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Author	Fumika Yamashina, Yuki Takeuchi, Kodai Fukunaga, Shingo Udagawa, Ee Suan Tan, Junhwan Byun, Chihiro Yamauchi, Akihiro Takemura
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2 *malabaricus*)

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4 Fumika Yamashina^a, Yuki Takeuchi^{b,c}, Kodai Fukunaga^a, Shingo Udagawa^a, Ee Suan Tan^a,
5 Junwhan Byun^a, Chihiro Yamauchi^c, and Akihiro Takemura^{c,*}

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7

8 ^aGraduate School of Engineering and Science, University of the Ryukyus, Senbaru 1, Nishihara,
9 Okinawa 903-0213, Japan

10 ^bOkinawa Institute of Science and Technology Graduate School, 1919-1 Tancha, Onna,
11 Okinawa 904-0495, Japan

12 ^cFaculty of Science, University of the Ryukyus, Senbaru 1, Nishihara, Okinawa 903-0213,
13 Japan

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18

19 *Corresponding author at: Department of Chemistry, Biology and Marine Science, University
20 of the Ryukyus, Nishihara, Okinawa 903-0213, Japan

21 *E-mail:* takemura@sci.u-ryukyu.ac.jp (A. Takemura)

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25

26 **Abstract**

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

46

47 **Keywords:**

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

49

50 1. Introduction

51 In vertebrates, most physiological and behavioral events are rhythmic and controlled
52 endogenously by biological clock systems (Pittendrigh, 1993). The survival and success of each
53 species are ensured by clock-related predictions of and adaptive entrainment to environmental
54 changes in habitats. It is generally accepted that the circadian rhythm oscillates over an
55 approximately 24-h cycle and is regulated by a core feedback loop, which consists of positive
56 [Circadian locomotor output cycles kaput (CLOCK) and Brain and muscle Arnt-like protein 1
57 (BMAL1)] and negative [PERIOD and CRYPTOCHROME (CRY)] transcription factors.
58 CLOCK and BMAL1 drive the rhythmic transcription of *period* (*Per*) and *cryptochrome* (*Cry*);
59 PERIOD and CRY interact negatively with CLOCK and BMAL1 (Gachon et al., 2004). This
60 molecular mechanism of the circadian system is conserved in vertebrates (Duston and Bromage,
61 1987; Norberg et al., 2004).

62 Recently, it was reported that clock genes are involved in the seasonal reproduction of
63 fish (Herrero and Lepesant, 2014; Takeuchi et al., 2015). In the tropical damselfish (the sapphire
64 devil, *Chrysiptera cyanea*), which is a long-day spawner (Bapary et al., 2009; Bapary and
65 Takemura, 2010) and has a photoinducible phase for ovarian development (Takeuchi et al.,
66 2015), manipulation of the photoperiod influenced the expression of *Per2*, *Cry1*, and *Cry2*, but
67 not *Per1*, in the brain (Takeuchi et al., 2015). This implies that variation in clock gene
68 expression according to the change in photoperiod contributes to seasonal reproduction. Similar
69 insight was obtained into the lunar-related reproduction of the goldlined spinefoot, *Siganus*
70 *guttatus*, which showed repeated lunar-dependent rhythmicity of *Cry1* and *Cry3* in its midbrain
71 during the spawning season (Fukushiro et al., 2011). A more recent study revealed that the
72 expression profiles of *Cry3* and *Per4* were moon phase-dependent under moonlight-disrupting
73 conditions in the diencephalon of the goldlined spinefoot, suggesting that they act as circalunar-
74 like clocks (Toda et al., 2014). These findings led to the hypothesis that the oscillation of clock

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

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

91

92 2. Materials and Methods

93 2.1. Animals and experimental regimes

94 The Malabar grouper (body mass 69.0–72.4 g) used in this study were obtained from Okinawa
95 Prefectural Sea Farming Center, Motobu, Okinawa, Japan, and transferred to Sesoko Station,
96 Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan. They were
97 reared in fiber-reinforced plastic stock tanks (3 metric ton capacity) with aerated running
98 seawater under a natural photoperiod and natural water temperatures (ranged from 19.4 to 30.6
99 °C), and fed commercial pellets (Himesakura, Higashimaru, Kagoshima, Japan) daily at 1000

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

103 The fish used for molecular cloning (n = 4) and to examine the tissue distribution (n =
104 3 – 6) of *mgCry2* were taken from the stock tanks at 1200 h, anesthetized on ice, and then
105 sacrificed by decapitation. The whole brain was taken from the skull for molecular cloning. In
106 addition to the whole brain, the retina and other peripheral tissues (heart, liver, spleen, gill,
107 intestine, and ovary) were sampled to examine the tissue distribution of *mgCry2*. The brain was
108 further separated into the diencephalon, telencephalon, optic tectum, pituitary, cerebellum, and
109 medulla oblongata. The tissues were immediately frozen in liquid nitrogen, and then stored at
110 –80°C until use. Total RNA was extracted from the tissues using TriPure Isolation Reagent
111 (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer’s instructions.
112 Following pretreatment with gDNA Eraser at 37°C for 15 min to avoid contamination with
113 genomic DNA, first-strand cDNA was synthesized from 1 µg of total RNA using Prime Script
114 RT reagent with the gDNA Eraser kit (Takara Bio, Kusatsu, Japan), according to the
115 manufacturer’s protocol.

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

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

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

134

135 2.2. Cloning

136 The primers used in this study were designed from the sequences of zebrafish (*Danio rerio*)
137 *Cry1a* and *Cry1b* (GenBank accession no. **NM_131790**), goldlined spinefoot *Cry1*
138 (**AB643455**), threespot wrasse (*Halichoeres trimaculatus*) *Cry1a* (**HQ893881**), and Atlantic
139 salmon (*Salmo salar*) *Cry1* (**BT058825**) (Table 1). The cDNA fragments encoding *mgCry2*
140 were amplified by polymerase chain reaction (PCR) in $10\ \mu\text{L}$ containing 50% Go Taq DNA
141 Polymerase Mixture (Promega, Madison, WI, USA), $0.3\ \mu\text{M}$ forward and reverse primers, and
142 1.6% cDNA under the following cycling conditions: 95°C for 3 min; 40 cycles at 95°C for 45
143 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
144 were fractionated by 2% agarose gel electrophoresis, subcloned into pGEM-T easy vector
145 (Promega), and sequenced using a 3730xl DNA Analyzer (Applied Biosystems, Waltham, MA,
146 USA).

147

148 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 (*mgEfl α*). Each PCR was carried out in a final volume of 10 μ L
160 containing 2 \times GoTaq qPCR Master Mix, the forward and reverse primers (0.3 μ M 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 *mgEfl α* levels in all tissues were measured in duplicate. The relative mRNA expression of
165 *mgCry2* to *mgEfl α* was calculated using the $\Delta\Delta$ Ct method (Figs. S1 and S2).

166 Verification of *mgEfl α* as an internal control gene was preliminary checked by comparing
167 transcript levels among candidate genes including *mg β -Actin*, *mgEfl α* , 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

174 application manual for nonradioactive ISH (Roche), with minor modifications (Takeuchi et al.,
175 2015). Every section was washed with phosphate-buffered saline and treated with 10 µg/mL of
176 proteinase K (Sigma-Aldrich) for 15 min at 37°C. Hybridization was performed with 500
177 ng/mL of DIG-labeled sense and antisense RNA probes at 55°C for 12h. A sense RNA probe
178 was used as a negative control. After hybridization, the slides were washed three times with 2×
179 saline sodium citrate containing 50% formamide and 1× saline sodium citrate, blocked with
180 1.5% blocking reagent (Roche, Basel, Switzerland) for 1 h at room temperature, and incubated
181 with an alkaline phosphatase-conjugated anti-DIG antibody (Roche) for overnight at 4°C. The
182 hybridization signal was detected using nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-
183 indolylphosphatase *p*-toluidine salt solution. Sections were rinsed in Tris-EDTA (TE) buffer,
184 mounted using 87% glycerol, and observed under a light microscope.

185

186

187 2.6. Statistics

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

193

194 3. Results

195 3.1. Cloning and characterization of Malabar grouper *Cry2*

196 The partial *mgCry2* cDNA sequence (**LC468787**) consisted of 512 bases with an open reading
197 frame (ORF) encoding a protein with 170 amino acids. The amino acid sequence shared 97%
198 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).

224

225 4. Discussion

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

237 The present study shows that under LD conditions, *mgCry2* fluctuated daily with an
238 increase during photophase and decrease during scotophase in the telencephalon and
239 diencephalon, suggesting that it is a light-inducible gene. In the European seabass, the
240 acrophase of *Cry1* and *Cry2* expression in the brain occurred at the beginning and end of the
241 light phase when fish were reared under LD conditions (del Pozo et al., 2012b). The daily
242 transcript profile of *Cry1*, but not of *Cry2*, in the brain matched that in the liver. In the European
243 seabass, it was also reported that the expression of a clock gene (*Per1*) in these two tissues
244 increased around the onset of the light phase (Sánchez et al., 2010), and that feeding time
245 influenced *Per1* transcription in the liver (feeding-entrained clock), but not in the brain (light-
246 entrained clock) (del Pozo et al., 2012a). Therefore, the synchronous oscillation of *Cry1* and
247 *Per1* suggests the existence of a negative feedback loop in the circadian system (del Pozo et al.,
248 2012b). The asynchronous oscillation of *Cry2* in the brain suggests a light-responsive function.

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

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

274 LH-RH and β -endorphin was evident in this area of the southern platyfish *Xiphophorus*
275 *maculatus* (Schreibman et al., 1979) and the mrigal carp *Cirrhinus mrigala* (Sakharkar et al.,
276 2006), respectively. This may be an indirect evidence suggesting a possible linkage between
277 clock genes and reproduction. Alternatively, clock genes in this area may be related to vision-
278 related behavior because a real-time imaging technique showed the abolishment of prey-capture
279 behavior on ablation of the pretectum (Muto et al., 2017).

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

289

290 **Conflict of interest**

291 The authors have declared no conflict of interest.

292

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300

301 **Authors' contributions**

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

308

309 **References**

310

311 Bapary, M.A.J., Fainuulelei, P., Takemura, A., 2009. Environmental control of gonadal
312 development in the tropical damselfish *Chrysiptera cyanea*. *Mar. Biol. Res.* 5, 462–469.
313 doi:10.1080/17451000802644722

314 Bapary, M.A.J., Takemura, A., 2010. Effect of temperature and photoperiod on the reproductive
315 condition and performance of a tropical damselfish *Chrysiptera cyanea* during different
316 phases of the reproductive season. *Fisheries Sci.* 76, 769–776. doi:10.1007/s12562-010-
317 0272-0

318 Davie, A., Minghetti, M., Migaud, H., 2009. Seasonal variations in clock-gene expression in
319 Atlantic salmon (*Salmo salar*). *Chronobiol. Int.* 26, 379–395.
320 doi:10.1080/07420520902820947

321 del Pozo, A., Montoya, A., Vera, L.M., Sánchez-Vázquez, F.J., 2012a. Daily rhythms of clock
322 gene expression, glycaemia and digestive physiology in diurnal/nocturnal European

323 seabass. *Physiol. Behav.* 106, 446–450. doi:10.1016/j.physbeh.2012.03.006

324 del Pozo, A., Vera, L.M., Sánchez, J.A., Sánchez-Vázquez, F.J., 2012b. Molecular cloning,
325 tissue distribution and daily expression of *cry1* and *cry2* clock genes in European seabass
326 (*Dicentrarchus labrax*). *Comp. Biochem. Physiol. A* 163, 364–371.
327 doi:10.1016/j.cbpa.2012.07.004

328 Duston, J., Bromage, N., 1987. Constant photoperiod regimes and the entrainment of the annual
329 cycle of reproduction in the female rainbow trout (*Salmo gairdneri*). *Gen. Comp.*
330 *Endocrinol.* 65, 373–384.

331 Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap.
332 *Evolution* 39, 783–791. doi:10.2307/2408678

333 Fukushima, M., Takeuchi, T., Takeuchi, Y., Hur, S.-P., Sugama, N., Takemura, A., Kubo, Y.,
334 Okano, K., Okano, T., 2011. Lunar phase-dependent expression of cryptochrome and a
335 photoperiodic mechanism for lunar phase-recognition in a reef fish, goldlined spinefoot.
336 *PLoS ONE* 6, e28643. doi:10.1371/journal.pone.0028643

337 Fukunaga, K., 2018. Lunar-rhythmic variation of gonadotropin-releasing hormones and
338 circadian clock genes, *Cryptochrome*, in the brain of the honeycomb grouper *Epinephelus*
339 *merra*. Master's thesis, University of the Ryukyus, Japan.

340 Gachon, F., Nagoshi, E., Brown, S., Ripperger, J., Schibler, U., 2004. The mammalian circadian
341 timing system: from gene expression to physiology. *Chromosoma* 113, 103–112.
342 doi:10.1007/s00412-004-0296-2

343 Herrero, M.J., Lepesant, J.M.J., 2014. Daily and seasonal expression of clock genes in the
344 pituitary of the European sea bass (*Dicentrarchus labrax*). *Gen. Comp. Endocrinol.* 208,
345 30–38. doi:10.1016/j.ygcen.2014.08.002

346 Hoskins, L.J., Volkoff, H., 2012. The comparative endocrinology of feeding in fish: Insights
347 and challenges. *Gen. Comp. Endocrinol.* 176, 327–335.

348 Levy, O., Appelbaum, L., Leggat, W., Gothlif, Y., Hayward, D.C., Miller, D.J., Hoegh-
349 Guldberg, O., 2007. Light-responsive cryptochromes from a simple multicellular animal,
350 the coral *Acropora millepora*. *Science* 318, 467–470. doi:10.1126/science.1145432

351 Martín-Robles, A.J., Whitmore, D., Sánchez-Vázquez, F.J., Pendón, C., Muñoz-Cueto, J.-A.,
352 2012. Cloning, tissue expression pattern and daily rhythms of *Period1*, *Period2*, and *Clock*
353 transcripts in the flatfish Senegalese sole, *Solea senegalensis*. *J. Comp. Physiol. B* 182,
354 673–685. doi:10.1007/s00360-012-0653-z

355 Migaud, H., Davie, A., Taylor, J.F., 2010. Current knowledge on the photoneuroendocrine
356 regulation of reproduction in temperate fish species. *J. Fish Biol.* 76, 27–68.
357 doi:10.1111/j.1095-8649.2009.02500.x

358 Murata, R., Kobayashi, Y., Karimata, H., Kishimoto, K., Kimura, M., Shimizu, A., Nakamura,
359 M., 2012. The role of pituitary gonadotropins in gonadal sex differentiation in the
360 protogynous Malabar grouper, *Epinephelus malabaricus*. *Gen. Comp. Endocrinol.* 178,
361 587–592. doi:10.1016/j.ygcen.2012.07.012

362 Muto, A., Lal, P., Ailani, D., Abe, G., Itoh, M., Kawakami, K., 2017. Activation of the
363 hypothalamic feeding centre upon visual prey detection. *Nat. Commun.* 8, 15029.
364 doi:10.1038/ncomms15029

365 Norberg, B., Brown, C.L., Halldorsson, O., Stensland, K., Björn, T., 2004. Photoperiod
366 regulates the timing of sexual maturation, spawning, sex steroid and thyroid hormone
367 profiles in the Atlantic cod (*Gadus morhua*). *Aquaculture* 229, 451–467.
368 doi:10.1016/S0044-8486(03)00393-4

369 Pittendrigh, C.S., 1993. Temporal organization: reflections of a Darwinian clock-watcher.
370 *Annu. Rev. Physiol.* 55, 16–54. doi:10.1146/annurev.ph.55.030193.000313

371 Sakharkar, A.J., Singru, P.S., Mazumdar, M., Subhedar, N., 2006. Reproduction phase-related
372 expression of beta-endorphin-like immunoreactivity in the nucleus lateralis tuberis of the

373 female Indian major carp *Cirrhinus mrigala*: correlation with the luteinising hormone cells-
374 ovary axis. J. Neuroendocrinol. 18, 319–329. doi:10.1111/j.1365-2826.2006.01421.x

375 Sánchez, J.A., Madrid, J.A., Madrid, J., Sánchez-Vázquez, F.J., Sánchez-Vázquez, F.J., 2010.
376 Molecular cloning, tissue distribution, and daily rhythms of expression of *per1* gene in
377 European sea bass (*Dicentrarchus labrax*). Chronobiol. Int. 27, 19–33.
378 doi:10.3109/07420520903398633

379 Schreibman, M.P., Halpern, L.R., Goos, H.J., Margolis-Kazan, H., 1979. Identification of
380 luteinizing hormone-releasing hormone (LH-RH) in the brain and pituitary gland of a fish
381 by immunocytochemistry. J. Exp. Zool. 210, 153–159. doi:10.1002/jez.1402100117

382 Takeuchi, Y., Hada, N., Imamura, S., Hur, S.-P., Bouchekioua, S., Takemura, A., 2015.
383 Existence of a photoinducible phase for ovarian development and photoperiod-related
384 alteration of clock gene expression in a damselfish. Comp. Biochem. Physiol. A 188, 32–
385 39. doi:10.1016/j.cbpa.2015.06.010

386 Toda, R., Okano, K., Takeuchi, Y., Yamauchi, C., Fukushiro, M., Takemura, A., Okano, T.,
387 2014. Hypothalamic expression and moonlight-independent changes of *Cry3* and *Per4*
388 implicate their roles in lunar clock oscillators of the lunar-responsive goldlined spinefoot.
389 PLoS ONE 9, e109119. doi:10.1371/journal.pone.0109119

390 Yamashina, F., 2016. Lunar-phase dependent expression pattern of cryptochrome genes in the
391 Malabar grouper. Master's thesis, University of the Ryukyus, Japan.

392 Velarde, E., Haque, R., Iuvone, P.M., Azpeleta, C., Alonso-Gómez, A., Delgado, M.J., 2009.
393 Circadian clock genes of goldfish, *Carassius auratus*: cDNA cloning and rhythmic
394 expression of period and cryptochrome transcripts in retina, liver, and gut. J. Biol. Rhythm.
395 24, 104–113. doi:10.1177/0748730408329901

396 Whitmore, D., Foulkes, N.S., 2000. Light acts directly on organs and cells in culture to set the
397 vertebrate circadian clock. Nature 404, 87–91. doi:10.1038/35003589

398 Zohar, Y., Muñoz-Cueto, J.-A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction
399 in teleost fish. *Gen. Comp. Endocrinol.* 165, 438–455. doi:10.1016/j.ygcen.2009.04.017
400

Table 1. Primes used in the present study.*

Primer	Sequence
Cloning	
<i>dgCryF</i>	5'-CHGTGTGGCCHGGDGGAG-3'
<i>dgCryR</i>	5'-AYRCCYTCYTCCCAGCTGAT-3'
Real-time PCR	
<i>mgCry2-realF</i>	5'-ATAGAGCGCCATCTGGAGAG-3'
<i>mgCry2-realR</i>	5'-CAAGTGCTTCGGGATTTTTG-3'
<i>mgEflα-realF</i>	5'-ACGTGTCCGTCAAGGAAATC-3'
<i>mgEflα-realR</i>	5'-GGGTGGTTCAGGATGATGAC-3'
<i>In situ</i> 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 *mgEflα* mRNA. Different letters indicate statistically significant differences ($P < 0.05$; Tukey-Kramer test). Each value was expressed as mean \pm 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 *mgEflα*. 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 *mgEfl α* . 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 tuberosus, PI; Pars intermedia, PPD, Proximal pars distalis. Inserted bar shows 200 μm .

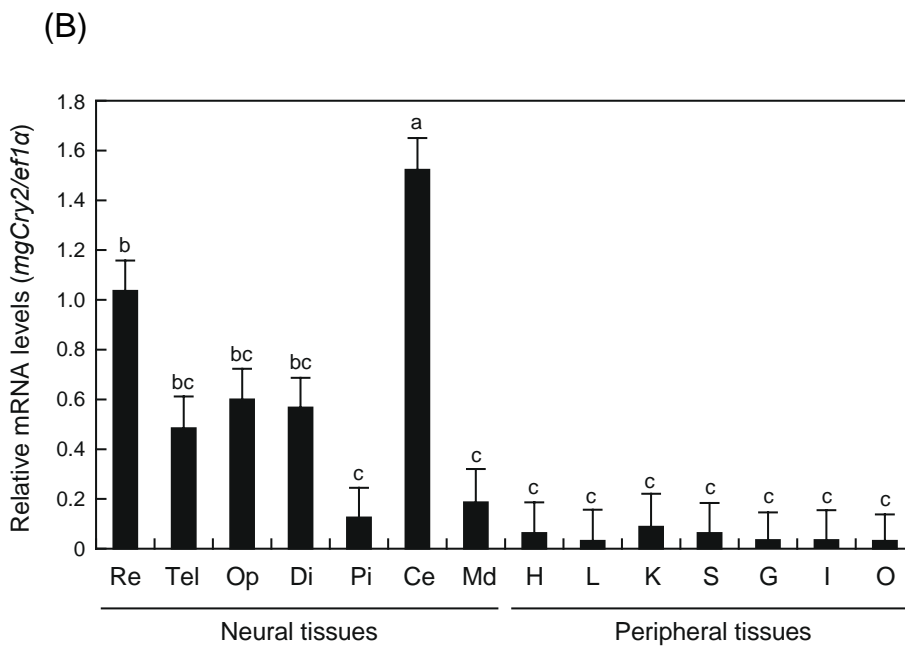
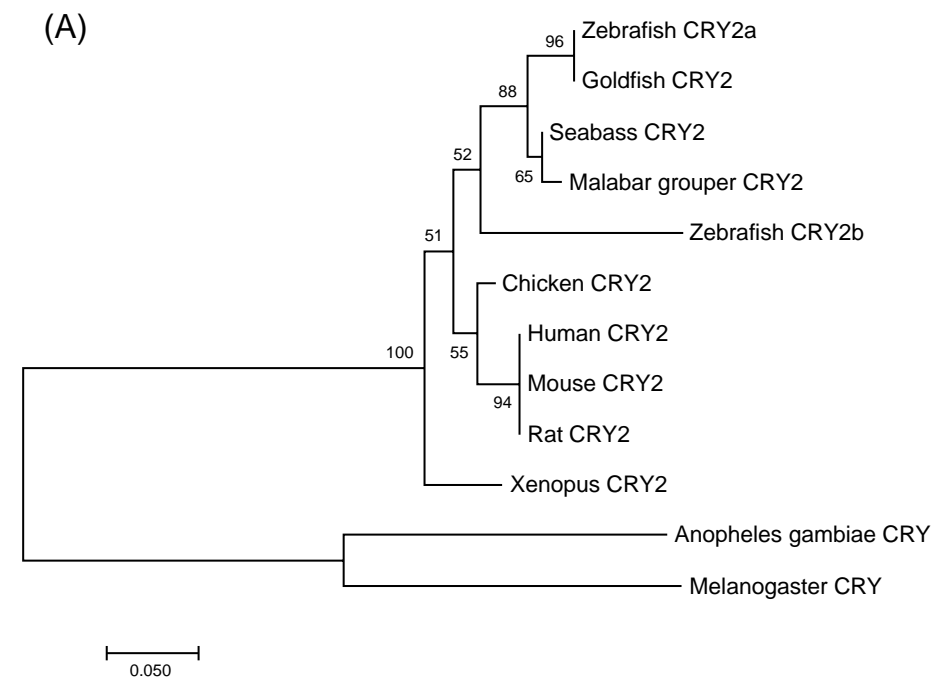


Figure 1

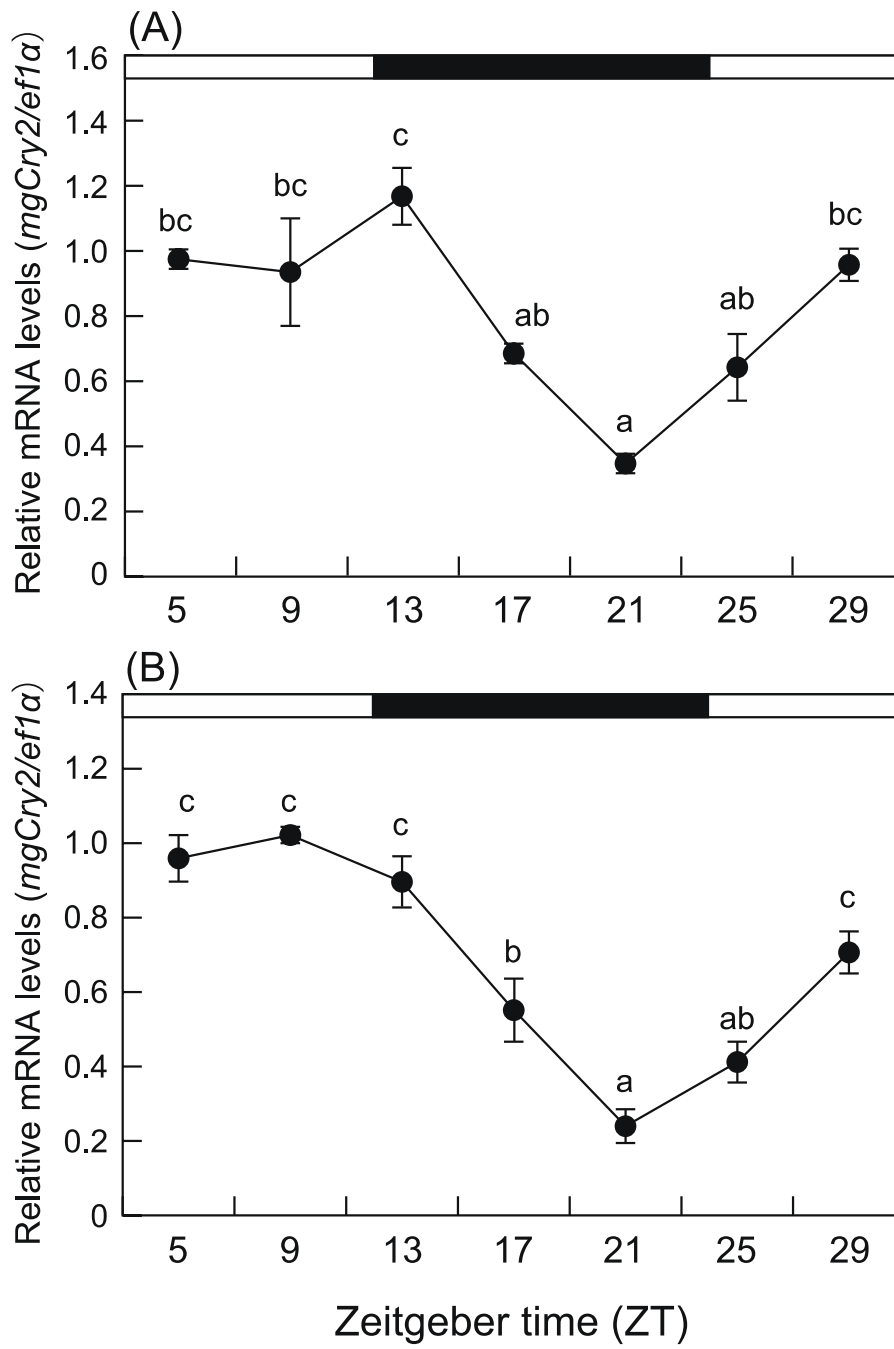


Figure 2

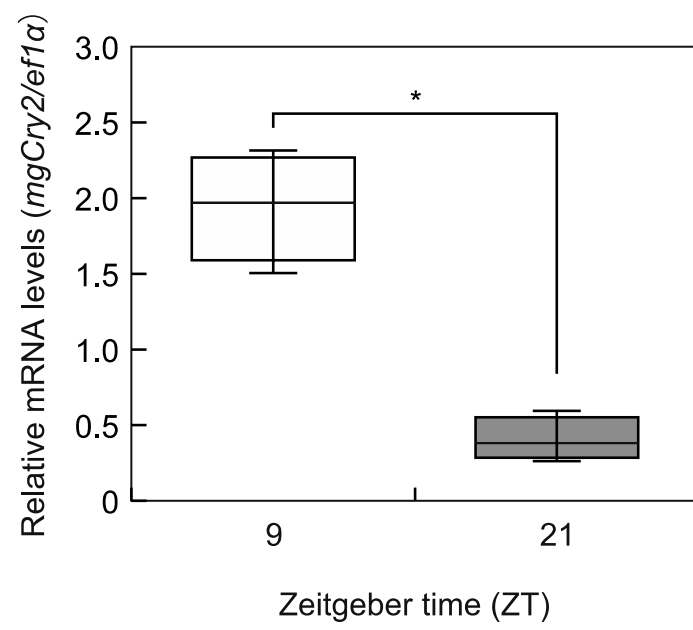


Figure 3

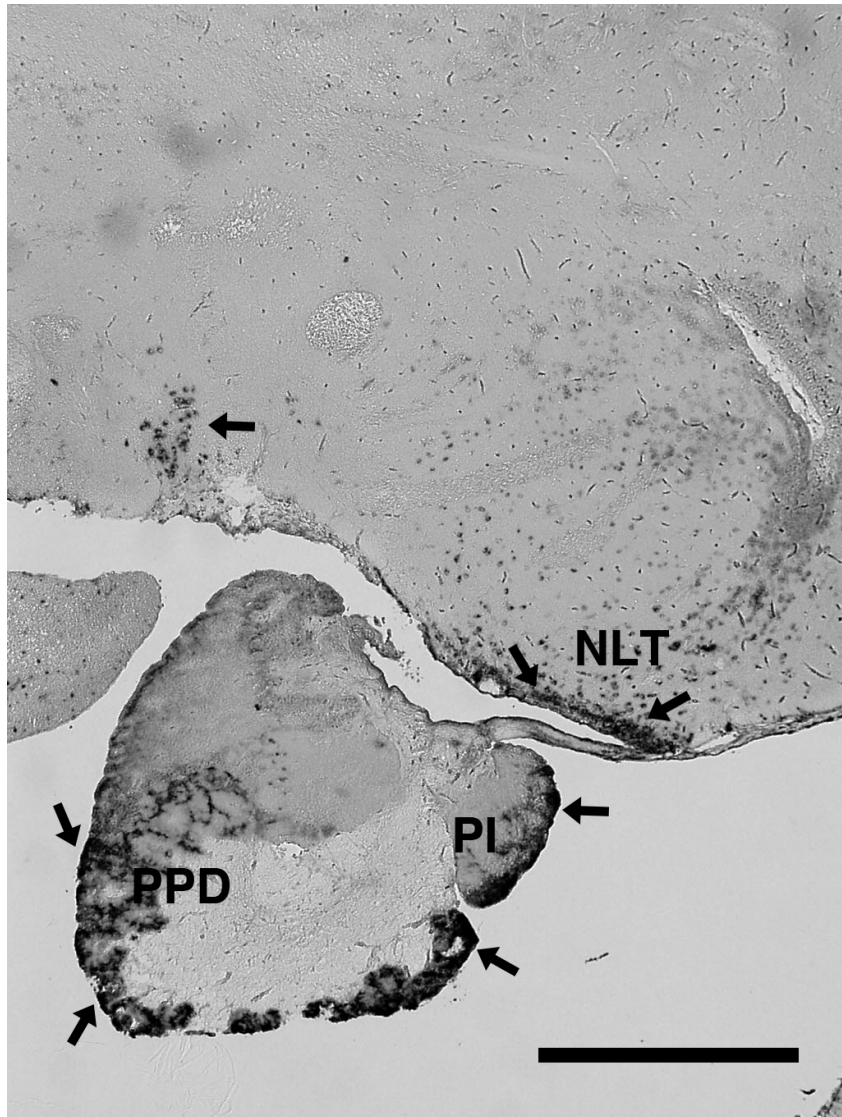


Figure 4