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Molecular cloning, tissue distribution and daily expression of *cry1* and *cry2* clock genes in European seabass (*Dicentrarchus labrax*)

Ana del Pozo *, Luisa M. Vera, Jose Antonio Sánchez, Francisco Javier Sánchez-Vázquez

Department of Physiology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100, Murcia, Spain

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ABSTRACT

Biological rhythms are driven by circadian oscillators, which are ultimately controlled by the cyclic expression of clock genes. Cryptochromes (CRY), blue light photoreceptors, belong to the negative elements of the transcriptional feedback loop into the molecular clock. This paper describes the cloning and characterization of two cryptochromes (*cry1* and *2*) in European seabass, which is considered an interesting chronobiology model due to its dual (diurnal/nocturnal) behavior. The cloned cDNA fragments encoded for two proteins of 567 and 668 amino acids, which included the FAD-binding and the DNA-photolyase domains. Moreover, both proteins had a high homology with cryptochrome proteins (Cry) of other teleost fish. These *cry1* and 2 genes were expressed in several tissues of seabass (brain, liver, heart, retina, muscle, spleen, gill and intestine). In addition, the daily expression of *cry1* was rhythmic in brain, heart and liver with the acrophase around ZT 03:15 h (after the onset of lights). Similarly, the *cry2* daily expression was rhythmic in liver, peaking at ZT 03:28 h, whereas in brain the acrophase was at ZT 11:08 h (shortly prior to the offset of lights). These findings provide new elements to help understanding the functioning of the molecular clock of seabass.

1. Introduction

All organisms are adapted to cyclic environmental changes, and are able to anticipate and predict them. Circadian rhythms are generated, regulated and maintained by interlocked autoregulatory feedback loops (positive and negative), whereby a group of circadian clock genes and their proteins interact (Wang, 2008). Cryptochromes (CRYs) belong to the DNA photolyase/cryptochrome flavoprotein family (Cashmore et al., 1999). These proteins are very much conserved across bacteria, plant and animal kingdoms, and are thought to act as blue light photoreceptors (Sancar, 2003). The first evidence of the existence of cryptochromes was found in *Arabidopsis thaliana* (Ahmad and Cashmore, 1993), where it is involved in specific light-dependent developmental and circadian entrainment. Later, two CRY proteins (CRY1 and CRY2) were identified in humans and mice (Hsu et al., 1996; Kobayashi et al., 1998), and another CRY protein in *Drosophila* (Emery et al., 1998). Recently, a larger number of cryptochrome (Cry) proteins have been described in fish, including Cry1a and b, Cry2a and b, Cry3 and Cry4 in zebrafish (*Danio rerio*) (Kobayashi et al., 1998) and Cry1, 2 and 3 in goldfish (*Carassius auratus*) (Velarde et al., 2009).

Despite the presence of cryptochromes during phylogeny, the photolyase function is only present in plant cryptochromes. Cryptochromes were defined as molecules with a protein sequence homologous to the photolyase sequence; they do not repair DNA but do have an enzymatic function that is activated by blue light (Sancar, 2003). Due to this function, cryptochromes were proposed as photoreceptors or phototranslators for blue light, mediating circadian photoreception (Hsu et al., 1996; Todo et al., 1996; Miyamoto and Sancar, 1998; Sancar, 2003). In vertebrates, CRY1 and CRY2 belong to the negative elements of the transcriptional feedback loop of the circadian clock, which drives the biological rhythms of the organisms. Vertebrate CRY protein joins Period protein (PER) to form a complex which binds to and blocks the protein complex formed by Circadian Locomotor Output Cycles Kaput and Brain and Muscle Aryl hydrocarbon receptor nuclear translocator (ARNT)-Like (CLOCK:BMAL1), inhibiting CRY transcription (Okamura et al., 2002; Iuvone et al., 2005). Among fish species, only the clock gene machinery of zebrafish is well known, differing from the mammalian mechanism in the larger number of cryptochrome (*cry*) and period (*per*) genes. Although both zebrafish *cry1* and *2* have two subtypes ("a" and "b"), the role of their corresponding proteins is similar to that of their homologues in mammals. In contrast, the Cry3 and 4 proteins (only found in fish) do not bind to the CLOCK:BMAL1 complex, and their function is still unknown (Kobayashi et al., 2000).

Besides being found in the master clock, cryptochrome (*Cry*) genes are expressed in a long list of peripheral tissues from mammals (Balsalobre, 2002; Muhlbauer et al., 2004; Peirson et al., 2006; Zvonic et al., 2006), and fish (Kobayashi et al., 2000; Velarde et al., 2009). Furthermore, the expression of several *cry* genes differs in zebrafish and goldfish and is tissue-dependent in the latter, where *cry* genes oscillate

* Corresponding author.
E-mail addresses: adelpozo@um.es (A. del Pozo), lmvera@um.es (L.M. Vera), jasanchez@um.es (J.A. Sánchez), javisan@um.es (F.J. Sánchez-Vázquez).

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with different acrophases depending on the tissue. However, *cry* genes expression was rhythmic in all cases, fitting a cosinor function in most (Kobayashi et al., 2000; Velarde et al., 2009).

European seabass (*Dicentrarchus labrax*) has great aquaculture and scientific interest, so that several genes of key endocrine axis (i.d. reproduction and food intake control) have been cloned and characterized (Cerda-Reverter et al., 1998; Gonzalez-Martinez et al., 2004; Isorna et al., 2008; Sauzet et al., 2008). In addition, this fish species is a prominent chronobiological model because of its dual behavior (diurnal/nocturnal), which allows seabass to change their feeding phase during the year and points to a flexible circadian system (López-Olmeda and Sánchez-Vázquez, 2010). However, the components of the seabass molecular clock and its function have been little explored. Only *per1* gene has been cloned, and its distribution and expression have been characterized in several tissues (Sánchez et al, 2010), including in brain and liver of diurnal and nocturnal seabass (del Pozo et al., 2012).

The aim of the present research was to clone two clock genes, *cry1* and *cry2*, in European seabass, as well as to study their tissue distribution and daily rhythm of expression in brain, heart and liver (central and peripheral oscillators, respectively).

2. Materials and methods

2.1. Fish-rearing conditions and sampling

Thirty-two adult seabass of 400 g were supplied by a local fish farm (Culmarex, S.A., Aguilas, Spain). Fish were reared in an aquaculture laboratory at the Naval Station of Algameca (Cartagena, Spain). Seabass were kept in two 500 L tanks with an open seawater system and constant water temperature of 20 °C, and they were randomly fed during the photophase. The photoperiod was set at 12 h light: 12 h dark (LD), with 300 lux illumination on the water surface during the photophase. Light onset and offset were at 9:00 h (zeitgeber time, ZT 00:00) and 21:00 h (ZT 12:00), respectively. After 2 weeks, 5 seabass were anesthetized and sacrificed every 3 h (ZT 00:00, ZT 03:00, ZT 06:00, ZT 09:00, ZT 12:00, ZT 15:00, ZT 18:00 and ZT 21:00) and brain, heart and liver samples were taken. The samplings were performed under light during the photophase and dim red light (<2.4 μW/cm2) during the darkphase. The fish were taken alternatively from different tanks at every time-point to minimize stress. Moreover, retina, spleen, gill, muscle and intestine samples were collected at ZT 06:00 to analyze the tissue distribution of *cry1* and *cry2* expression. TRIzol® (Invitrogen) was immediately added to the samples, which were frozen in dry ice and stored at −80 °C for future analysis.

Fish were reared and manipulated following Spanish legislation on Animal Welfare and Laboratory Practices, the experimental protocol was approved by the National Committee and the Committee of the University of Murcia on Ethics and Animal Welfare and the legislation concerning the protection of animals used for experimental purposes (Directive 2010/63/EU).

2.2. Cloning of seabass cry1 and cry2

RNA from the whole brain was isolated using a commercial kit, PureLink Micro-to-Midi Systems (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. A multialignment of *cry* mRNA from several species identified conserved regions, which was used to design a degenerate primer set (Forward primer: 5′ CARGA GGGVTGGATCCACCA 3′ and reverse primer: 5′ TACATYTAYGABC CBTGGAA 3′) to amplify the *cry* cDNA fragments. The cDNA was synthesized from 1 μg of total RNA from European seabass whole brain homogenates, using the Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. This fragment was amplified by 35 cycles of PCR (30 s at 94 °C for denaturing, 30 s at 55 °C for annealing and 1 min at 72 °C for extension). The cDNA fragments were identified as putative *cry* genes of seabass by nucleotide BLAST analysis (National Center for Biotechnology Information, NIH, USA, http://blast.ncbi.nlm.nih.gov/Blast.cgi), using the *cry* genes of different fish species. The *cry* specific primers and nested primers for 3′ and 5′-RACE were designed from these partial cDNA fragments. Then, these fragments were lengthened to obtain the full-length by Rapid amplification of cDNA ends (RACE), using the SMART RACE cDNA amplification kit (Clontech Laboratories, Mountain View, California, USA), according to the manufacturer's protocol and the 3' and 5'-RACE described above. The first PCR conditions for the RACE reaction were: (Ahmad and Cashmore, 1993) 5 cycles at 94 °C for 5 s, 72 °C for 3 min; (Amores et al., 1998) 5 cycles at 94 °C for 5 s, 70 °C at 10 s, and 72 °C for 3 min; and (Balsalobre, 2002) 25 cycles at 94 °C for 5 s, 68 °C for 10 s, and 72 °C for 3 min. The nested PCR involved 25 cycles at 94 °C for 5 s, 68 °C for 10 s and 72 °C for 3 min. Finally, the resulting RACE products were cloned in TOPO-TA vector (Invitrogen) and then sequenced. Coding sequences were submitted in GenBank database (accession no. JX046479 for cry1 and JX046478 for cry2).

The amino acid sequences were deduced from the cDNA sequences and compared to counterpart sequences currently available in GenBank (http://www.ncbi.nlm.nih.gov), using the BLASTX program (National Center for Biotechnology Information, NIH, USA, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Furthermore, an alignment was performed from the cry protein sequence of seabass and other cry proteins of teleosts (zebrafish, Somalian cavefish: *Phreatichthys andruzzii*, and Atlantic salmon: *Salmo salar*) available in GeneBank using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The accession numbers of the proteins used in both analyses are listed in Table 1.

The protein domains were predicted with the simple modular architecture research tool (SMART) program version 4.0 (Letunic et al., 2006; Schultz et al., 1998) (http://smart.embl-heidelberg.de/) and ScanProsite (http://expasy.org/tools/).

2.3. Phylogenetic analysis

Another alignment of CRY protein sequences was performed by the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.

Table 1
Homology of Cry protein sequences of different fish species with the deduced Cry1 and 2 protein sequences of seabass (*Dicentrarchus labrax*) by BLASTX.

Scientific names of species	Common names of species	Gene name	Seabass cry1 indentity (%)	Seabass Cry2 indentity (%)	GenBank accession number
Danio rerio	Zebrafish	Cry1a	87	–	CAH69046.1
Danio rerio	Zebrafish	Cry1a isoform 2	87	–	NP_571864.1
Danio rerio	Zebrafish	Cry1a isoform 1	–	88	NP_001070765.1
Danio rerio	Zebrafish	Cry1b	87	87	NP_571865.4
Danio rerio	Zebrafish	Cry2a	81	–	CAQ13306.1
Phreatichthys andruzzii	Somalian Cavefish	partial Cry1b	87	89	ADL62680.1
Phreatichthys andruzzii	Somalian Cavefish	Cry1a	84	–	ADL62679.1
Phreatichthys andruzzii	Somalian Cavefish	partial Cry2a	–	–	ADL62681.1
Salmo salar	Atlantic Salmon	Cry1	73	78	ACN10538.1

Table 2
Accession numbers of CRY proteins with which the protein alignment was performed for the phylogenetic analysis by CLUSTALW2.

Scientific names of species	Common names of species	Gene name	Accession number
Homo sapiens	Human	CRY2 isoform1	NP_066940.2
Homo sapiens	Human	CRY1	NP_004066.1
Mus musculus	Mouse	CRY1	NP_031797.1
Mus musculus	Mouse	CRY2	NP_034093.1
Rattus norvegicus	Dumbo Rat	CRY2	NP_596896.1
Rattus norvegicus	Dumbo Rat	CRY1	NP_942045.2
Xenopus laevis	Common Platanna	CRY1	NP_001081129.1
Xenopus laevis	Common Platanna	CRY2	NP_001083936.1
Amphiprion melanopus	Clownfish	CRY	ADI59662.1
Danio rerio	Zebrafish	Cry1a isoform 1	NP_001070765.1
Danio rerio	Zebrafish	Cry1b	NP_571865.4
Danio rerio	Zebrafish	Cry2a	CAQ13306.1
Danio rerio	Zebrafish	Cry2b	NP_571867.1
Danio rerio	Zebrafish	Cry3	AAH46088.1
Carassius auratus	Goldfish	Cry1	ABU93790.1
Carassius auratus	Goldfish	Cry2	ABU93791.1
Drosophila melanogaster	Drosophila	CRY	NP_732407.1

html), using several CRY proteins from fish and mammal proteins (the protein accession numbers of NCBI databases used for phylogenetic analysis are listed in Table 2). The parameters were: gap extension penalty = 0.05, gap distance = 4 and gap open penalty = 10. To complete the phylogenetic analysis, a phylogeny inference software package, PHYLIP 3.68 (Felsenstein, 1989), was used. A bootstrap analysis was performed by the SEQBOOT program using 1000 replicating data sets and 33 random seeds. The PROTDIST program computed the genetic distances measured for protein sequences by means of a Jones-Taylor-Thornton matrix with default setting. The NEIGHBOR program accepted these distance matrices as input, generating 1000 unrooted trees from 33 random seeds, without the assumption of a clock. The CONSENSE algorithm computed consensus trees by means of the majority-rule consensus tree to finally obtain a reliable tree. Finally, the TreeView software (version 1.40) displayed and printed the phylogenetic tree.

2.4. Tissues distribution of cry1 and cry2 gene expression and their daily expression

All samples were homogenized in TRIzol® (Invitrogen) by means of a tissue homegenizer (Polytron®, PT1200, Kinematica, Switzerland). RNA was extracted using PureLink Micro-to-Midi Systems (Invitrogen) according to the manufacture's protocol. Total RNA concentration was measured by spectrometry. DNAase I amplification grade (1 unit/μg RNA, Invitrogen) was added to avoid genomic DNA contamination. cDNA synthesis was performed with Superscript III Reverse Transcriptase and Random primers (Invitrogen) in a 20 μL reaction volume.

To analyze the distribution of *cry1* and *cry2* expression in different tissues, cDNA of *cry1*, *cry2* and *β-actin* from brain, heart, liver, retina, spleen, gill, muscle and intestine samples at ZT 06:00 h were amplified by PCR with specific primers (*cry1* forward: TCTTTGTGGTTGCCCCTCAT; *cry1* reverse: CCTGAACCAGTGGATGGTGTT; *cry2* forward: CGCCAACCT TCGGAAGCT; *cry2* reverse: TGGCAGGTTGGCCTCTGA). The products of the PCR were run through an agarose matrix by electrophoresis.

The cDNA of brain, heart and liver samples at every time-point was used as substrate for a Quantitative real-time PCR (qRT-PCR). The qRT-PCR was carried out using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a 20 μL final reaction volume, where forward (*cry1*: TCTTTGTGGTTGCCCCTCAT and *cry2*: CGCCAACCTTCGGAAGCT) and reverse (*cry1*: CCTGAACCAGTGGAT GGTGTT and *cry2*: TGGCAGGTTGGCCTCTGA) specified primers were included according to the manufacturer's protocol. All samples were run in triplicate. An ABI Prism 7500 apparatus (Applied Biosystems) supported the ABI Sequence Detection System 7000 software (Applied Biosystems), which reproduced a cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s and finally a cycle at 60 °C for 1 min. All specified primers were designed with Primer Express software (Applied Biosystems) and synthesized by Invitrogen. The standard curve method tested the amplification efficiency, specificity of primers, and the amount of cDNA/sample. The relative expressions of *cry1* and *cry2* were calculated by the C_T method with efficiency correction, using *β-actin* gene as the reference gene (accession no. AJ493428), which has been validated for seabass by Patruno et al. (2008). The *β-actin* specific primers were: forward 5′ ACC CAG TCC TGC TCA CAG AG and reverse 5′ GGG AGT CCA TGA CAA TAC CAG TG.

2.5. Statistical analysis

Differences in *cry1* and *cry2* relative expression for each tissue (brain, heart and liver) along 24 h were analyzed by one-way ANOVA (ANOVA I) with SPSS 15.0 software followed by a Tukey test. Moreover, the Cosinor (CSR 3.0.2) analysis was performed to determine whether the rhythmic expression of *cry1* and *cry2* in brain, heart and liver, fitted the cosine function ($Y = M + A^*[\mathrm{Cos}\ (\Omega t + \Phi)]$) where M is mesor, A is amplitude, Ω is angular frequency ($2\pi/24$ for the circadian rhythms) and Φ is acrophase. The significance level was fixed at $p<0.05$ for all the statistical analyses.

3. Results

3.1. Cloning of seabass cry1 and cry2

For cloning, degenerated PCR primers were designed from conserved regions in Cry protein of several species. Thus, the seabass cDNA partial sequences were sequenced and compared to other fish *crys* by BLAST (National Center for Biotechnology Information, NIH, USA), identifying both sequences as putative *cry* genes. Primers were designed from these nucleotide sequences to lengthen them by RACE to obtain full-length cDNA sequences. These full-length cDNA sequences of both seabass *cry* genes were compared with the Cry protein sequences of different fish species from the NCBI databases by BLASTX. The seabass *cry1* and *2* showed high homology with other Cry proteins from several teleost fish. The homology percentage and the accession number of the used database proteins are presented in Table 1.

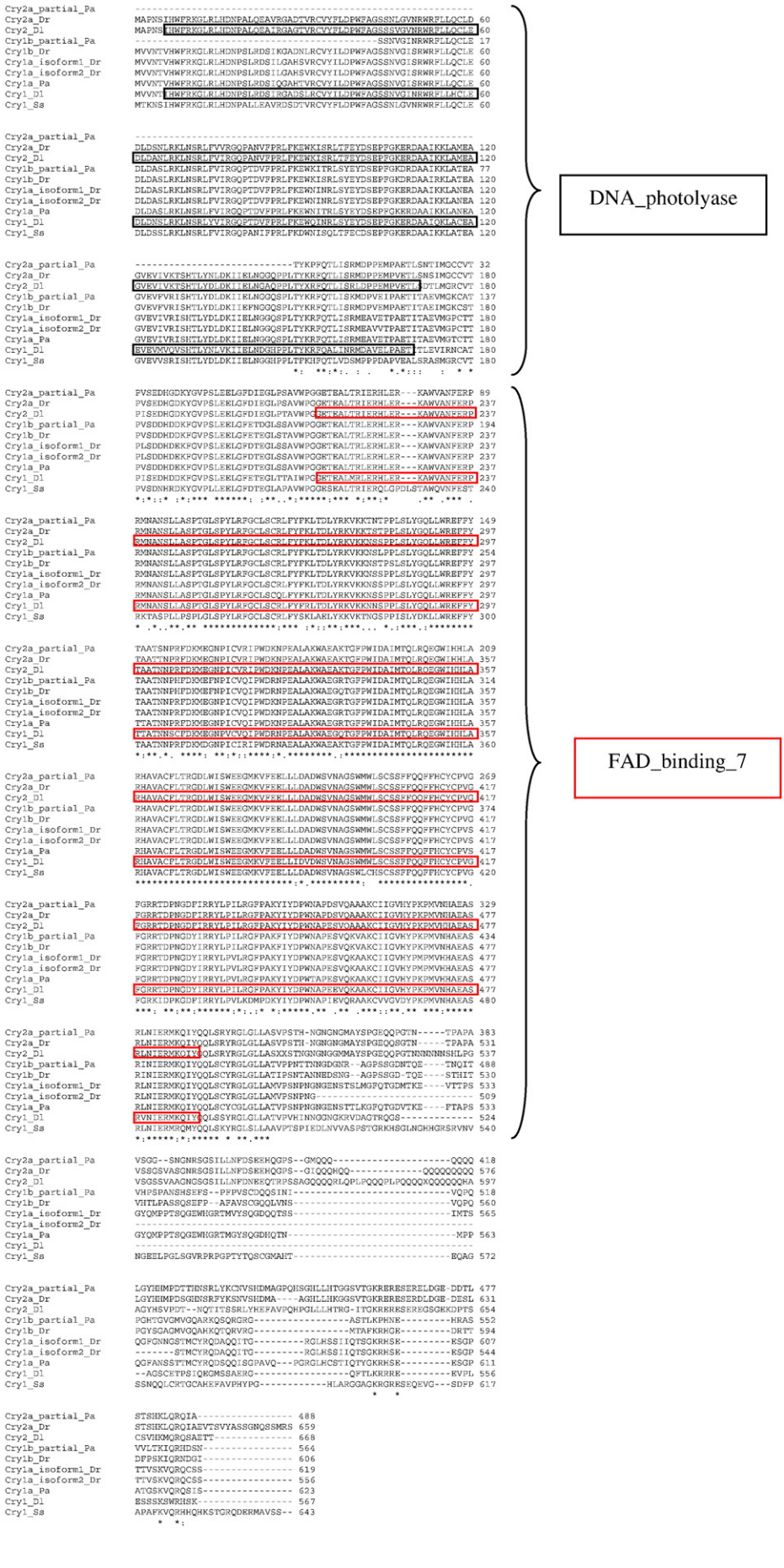
Moreover, the complete cDNA sequences codified two proteins of 567 and 668 aminoacids, respectively. Two domains, FAD-binding and DNA-photolyase, were indentified within both proteins (SMART, $p<0.01$). The FAD-binding domain was included between the 5–169 and 5–170 positions for the Cry1 and Cry2 protein sequences, respectively, whereas, the DNA-photolyase domain was included between the 212–488 positions in both Crys protein sequences.

Consequently, the ClustalW2 analysis aligned the deduced protein sequence of seabass Cry1 and 2 with other Cry1 and 2 fish proteins from the database, showing high homology (Fig. 1).

3.2. Phylogenetic analysis

Evolutionary relations of different *Cry* subtypes were found between seabass and other vertebrates. The phylogenetic analysis separated CRY1 and CRY2 of all species in two sides of the tree (Fig. 2). The

Fig. 1. Multiple sequence alignment of deduced aminoacid sequences from seabass Cry1 and 2 with protein sequences from other fish of the database by ClustalW program. "*" indicates residues in that column are identical in all sequences; "." indicates semi-conserved substitutions; ":" indicates conserved substitutions. Filler squares around the sequence fragment show the DNA-photolyase domain within both seabass Cry1 and 2 protein sequences; similarly, the empty squares bring out the FAD-binding7 domain in both sequences. Abbreviations: Pa = *Phreatichthys andruzzii*, Dr = *Danio rerio*, Ss = *Salmo salar*, Dl = *Dicentrarchus labrax*. The protein accession numbers are cited in Table 1.


DNA_photolyase
FAD_binding_7

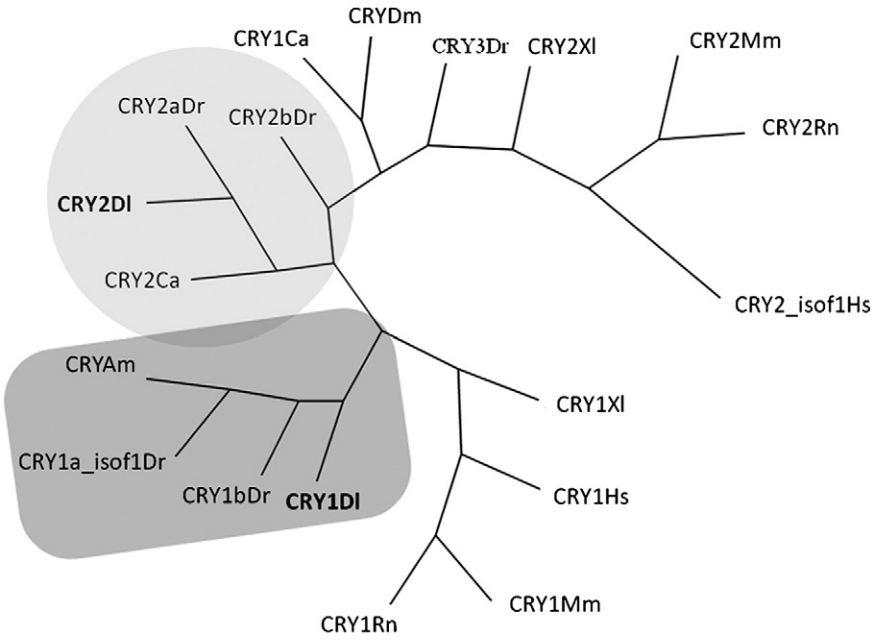


Fig. 2. Phylogenetic tree showing the relationship of seabass Cry1 and 2 proteins with CRY1 and 2 of other vertebrates. The tree was created by the Neighbor-Joining method of the PHYLIP package (version 3.68). Abbreviations: isof = isoform, Hs = *Homo sapiens*; Mm = *Mus musculus*; Rn = *Rattus norvegicus*; Dr = *Danio rerio*; Dm = *Drosophila melanogaster*; Xl = *Xenopus laevis*; Ca = *Carassius auratus*; Dl = *Dicentrarchus labrax*. The protein accession numbers of NCBI databases are listed in the Table 2.

CRY1 and CRY2 of tetrapods were grouped at both ends of the phylogenetic tree. In the middle, the Cry1 and Cry2 of teleost fish were divided into two different groups, in which both seabass (*D. labrax*) Crys were included. Thus, seabass Cry1 was closely related to zebrafish (*D. rerio*) Cry1b and Cry1a and clownfish (*Amphiprion melanopus*) Cry1, whereas seabass Cry2 was closely related to zebrafish Cry2a, followed by goldfish Cry2 and zebrafish Cry2b.

3.3. Tissues distribution of *cry1* and *cry2* gene expression and their daily expression.

Both *cry1* and *cry2* of seabass were expressed in all analyzed tissues (brain, liver, heart, retina, muscle, spleen, gill and intestine) (Fig. 3). Moreover, the *cry1* expression showed a daily rhythm in brain, liver and heart, peaking in the tree tissues at ZT 03:00 h, which differed statistically from the expression levels observed during the rest of the day (ANOVA I, $p<0.01$) (Fig. 4). This rhythm fitted a cosinor curve (COSINOR, $p<0.01$), showing the acrophase around ZT 03:15 h, a similar mesor and amplitude. On the other hand, the *cry2* expression was rhythmic in brain and liver (not in heart), fitting a cosinor curve (COSINOR, $p<0.01$) (Fig. 5), although the acrophases differed among tissues, at ZT 11:08 h in brain and ZT 03:28 h in liver. The cosinor parameters (mesor, amplitude and acrophase) and statistically significance of *cry 1* and *2* genes expression rhythms in seabass brain, liver and heart have been represented in Table 3.

4. Discussion

In the present study, two cryptochrome genes (*cry1* and *cry2*) were cloned and characterized in seabass. These cDNA sequences codified two Crys proteins, 1 and 2 (of 597 and 668 aminoacids, respectively) which included two typical domains of CRY proteins, DNA-photolyase and FAD-binding. Animal cryptochromes belong to the photolyase-like flavoproteins family, which, together with the photolyase DNA repair enzymes, compose the cryptochrome/photolyase protein family. Cryptochromes and photolyase proteins share sequence homology as well as FAD (flavin adenine dinucleotide) cofactor and DNA-binding activity, although cryptochromes lack DNA-repair activity (Brudler et al., 2003; Song et al., 2007; Oztürk et al., 2007).

The deduced protein from the *cry1* and *2* cDNA sequences of seabass showed high homology (> 78%) with Cry1 and 2 proteins from other teleost fish species such as zebrafish, Somalian cavefish and Atlantic salmon. In mammals, only two *Cry* (Ahmad and Cashmore, 1993; Amores et al., 1998) genes have been discovered so far (Kobayashi et al., 1998), while in fish species several *cry* paralogues have been described. Thus, as many as 6 *cry* genes (*cry1a*, *cry1b*, *cry2a*, *cry2b*, *cry3* and *cry4*) have been found in zebrafish (Kobayashi et al., 2000), 3 *crys* genes (*cry1*, *cry2* and *cry3*) in goldfish (Velarde et al., 2009) and 5 *crys* genes (*cry1a*, *cry2b*, *cry3*, *cry4*, *cry5*) in cavefish (Cavallari et al., 2011). The existence of the *Cry* paralogues in vertebrates has been explained by the existence of two rounds of whole-genome duplication (2R hypothesis) during the emergence of vertebrates (Ohno, 1970; Panopoulou and Poustka, 2005). In addition, a third round of whole-genome duplication (3R hypothesis) would have generated a larger number of *cry* genes in fish than in mammals, which would have occurred in the teleost linage after its divergence from the tetrapod linage but before its radiation (Amores et al., 1998; Woods et al., 2005). However, the 3R hypothesis alone is not able to elucidate the total number of *cry* genes in zebrafish and cavefish, so more studies are necessary to clarify this point (Wang, 2008).

In the present study, the phylogenetic analysis of seabass crys showed that CRY1s of all species are clustered together and separately from CRY2s, which form another cluster. Furthermore, the fish Crys (seabass included) were gathered within both subclusters (for Cry1 and Cry2) and separated from the CRYs of other taxa, suggesting that they appeared later in evolution. Similarly, Kobayashi et al. (2000) performed a phylogenetic analysis of the photolyase/cryptochrome family, and described a subcluster within the animal CRY cluster composed of all CRYs1 and 2 from fish and mammals and Cry3 of zebrafish (zCry3). In this subcluster, zCry1s and CRY1 of mammals (mCRY1s) were closely related, whereas, zCry2s were closer to CRY1s of both fish and mammals species than to mCRY2s. However, zCry3 diverged previously and zCry4 would not be included in this subcluster. Therefore, *cry* genes could be classified in three groups: the first one shared by *cry1s* and *cry2s*, and other two groups only with one member: *cry3* and *cry4*, respectively. Only Cry1 and 2 had the capacity to self-inhibit their own transcription by means of blocking the CLOCK:BMAL1 complex, as in mammal *Crys*. The function of Cry3 and Cry4 remains

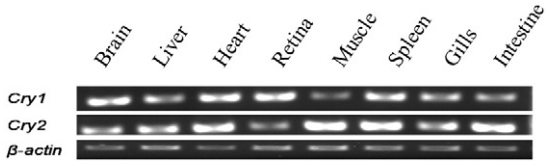


Fig. 3. *Cry1* and *2* expressions in several seabass tissues. A PCR screening was performed in brain, liver, heart, retina, muscle, spleen, gill, intestine. The samples were taken at ZT 00:00 h.

unknown, but they could be classified as *Drosophila* type CRY or as a separated group (Kobayashi et al., 2000; Kume et al., 1999). Among all zCrys, the zCry4 structure is the closest to *Drosophila* CRY (dCRY), seeming the candidate for a zebrafish circadian photoreceptor with a similar function to that of dCRY. Nevertheless, more *in vivo* experiments should be performed to clarify the zCry3 and 4 functions (Kobayashi et al., 2000).

PCR screening showed that *cry1* and *2* gene expression was present in all the seabass tissues analyzed in this investigation (brain, liver, heart, retina, muscle, spleen, gill and intestine), which also expressed *per1* gene (Sánchez et al., 2010). To date, circadian clocks in peripheral tissues have been reported in *Drosophila*, zebrafish, mammalian cell lines and tissues, supporting the hypothesis on the existence of decentralized clocks (Tamai et al., 2005). Moreover, *Drosophila* and adult zebrafish tissues, as well as zebrafish cell lines and embryos,

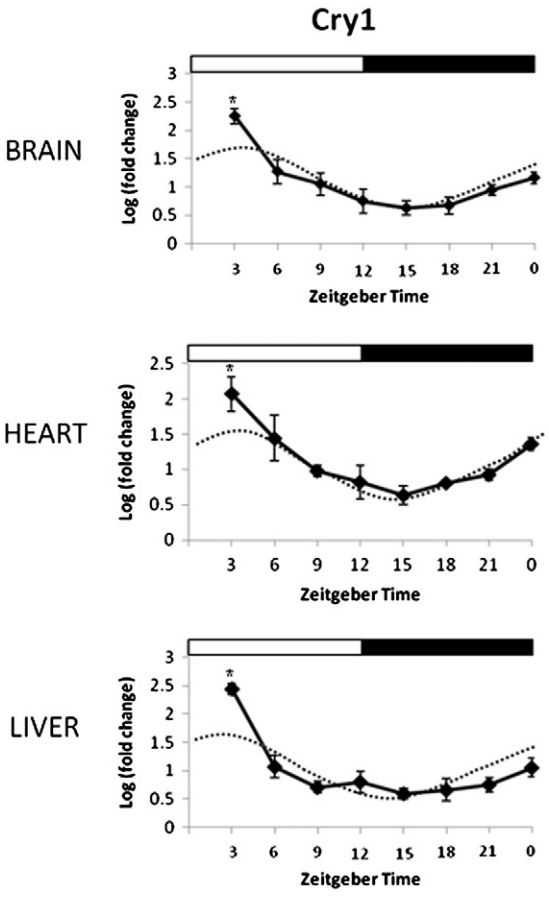


Fig. 4. Relative expression of *cry1* in seabass brain, heart and liver. Every point represents the mean *cry* expression of 3–4 fish and the error bars show the standard error about the mean (SEM). The black and white bars above the graphs show the dark and light phases, respectively. The zeitgeber time (ZT, in hours) is represented on the horizontal axis, while the relative expression as fold change (log_{10}) is plotted on the vertical axis. "*" indicates the time point which differed by ANOVA ($p<0.01$). The dotted line denotes the cosinor adjustment (COSINOR, $p<0.01$).

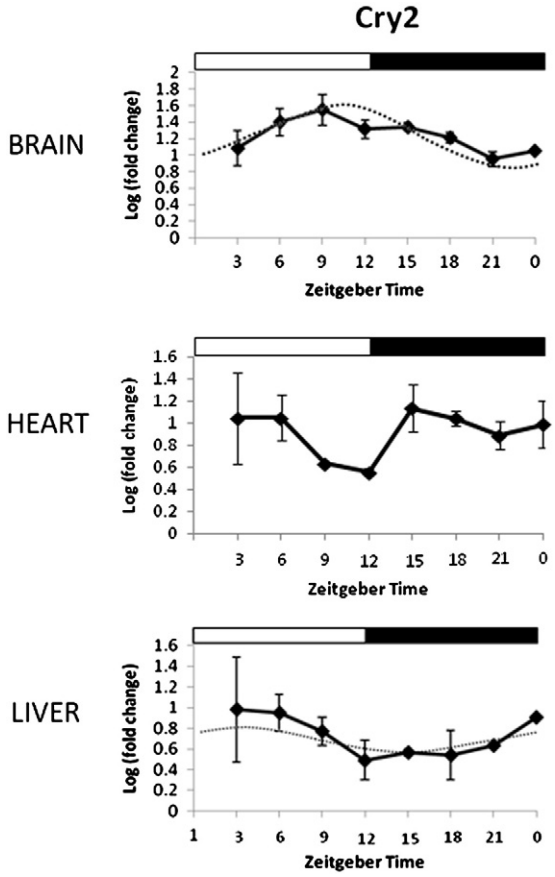


Fig. 5. Relative expression of *cry2* in seabass brain, heart and liver. Every point represents the mean *cry* expression of 3–4 fish (except at ZT 00:00 h and 09:00 h in liver with 1 sample) and the error bars show the standard error about the mean (SEM). The black and white bars above the graphs show the dark and light phases, respectively. The zeitgeber time (ZT, in hours) is represented on the horizontal axis, while the relative expression as fold change (log10) is plotted on the vertical axis. "*" indicates the time point which differed by ANOVA ($p<0.01$). The dotted line denotes the cosinor adjustment (COSINOR, $p<0.01$).

are directly light-responsive (Whitmore et al., 1998; Schibler and Sassone-Corsi, 2002). In this research, the *cry1* daily rhythms were similar in seabass liver, heart and brain, peaking shortly after the lights were switched on. Also, the *cry2* oscillated rhythmically in liver with a similar acrophase to *cry1*, while in brain the acrophase of *cry2* was at the end of the light phase. Similarly, the rhythmic expression of different *cry* genes displayed dissimilar acrophases in several tissues of zebrafish (Kobayashi et al., 2000) and goldfish, which also showed different acrophases for the same *cry* subtype in different tissues (Velarde et al., 2009). Sometimes the *cry* expression is not rhythmic in some tissues, as reported in *cry1* expression in goldfish gut and liver (Velarde et al., 2009), and as we observed in *cry2* expression in seabass heart. On the other hand, the maximum expression peak of *per1* in several tissues (brain, retina, liver and gut) has been observed close to light onset in several species (Vallone et al., 2004; Lahiri et al., 2005; Velarde et al., 2009; Sánchez and Sánchez-Vázquez, 2009), including seabass brain, liver and heart (Sánchez et al., 2010; Del Pozo et al., 2012). Therefore, seabass *per1*, *cry1* and *cry2* expressions in all tissues, where their rhythmicity has been reported (except *cry2* in brain), were in phase, and so both the proteins encoded by these genes (Per1 and Cry1 and Cry2) could join together and form the Per-Cry complex, which, in turn, would inhibit the *cry* transcription, closing the negative loop of the molecular mechanism that directs the circadian clock.

Table 3
Cosinor analysis of *cry1* and *cry2* genes expression rhythms in seabass brain, liver and heart.

Cosinor parameters	*cry1*			*cry2*		
	Brain	Liver	Heart	Brain	Liver	Heart
Acrophase (h)	ZT 03:21 (1:50, 4:48)	ZT 03:03 (0:52, 5:16)	ZT 03:22 (1:42, 5:03)	ZT 11:08 (8:20, 14:03)	ZT 03:28 (2.10, 4.46)	n.s.
Mesor	$\log_{10}$ 1.12 (0.99, 1.26)	$\log_{10}$ 0.99 (0.80, 1.18)	$\log_{10}$ 1.09 (0.93, 1.25)	$\log_{10}$ 1.20 (1.07, 1.32)	$\log_{10}$ 0.73 (0.67, 0.79)	n.s.
Amplitude	$\log_{10}$ 0.55 (0.36, 0.74)	$\log_{10}$ 0.57 (0.32, 0.83)	$\log_{10}$ 0.59 (0.36, 0.81)	$\log_{10}$ 0.32 (0.13, 0.50)	$\log_{10}$ 0.25 (0.18, 0.33)	n.s.

Notes: ZT = Zeitgeber time; the significant limits for each parameter are bracketed; n.s. indicates that the rhythm was not significant. The statistical significance threshold was set at $p<0.05$.

In summary, two *cry* genes of seabass, *cry1* and *cry2*, which were expressed in several tissues (brain, liver, heart, retina, muscle, spleen, gill and intestine), were cloned. *Cry1* expression was rhythmic in brain, liver and heart, whereas *cry2* displayed a daily rhythm in brain and liver. In all the tissues investigated, the acrophases occurred shortly after lights onset, except *cry2* expression in brain with an acrophase at the end of the light phase. These findings provide the basis for understanding how the molecular clock of seabass is assembled. Actually, *per1* expression differs in diurnal and nocturnal seabass (Del Pozo et al., 2012). However, further research will be required to test such differences in *cry* genes and the role of other clock genes (e.g. *clock* and *bmal*) in the dual phasing behavior.

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