Melatonin synthesis enzymes in pineal, retina, liver and gut in the goldfish (*Carassius auratus*): cloning, expression and transcriptional regulation of daily rhythms by lighting conditions

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Short running title: Transcriptional regulation of *Aanat-2* and *Hiomt-2* in goldfish

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ABSTRACT (MAX 350 WORDS)

Melatonin is currently proposed to be synthesized in non-photosensitive organs of vertebrates, besides its well-known sites of synthesis, the pineal gland and the retina. However, very few studies have demonstrated gene expression of MEL synthesizing enzymes in extrapineal and extraretinal locations. In the present study, present study focuses on the circadian expression of the two key enzymes of the melatoninergic pathway, the AANAT and the HIOMT in central and peripheral locations of the goldfish we give report of the full-length cloning of the two enzymes catalyzing the final steps of melatonin biosynthesis, the arylalkylamine *N*-acetyltransferase (AANAT) and the hydroxyindole-*O*-methyltransferase (HIOMT), in a teleosts fish, the goldfish (*Carassius auratus*). Both enzymes showed high similarity with other teleost sequences, corresponding to the goldfish AANAT-2 and HIOMT-2. Two forms of AANATs were widely known to exist in teleosts, but this is the first time that two isoforms of HIOMT are deduced from a phylogenetic analysis. Both enzymes were detected in several peripheral locations, including liver and gut, being present results the first to find HIOMT in non-photosensitive structures of a fish species. No studies exist on transcriptional regulation of the expression of MEL biosynthesis enzymes in non photosensitive structures in fish The daily expression pattern of both genes in pineal gland, retina, liver and gut was investigated using quantitative real time RT-PCR and cosinor analysis. This is the first time that a rhythmic expression of AANAT and HIOMT are found in digestive tissues of a vertebrate species, supporting a functional melatonin synthesis pathway in liver and gut of the goldfish. Besides, the transcriptional regulation of *Aanat-2* in pineal and peripheral locations of goldfish maintained under different lighting conditions was investigated expression of gAANAT-2 is analyzed under different lighting conditions including continuous light and darkness, revealing, as expected, light-dependent rhythms in pineal gland and retina, but also in liver. Nevertheless, the persistence in hindgut of these *Aanat-2* rhythms in constant conditions suggests that the expression of this transcript is under circadian clock and entrained by non-photic cues. Our results reinforce the existence of melatonin synthesis in gut and liver of the goldfish, while the rhythmic expression profiles

reported point to a regulation of both genes in gut and liver by peripheral oscillators entrainable to non-photic cues.

Key words: Arylalkylamine *N*-acetyltransferase, Hydroxyindole-*O*-methyltransferase; transcriptional regulation, pineal, retina, liver, gut, fish.

INTRODUCTION

The melatonin (N-acetyl-5-methoxytryptamine, MEL) synthesis is rhythmically produced on a daily basis with high levels during the night in all vertebrates studied. This rhythmic pattern is a highly conserved feature and plays a key role in the circadian organization of vertebrates, synchronising physiological and behavioural processes to both daily and seasonal cyclic variations (Reiter, 1993; Falcón et al., 2007). This indoleamine is mainly synthesized in photosensitive structures like the pineal organ and retina, but it has been also found in several non-photosensitive tissues including the gastrointestinal tract, skin, platelets, immune system, and others, where it can exert many different actions (Pandi-Perumal et al., 2006). The phylogenetic evolution of the melatoninergic system in teleosts is highly interesting, as recently reported (Falcón et al., 2009). Particularly, the goldfish (*Carassius auratus*) is a teleost recently revealed as a good model to investigate the circadian system functioning on the basis of its robust oscillations of clock genes in both, photoreceptor tissues (retina) and peripheral locations (liver and gut) (Velarde et al., 2009a).

The MEL biosynthesis from serotonin involves the sequential activity of two enzymes, the arylalkylamine *N*-acetyltransferase (AANAT; EC 2.1.3.87), which acetylates serotonin into *N*-acetylserotonin, and the hydroxyindole-*O*-methyltransferase (HIOMT; EC 2.1.1.4), which forms MEL by methylation of *N*-acetylserotonin. A highly conserved feature in vertebrates is the daily rhythmic pattern of MEL synthesis, which is determined by rhythms in AANAT activity, protein amount and, in some cases, gene expression, resulting in a nocturnal increase of this neurohormone (Iuvone et al., 2005, Klein, 2007). Pineal and retinal expression of *Aanat*s has been reported in several teleost adults (Coon et al., 1998;

Zilberman-Peled et al., 2004; 2006) and in photoreceptive organs of embryos of chum salmon (Shi et al., 2004), turbot (Vuilleumier et al., 2007) and in the sole (Isorna et al., 2009), and a species-dependent expression pattern has been evidenced. In contrast to avian and mammals, where only a single *Aanat* gene has been identified, two *Aanat* subfamilies (*Aanat*-1 and *Aanat*-2) exist in teleost. The subfamily *Aanat-1*, the orthologue of *Aanat* of tetrapods, with two subtypes reported in some teleost (Falcón et al., 2009) that are expressed preferentially in the retina. The *Aanat-2* is preferentially expressed in the pineal organ of most teleost such as the pike (Coon et al., 1999), gilthead seabream (Zilberman-Peled et al., 2004) and chum salmon (Shi et al., 2004), but is also present in the retina of zebrafish embryos (Gothilf et al., 1999). The HIOMT activity displays none or slight variations throughout the 24-h light/dark cycle, being the *O*-methylation rate determined by substrate availability (Ribelayga et al., 2000). Gene expression studies shows daily variation in *Hiomt* expression along the light/dark cycle in quail (Fu et al., 2001) and some mammals (Gauer and Craft, 1996; Ribelayga et al., 1999). To date only one study is available on the HIOMT gene expression in fish (the turbot, Vuilleumier et al., 2007), but the possible daily rhythmicity has not been investigated.

In spite of the broadly acceptance of MEL synthesis in non-photosensitive organs in vertebrates, very few studies have demonstrated gene expression of MEL synthesizing enzymes in extrapineal and extraretinal locations. In fish, low levels of *Aanat* expression have been detected in the ovary of zebrafish (Bégay et al., 1998) and in different peripheral tissues of rainbow trout (Fernández-Durán et al., 2007). The *Hiomt* transcripts have been identified in extrapineal and extraretinal locations such as gonad, stomach, gut, skin and immune cells of birds and mammals, suggesting a possible MEL peripheral synthesis (Conti et al., 2000; Fu et al., 2001; Stefulj et al., 2001; Slominski et al., 2008). To date, no data exist about *Hiomt* expression in extrapineal or extraretinal locations in fish.

Many studies investigated the environmental regulation of pineal AANAT activity in fish, but the transcriptional regulation by light has been only scarcely examined in the trout retina (Besseau et al., 2006) and in the pineal gland of the gilthead seabream, (Zilberman-

Peled et al., 2006). No studies exist on transcriptional regulation of the expression of MEL biosynthesis enzymes in non photosensitive structures in fish.

The present study focuses on the circadian expression of the two key enzymes of the melatoninergic pathway, the AANAT and the HIOMT in central and peripheral locations of the goldfish. First, we give report of the full-length cloning of *Aanat-2* in this species and demonstrate for the first time the existence of a second *Hiomt gene* in fish. Second, the expression pattern and daily rhythms of both enzymes in central and peripheral locations were analyzed. Finally, the transcriptional regulation of *Aanat-2* in pineal and peripheral locations of goldfish maintained under different lighting conditions was investigated.

MATERIALS AND METHODS

Animals and Tissue Sampling

Goldfish (*Carassius auratus*) with a body weight of 2-4 g were kept in 60 l tanks with filtered and aerated water, and fed randomly twice a day on a commercial flake diet (1.8 % body weight, Tetra). Random feeding times were provided by random number generator software (Microsoft Excel). Animals were separated into four experimental groups, each one under the following daily light/dark conditions: 12L:12D, 12D:12L, 24D (24-h darkness) and 24L (24-h light) for 30 days.

For the cloning and tissue distribution study, fish under a natural photoperiod were sacrificed at zeitgeber time (ZT) 4. Pineal gland, neural retina, hypophysis, liver, gut, gall bladder, heart, kidney, gonad, gills, muscle and skin were collected and immediately immersed in RNA*later* (Sigma, Saint Louis, MO, USA) for tissue preservation. For the study of daily variations in gene expression, fish from every group were sacrificed every six hours beginning at ZT2 or Circadian time 2 (CT2) until ZT2 or CT2 of the following day. Neural retina, liver, foregut, hindgut and midbrain area including the pineal gland were rapidly removed, immersed in TRI® Reagent (Sigma) and frozen in dry ice. All samples were stored at -80ºC until used. All animal experiments were conducted in accord with the NIH *Guide for the Care and Use of Laboratory Animals* and complied with the Spanish legal requirements.

Cloning of Arylalkylamine N-acetyltransferase 2 (*Aanat*-2) and Hidroxyindole-O-methyltransferase 2 (*Hiomt*-2).

Total RNA from goldfish pineal glands and retinas was extracted by the TRI® Reagent method (Sigma). The quality and quantity of the isolated RNA were assessed spectrophotometrically (260/280 nm ratio, GeneQuant, Amersham Biosciences). An aliquot of 1 µg total RNA was reverse transcribed using oligo-dT, RNase inhibitor and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in a 25 µl reaction volume. The reaction was carried out for 50 min at 42ºC, followed by 15 min at 72ºC to inactivate the reverse transcriptase.

AANAT cDNA amplification was carried out by PCR with specific primers Fw1 and Rv1 (Table 1) developed from a partial cDNA sequence available (GenBank accession No. AB167078). For *Hiomt* cloning, degenerated primers were designed against highly conserved regions among available sequences (Deg_Fw1 and Deg_Rv1) (Table 1). Reactions were performed in a total volume of 25 µl. PCR conditions for *Aanat* were set at 95ºC for 3 min, 30 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s, and 1 cycle of 72ºC for 7 min. For HIOMT, touchdown PCR conditions were set with annealing temperatures decreasing from 55ºC to 47ºC (16 cycles) and 47ºC (15 cycles). The expected-sized fragments were obtained (190 bp and 306 bp respectively), gel purified using EZNA gel extraction kit (Omega Bio-tek), and subcloned into pGEM®-T EASY vectors (Promega, Madison, WI, USA). Vectors containing the amplified products were transformed into *Escherichia coli* JM109 cells. Positive clones were purified and sequenced with two T-vector specific primers (sp6 and T7).

Full-length cDNA from both genes was obtained by 5'-, 3'-RACE. For 3'-RACE, a new template was generated using homemade JAP/ADAPTER primers (5'-CAGTCGAGTCGACATCGAT(12)-3') and 5 µg of pineal gland total RNA. For 5'-RACE, specific primers for cDNA synthesis were also used (Table 1). With these new templates, a first set of PCR was performed using the homemade universal primers and primers designed

from the cloned fragments of AANAT and HIOMT (touchdown PCR with annealing temperatures decreasing from 55ºC to 50ºC in 10 cycles followed by 20 cycles of 50ºC). PCR products were submitted to a nested PCR with universal primers and the second set of designed primers (touchdown PCR with annealing temperatures from 60ºC to 55ºC followed by 20 cycles of 55ºC). The amplified products were ligated, transformed and sequenced as above described.

Protein Alignments and Phylogenetic Trees

Nucleotide sequences were obtained form GeneBank and Ensembl Genome Browser (http://www.ensembl.org) and manually curated to obtain the corresponding amino acid sequences. Only available full-length amino acid sequences of AANAT and HIOMT were aligned using ClustalX Program (ver. 2.0.11; Larkin et al., 2007). The degree of support for internal nodes of phylogenetic trees was assessed using 1000 bootstrap replications of Neighbour Joining method (NJ). The Dendroscope ver. 2.2.2 software program was used to draw the final tree (Huson et al., 2007).

Tissue Distribution

Total RNA from goldfish retina, pineal gland, hypophysis, liver, gut, gall bladder, heart, kidney, gonad, gills, muscle and skin was extracted and reverse transcribed as described above. Specific primers (Table 1) from the sequences obtained were designed, and PCR of goldfish *Aanat*-2 (g*Aanat*-2), *Hiomt* (g*Hiomt*-2) and g18S were performed (annealing temperature for g18S and gHIOMT-2 set at 54ºC, for gAANAT-2 set at 52ºC). Amplified PCR products were checked by 1.5% agarose electrophoresis in the presence of a DNA size marker (1Kb, Invitrogen). Products from retina, pineal gland, liver and gut were gel-extracted and sequenced in order to confirm the presence of g*Aanat*-2 and g*Hiomt*-2 in the mentioned tissues.

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

Total RNA from neural retina, midbrain area containing the pineal gland, liver, foregut and hindgut was extracted with the TRI® Reagent method (Sigma), treated with DNase (1h, 37ºC, Promega) and retro-transcribed with random primers and SuperScript II Reverse Transcriptase (Invitrogen) using 1.5 µg of RNA for pineal gland and retina, and 5 µg of RNA for liver and gut.

Amplification reactions were carried out in a final volume of 15 µl containing ABsolute SYBR green fluorescein (Thermo Scientific) and 70 nmol of the gene-specific forward and reverse primers (Table 1). PCRs were performed in an iCycler (Bio-Rad) in the following conditions: 1 cycle of 95ºC, 40 cycles of 95ºC for 30 s, 54ºC for 30 s and 72ºC for 15 s in the case of g*Hiomt* and 18S; annealing temperature for g*Aanat*-2 was 52ºC. All samples were assayed in duplicates to ensure consistency. As an internal control, experiments were duplicated with 18S and the gene expression levels were expressed as the change with respect to the corresponding 18S-RNA calculated threshold cycle (Ct) levels. All analyses were based on the Ct values of the PCR products. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve.

Statistical Analysis

The variations in mRNA levels among different 24-h time points were statistically analysed with one-way ANOVA. The rhythmicity in gene expression was checked by cosinor analysis by fitting periodic sinusoidal functions to the expression values for the genes across the five time points using the formula $f(t)=M+A\cos(t\pi/12-\varphi)$, where $f(t)$ was the gene expression level at a given time, the mesor (M) is the mean value, A is the sinusoidal amplitude of oscillation, t is time in hours, and φ is the acrophase (time of peak expression). Non-linear regression allows the estimation of M, A, and φ, and their standard error (SE) (Delgado et al., 1993). Significance of cosinor analysis was defined by the noise/signal of amplitude calculated as the ratio SE$(A)/A$, considering ratio values lower than 0.3 as

statistically significant. Expression was considered to display a daily rhythm if it had both $P<0.05$ by ANOVA and $SE(A)/A <0.3$ by cosinor analysis.

RESULTS

Cloning of Goldfish *Aanat-2* and *Hiomt-2* Genes.

The cloning procedure used herein allowed obtaining the complete cDNAs of goldfish g*Aanat-2* gene. Full-length cDNA includes an open reading frame (ORF) of 626 base pair (bp) encoding a protein of 208 amino acids (Fig. 1a). Two putative phosphorylation sites for protein kinase A (PKA) were identified, including the conserved PKA site (RRHT) of vertebrates at the *N*-terminal region. The protein also contains two acetyl coenzyme A binding motifs (Motif A and B), three highly conserved regions (C/c-1, D/c-1, D/c-2) and four highly conserved cysteine residues necessary for disulfide bonds formation (Fig. 1A). This sequence of gAANAT-2 was compared to the available full-length amino acid sequences in other species (Figure 1b). The alignment of the deduced goldfish protein with other teleost AANAT-2 sequences showed a high similarity (zebrafish 95%; seabream 91%, rainbow trout 91%, pike 89%, turbot 87%) while homology with AANATs-1 was lower (turbot 79%, seabream 72%, zebrafish 70%). These results confirm that the isolated sequence of goldfish by us belongs to the *Aanat-2* subfamily and may be properly designated as *gAanat-2.*

The isolated goldfish *Hiomt* cDNA showed an ORF of 1044 bp encoding a protein of 348 amino acids and includes a putative casein kinase II phosphorilation site (Fig. 2a). The protein sequence deduced from the goldfish *Hiomt* cDNA sequence was compared with other vertebrate sequences using the ClustalX algorithm (Fig. 2b). Results show a high sequence similarity with the rest of vertebrates (higher than 46 %). Similarly to the case of *Aanat*, teleosts have more than one sequence for *Hiomt*, which are nested in two separate branches of the phylogenetic tree with a very high internal homology (91-70%) in each branch. This homology grade is comparable to the sequences of placentalian *Hiomt* (92-70%). By other hand, the sequence resemblance between the two paralog sequences of the same teleost species drops to 56-54%. From these results, and similarly to the g*Aanat*, it can

be proposed the existence of two teleost HIOMT isoenzymes, *Hiomt-1* and *Hiomt-2,* named attending to its higher and lower similarity with tetrapods HIOMT (Fig. 2b). The goldfish *Hiomt* sequence shows the maximal similarity (91%) with *Hiomt-2* of zebrafish (zgc: 162232), indicating that we cloned the isoenzyme 2 of goldfish.

Central and Peripheral Distribution of AANAT-2 and HIOMT-2 mRNA in Goldfish.

RT-PCR analysis was performed in order to localize mRNAs in different central and peripheral tissues. Particularly, the mRNA expression pattern for both genes, g*Aanat-2* and *gHiomt-2,* was studied in goldfish retina, pineal gland, hypophysis, liver, gut, gall bladder, heart, kidney, gonad, gills, muscle and skin and it is shown in Figure 3. A housekeeping gene (18S) served to monitor the quality of the cDNAs from each tissue. PCRs were carried out up to 30 cycles. The pineal gland showed strong expression for both enzymes, as expected. Peripherally, AANAT has a wide distribution range and its expression was detected in kidney, liver, foregut, hindgut, retina and gallbladder (Fig. 3). HIOMT-2 transcript expression was detected in the pineal, retina and gonads with strong signals, while low expression levels were obtained in liver, foregut, hindgut and heart. No positive results were found in the rest of the tested tissues.

Daily rhythmic expression and transcriptional regulation of goldfish *Aanat2*

The Figures 4-7 show daily variations of *gAanat-2* expression in the pineal gland, retina, liver and hindgut of goldfish maintained in a 12L:12D photocycle, a phase shifted photocycle (12D:12L) and under a constant regime of darkness (24D) or lighting (24L) conditions. The significance of daily cyclic oscillations was tested by cosinor analysis and one-way ANOVA, and the parameters defining the significant gene expression rhythms are summarized in Table 2. The foregut was the only tissue that did not display significant rhythms at any of the lighting conditions investigated (data not shown). Very high levels of the transcript (mesor values) were found in the pineal compared to the rest of the studied organs (Table 2). Moreover, the pineal gland presented significant rhythms in both

photoperiodic conditions tested, with peaks of expression at the second half of scotophase, corresponding to acrophases at ZT21 in 12L:12D and ZT19 in 12D:12L. Such high expression levels of the transcript were abruptly damped in 24L or 24D conditions, the rhythm disappears in total darkness, but persists under constant light with low amplitude peaking around the subjective midnight (CT 15, Fig. 4).

The g*Aanat-2* expression in the retina showed a significant daily rhythm in goldfish maintained under 12L:12D photoperiod and in both constant conditions, 24L and 24D, but such rhythmic oscillation disappeared under a phase shifted photoperiod (12D:12L) (Figure 5). The mesor and amplitude are significantly higher in 12L:12D compared to the other daily expression rhythms found under 24D and 24L conditions. The acrophase in 12L:12D is coincident to that for the pineal gland, and it is slightly advanced in 24D and 24L (around CT16 and CT10 respectively).

The Figure 6 shows the 24-h changes for g*Aanat-2* expression in goldfish liver. The levels of the transcript (mesor values) were higher in liver compared to the rest of the peripheral organs, including the retina. Moreover, significant rhythms were found at all the experimental conditions tested. The highest expression levels were found under both photoperiodic regimes (12L:12D and 12D:12L), and the exposition to constant conditions (24D and 24L) markedly reduced (around 200-350 fold) such expression levels, but significant rhythms (from ANOVA and cosinor analysis) were surprisingly maintained. The acrophases of the daily rhythms in this peripheral location were located around midday in 12L:12D (ZT8) and 24D (CT7), but it were conserved respect to pineal rhythms at CT20 and ZT20 in 24L and in 12D:12L, respectively.

The rhythmic expression patterns of *gAanat-2* transcripts in hindgut are presented in Figure 7, where significant 24-h rhythms (noise/signal <0.3) can be observed under the four different lighting conditions investigated. The mean expression values are very similar in all the groups and at the same order than that found in retina under 12L:12D conditions (Table 2) The acrophases of the rhythms were also highly conserved among the different experimental conditions, and peaked at the second half of the scotophase or the subjective

night (ZT20 and CT19), except for the phase shifted photocycle (12D:12L) where the peak was slightly advanced (ZT16). The amplitude of the g*Aanat-2* expression rhythms in the hindgut (around 2-fold) was similar to that exhibited by liver and retina, and it was not significantly modified by the maintenance at the different lighting conditions (Table 2).

Daily rhythmic expression of goldfish Hiomt-2

The 24-h variations of g*Hiomt-2* transcript levels were examined by means of qRT-PCR in retina, pineal gland, liver, foregut and hindgut from goldfish maintained under a 12L:12D photocycle for 30 days. The highest levels of g*Hiomt* transcript were found in the pineal gland and the hindgut, with lower levels in the foregut, liver and retina (Fig. 8). Statistical analysis (cosinor and ANOVA) revealed significant daily rhythms of HIOMT-2 mRNA in pineal gland, liver and hindgut, with the acrophases at the second half of dark phase (ZT19) for pineal and hindgut, and at the first half of the dark phase (ZT 13) in liver (Table 3). The amplitude of the HIOMT-2 mRNA rhythm is around 100-fold in pineal and hindgut, decreasing to 6-fold for liver. Parameters defining such gene expression rhythms are summarized in Table 3.

DISCUSSION

Cloning of Goldfish *Aanat*-2 and *Hiomt-2* Genes

The present study reports the full-length cDNA sequences of the two key enzymes of MEL biosynthesis, *Aanat-2* and *Hiomt-2* in the goldfish. According to multiple alignment analyses, the putative protein sequence deduced from the cloned cDNAs show a high degree of homology with AANAT-2 sequences available on the database. The low identity (maximum values of 79%) of the goldfish AANAT protein sequence with other fish AANAT-1 sequences supports that the protein cloned in the present study corresponds with the AANAT-2. This goldfish AANAT-2 deduced amino acid sequence shows more that 90% identity with *Aanat-2* sequences from other fish species such as zebrafish, seabream, rainbow trout or pike. Moreover, characteristic binding motifs, phosphorylation sites and

conserved regions of the AANAT family (Ganguly et al. 2002; Klein, 2007) are identified in the goldfish sequence, supporting the homology of the encoded protein.

In contrast to the significant number of studies available on *Aanat*, very few are focused on vertebrate *Hiomt*. In the present study, full-length cDNA encoding goldfish HIOMT-2 is reported, as confirmed by the high homology obtained in the multiple alignment analysis performed with sequences from other vertebrates. The presence of several highly conserved domains corresponding to the catalytic as well as noncatalytic sites of the protein (Vuilleumier et al., 2007) support that the protein cloned in the present study corresponds with the HIOMT. The deduced aminoacid sequence of goldfish HIOMT displays a high degree of similarity with a second highly divergent *hiomt* gene. Then, our results show for the first time that a second *hiomt* also exist in teleost, as occurs with *aanat*. A whole genome duplication close to the origin of teleost (Hoegg et al., 2004; Volff 2005), and subsequent mutations in the duplicated genes have resulted in two subfamilies of HIOMT, as for AANAT, in these vertebrates. In contrast, only one *Hiomt* is reported in the turbot, probably as a result of loss of the second *Hiomt* paralogue during ancestral lineage of this species, as authors suggest (Vuilleumier et al., 2007).

Distribution of AANAT-2 and HIOMT-2 mRNA in Goldfish

The distribution of the AANAT-2 transcript in the goldfish displays an intense signal in pineal, as occurs in pike and trout (Bégay et al., 1998; Coon et al., 1999), the gilthead seabream (Zilberman-Peled et al., 2004) and the sole (Isorna et al., 2009). Moreover, as occurs in zebrafish (Gothilf et al., 1999), a positive signal for AANAT-2 transcript is also found in goldfish retina, in spite of the general acceptance that *Aanat-2* is more specifically expressed in the pineal organ of teleost (Klein, 2007, Falcon, 2009). One interesting evidences from present results is the peripheral expression of both enzymes, AANAT-2 and HIOMT-2, which supports MEL synthesis in such peripheral locations. Peripheral AANAT expression has been reported in rodents (Gauer et al., 1999: Stefulj et al., 2001), birds (Kato et al., 1999) and frogs (Isorna et al., 2006). Evidences for AANAT-2 expression in fish was

reported in rainbow trout peripheral tissues (Fernández-Durán et al., 2007), but not in others species (pike, trout, zebrafish, Bégay et al., 1998; Coon et al., 1998, 1999; Gothilf et al., 1999). This apparent diversity among fish species could be explained by the different sensitivities of methodologies (Northern blot analysis versus quantitative real time RT-PCR) and/or by the different pattern of distribution between AANAT-1 and AANAT-2 in periphery. In our study AANAT-2 mRNA was detected in peripheral locations at 25 PCR cycles, which support the physiological presence of such AANAT transcripts. The functional role played by AANAT-2 in these locations, is of great interest and several possibilities, apart from the MEL synthesis, can be suggested, as the reduction of the oxidative formation of aldehydes of serotonin and other arylalkylamines (Bernard et al., 1995; Coon et al., 2002) and/or a detoxification role, as it is considered the original function of this enzyme (Besseau et al., 2006; Klein, 2007). Nevertheless, in addition to the expected *Hiomt* expression in goldfish pineal and retina, where MEL synthesis is well documented *in vivo* and *in vitro* (Iigo et al., 1991; 1997), we found positive signals for HIOMT transcripts in gonads and, to a lesser extent, in liver and gut. These are the first results identifying *Hiomt* transcripts in non photosensitive organs in fish, and accords with previous studies in rat (Stefulj et al., 2001) and quail (Fu et al., 2001). The high level of *gHiomt-2* expression detected in peripheral locations, as gonads, liver, foregut and hindgut support, but do not demonstrate, a local MEL synthesis, and its physiological relevance deserves to be investigated. Recent results regarding a regulatory role of MEL on gut contractile activity in goldfish (Velarde et al., 2009b) would support such a local autocrine/paracrine signalling role of gastrointestinal MEL in fish.

***Daily rhythmic expression* and transcriptional regulation**

Present results demonstrate daily rhythms in the expression of goldfish *Aanat-2* in pineal and peripheral sites (retina, liver and hindgut) under 12L:12D conditions with significant differences dependent on locations. The rhythmic oscillation in pineal g*Aanat-2* expression peaked during dark phase (ZT20) in L/D conditions, in agreement with previous

findings in pike (Coon et al., 1998), zebrafish (Begay et al., 1998; Gothilf et al., 1999) or gilthead seabream (Ziberman-Peled et al., 2006), but the amplitude of the rhythms varies in a species-dependent manner. The goldfish pineal exhibits low amplitude rhythms of *gAanat-2* as in the gilthead seabream (Zilberman-Peled et al., 2006), suggesting that post-transcriptional regulation of the goldfish AANAT-2 has more impact on the robust MEL rhythm (Iigo et al., 1991) than the transcriptional regulation. The daily rhythmic pattern in *Aanat-2* is not a constant feature in teleost, as the AANAT-2 mRNA levels in trout pineal are constant throughout the 24-h cycle (Bégay et al., 1998) in spite of pineal rhythmic MEL production. This species difference reinforces the distinct relevance of post-translational regulation to ensure daily rhythmic AANAT activity and MEL synthesis. A very interesting result in the present study is that light schedules 180º out of phase entrain the rhythm of *gAanat*-2. To date no report demonstrates such phase shifting entrainment effects of light on pineal MEL synthesis in goldfish. On the other hand, the significant decrease in the mesor level of *gAanat-2* expression under 24-D and 24-L indicates that pineal AANAT-2 is under photic regulation in goldfish, but the persistence of the rhythm in 24-L suggest that expression of this transcript is under circadian clock, as occurs in the pike (Bégay et al., 1998). Nevertheless, the loss of AANAT-2 transcript rhythm under 24-D could be related to the proteolysis-based mode of regulation underlying the suppressive effect of light on AANAT activity *in vivo*.

The *Aanat-2* expression levels in the goldfish retina are highly lower than in the pineal, but significant rhythms with similar acrophases were found. These low expression levels are in agreement with the general acceptance that AANAT-2 is preferentially expressed in the pineal, whereas the most abundant isoform in the retina is the AANAT-1 (Klein, 2007; Falcón, 2009). Nevertheless, our results indicate that in the goldfish retina an AANAT-2, with parallel rhythmic expression to ocular MEL rhythms (Iigo et al., 1997) could eventually produce MEL, in addition to a possible role on reduction of cytotoxic compounds formation, as suggested (Klein, 2006). Further information on gAANAT-1 is needed to delve into the role of both enzymes in the retina of this species. The amplitude of the oscillations in

the g*Aanat*-2 expression is dampened under constant conditions, but significant rhythms persist, suggesting the existence of a circadian clock driving the g*Aanat*-2 expression in the retina, as in the goldfish pineal. This circadian regulation of AANAT-1 expression does not exist in the trout retina, where the gene expression is driven by environmental lighting (Besseau et al., 2006). All these results, together with the recent finding of clock genes expression in goldfish retina (Velarde et al., 2009a) support the existence of an ocular circadian clock in this species, as occurs in the pineal (Iigo et al., 1991).

Present results show for the first time in vertebrates the existence of circadian rhythms in *Aanat-2* and *Hiomt-2* transcripts in liver and hindgut. In goldfish liver, the expression level and the amplitude of oscillations are higher in a LD photoperiodic regime than in the absence of photocycle, when the rhythms damp but persist. These results indicate that photocycle is needed for the maintenance of this rhythm, but a circadian clock drives the *Aanat*-2 expression rhythm in both, liver and hindgut. The expression rhythms in hindgut seem to be light-independent, as the acrophases occur at ZT20 (CT20) in all the photic conditions tested except in the phase-shifted photoperiod (12D:12L). Recent findings of clock genes in liver and gut of the goldfish (Velarde et al., 2009a), and present results on rhythms of AANAT-2 and HIOMT-2 expression suggest that such local MEL synthesis can be regulated by peripheral clocks entrainable to non-photic cues. Feeding seems to be a good candidate to exert such a role. In fact, the existence and characterization of food-entrainable oscillators is a great interesting question, and it would constitute an adaptive system allowing the organisms to anticipate changes in metabolic requirements (Mendoza, 2007). To date, there are no previous reports concerning rhythmic expression of enzymes involved in MEL synthesis in vertebrate peripheral locations, but present results point to liver and hindgut as peripheral organs exhibiting functional clocks in goldfish. Both, the very low expression levels of AANAT-2 and the lack of rhythms in *gAanat-2* and *gHiomt-2* in foregut emphasize the relevance of the different intestinal segments concerning the melatoninergic system in goldfish.

Finally, present study shows significant rhythms of g*Hiomt-2* transcript in pineal, liver and hindgut, but not in retina or foregut, under a LD cycle, being of high amplitude in pineal and hindgut with acrophases through the dark phase. The HIOMT activity and its possible rhythmicity in goldfish have not been investigated to date. Present results indicate firstly that in terms of HIOMT, a physiological rhythmic MEL synthesis in the gut can occur. Second, the similar pronounced rhythmic pattern of HIOMT expression in pineal and gut could suggest that a distinct posttranscriptional regulation of HIOMT underlies the differences in rhythmic MEL production in these two locations. To our knowledge, this is the first time that circadian rhythms of HIOMT expression are reported in peripheral tissues, and then, further experiments are needed to investigate possible correlations between mRNA variations and enzymatic activity.

In summary, cloning of the full-length cDNAs encoding g*Aanat2* and g*Hiomt-2 genes*, highly expressed not only in goldfish pineal gland, but also in peripheral tissues, provides, for the first time, evidences that the serotonin-*N*-acetylserotonin-MEL pathway may be functional in goldfish liver and gut. Both genes display circadian light-dependent variations in pineal gland, retina and liver. However, a rhythm entrained to non-photic cues is suggested in hindgut. The functional role of the rhythms in AANAT-2 mRNA in liver and gut as part of the peripheral endogenous circadian system in the goldfish is questioned and needs further investigations.

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FIGURE LEGENDS

Figure 1. Sequence analysis of goldfish AANAT2. (a) Complete nucleotide and deduced amino acids sequences of *Carassius auratus* AANAT2. The analysis was performed using Gene Runner software (Hastings Software, USA). The start and stop amino acids are marked in bold, underlined letters. PKA: putative cyclic nucleotide-dependent kinase site. C/c-1, D/c-1, D/c-2: highly conserved regions. Motif A/B: acetyl coenzyme A binding domains. The Cys residues for disulphide bond formation are marked with a star. (b) Dendrogram showing the phylogenetic relationships of goldfish AANAT-1 and AANAT-2 with AANATs peptides of other species. Peptide sequences were used to generate the dendrogram. All residues up to and including the N-terminal PKA site, and residues including and following the C-terminal PKA site were removed prior to alignment. Sequences were aligned using Clustal W and the phylogenetic tree was generated using the neighbor-joining method. Branch length is proportional to evolutionary distance. Scale bar indicates 0.01 (1%) the rate of amino acid substitution per residue. Number in branches indicates the robustness (significance) of each internal node after 1000 bootstrap replications. Numbers below 500 are omitted, indicating the low supported nodes.

Figure 2. (a) Complete nucleotide and deduced amino acids sequences of *Carassius auratus* HIOMT. The analysis was performed using Gene Runner software (Hastings Software, USA). The start and stop amino acids are marked in bold, underlined letters. CkII: putative casein kinase II phosphorilation site. (b) Phylogenetic tree of HIOMT obtained by NJ method. Branch length is proportional to evolutionary distance. Scale bar indicates 0.01 (1%) the rate of amino acid substitution per residue. Number in branches indicates the robustness (significance) of each internal node after 1000 bootstrap replications. Numbers below 500 are omitted, indicating the low supported nodes.

Figure 3. Distribution of *gAanat-2* and g*Hiomt-2* mRNA expression in different central and peripheral tissues of goldfish (*Carassius auratus*) as revealed by quantitative real time RT-

PCR assays. The rRNA-18S was used as reference gene. The size of RT-PCR products in bp was indicated at the left of the figure. Each lane contains 1 µg of total RNA from a pool of 5 samples. R: retina, P: pineal gland, HP: hypophysis, L: liver, FG: foregut, HG: hindgut, H: heart, K: kidney, GB: gallbladder, G: gonad, GL: gills, M: muscle, S: skin, ---: negative control, 1*kb* (kilobases*)*: ladder.

Figure 4. Daily variations of g*Aanat*-2 expression in the pineal of goldfish (*Carassius auratus*). Fish were maintained in laboratory conditions under (a) light:dark photoperiod (12L:12D), (b) inverted dark:light photoperiod (12D:12L), (c) constant darkness (24D) and (d) constant light (24L). Each point represents the mean ± SEM of the transcript amount at a given time point. Transcript levels were quantified using quantitative real-time RT-PCR and normalized to 18*S* mRNA. The asterisks indicate significant daily rhythms by one way analysis of variance and cosinor analysis. The open bar at the bottom of the graphs represents the period of light, while the closed bars represents periods of darkness.

Figure 5. Daily variations of g*Aanat*-2 expression in the retina of the goldfish (*Carassius auratus*). Fish were maintained in laboratory conditions under (a) light:dark photoperiod (12L:12D), (b) inverted dark:light photoperiod (12D:12L), (c) constant darkness (24D) and (d) constant light (24L). Each point represents the mean ±SEM of the transcript amount at a given time point. Transcript levels were quantified using quantitative real-time RT-PCR and normalized to18*S* mRNA. The asterisks indicate significant daily rhythms by one way analysis of variance and cosinor analysis. The open bar at the bottom of the graphs represents the period of light, while the closed bars represents periods of darkness.

Figure 6. Daily variations of g*Aanat*-2 expression in the liver of goldfish (*Carassius auratus*). Fish were maintained in laboratory conditions under (a) light:dark photoperiod (12L:12D), (b) inverted dark:light photoperiod (12D:12L), (c) constant darkness (24D) and (d) constant light (24L). Each point represents the mean ±SEM of the transcript amount at a given time point.

Transcript levels were quantified using quantitative real-time RT-PCR and normalized to 18*S* mRNA. The asterisks indicate significant daily rhythms by one way analysis of variance and cosinor analysis. The open bar at the bottom of the graphs represents the period of light, while the closed bars represents periods of darkness.

Figure 7. Daily variations of g*Aanat*-2 expression in the hindgut goldfish (*Carassius auratus*). Fish were maintained in laboratory conditions under (a) light:dark photoperiod (12L:12D), (b) inverted dark:light photoperiod (12D:12L), (c) constant darkness (24D) and (d) constant light (24L). Each point represents the mean ±SEM of the transcript amount at a given time point. Transcript levels were quantified using quantitative real-time RT-PCR and normalized to 18*S* mRNA. The asterisks indicate significant daily rhythms by one way analysis of variance and cosinor analysis. The open bar at the bottom of the graphs represents the period of light, while the closed bars represents periods of darkness.

Figure 8. Daily variations of g*Hiomt-2* expression in pineal, retina, liver, foregut and hindgut in goldfish (*Carassius auratus*) maintained under under 12L:12D photoperiod. Levels of transcripts were quantified using quantitative real-time and normalized to 18*S* mRNA. Each point represents the mean ±SEM of the transcript amount at a given time point. Transcript levels were quantified using quantitative real-time RT-PCR and normalized to 18*S* mRNA. The asterisks indicate significant daily rhythms by one way analysis of variance and cosinor analysis. The open bar at the bottom of the graphs represents the period of light, while the closed bars represents periods of darkness.