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Cloning and expression of arylalkylamine *N*-acetyltranferase-2 during early development and metamorphosis in the sole *Solea senegalensis*

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ABSTRACT

The arylalkylamine *N*-acetyltransferase (AANAT) is a key enzyme in the rhythmic production of melatonin. Two *Aanats* are expressed in teleost fish, one retinal specific, *Aanat1*, and the other one pineal specific, *Aanat2*, being the latter the main enzyme responsible of the plasma nocturnal melatonin increase in fish. In anurans melatonin has been involved in metamorphosis through antagonizing thyroid hormone function; however, no available data reports a relationship between melatonin system and metamorphosis in fish. In this study, we have cloned the AANAT2 (*SsAanat2*) in a flatfish, *Solea senegalensis*, and studied its sites of expression and developmental expression pattern by *in situ* hybridization and Real Time PCR. These studies allowed us to demonstrate a specific signal in the pineal gland of sole larvae from 2 days post-fertilization (dpf), which was evident until post-metamorphosis. Immunohistochemical analysis on the hybridized slides showed that the sole pineal *Aanat2* expressing cells corresponded to pineal photoreceptor cells. Real Time PCR was performed in animals kept under natural photoperiod and sampled at different stages from 0 to 21 dpf (including pre-, early-, middle- and late-metamorphic stages) and at midlight (ML) and middark (MD) daytimes. Sole *Aanat2* expression was higher at MD than at ML from 2 dpf and at most developmental stages analyzed. The highest AANAT2 mRNA abundance was observed at 2 and 4 dpf. A significant 60-fold reduction in *Aanat2* expression was seen just before metamorphosis demonstrating, for the first time in a vertebrate species, that the expression of pineal AANAT and thyroid hormones levels exhibit an inverse pattern during metamorphosis.

1. Introduction

Melatonin is a time-keeping hormone that synchronizes many physiological and behavioural processes entraining them to the daily and seasonal variations of photoperiod (Reiter, 1993; Falcón et al., 2007a). This neurohormone is synthesized and released rhythmically during the dark phase by the retina and the pineal gland, two organs of neural origin that are photosensitive in fish (Coon and Klein, 2006; Falcón et al., 2007a). In most vertebrate species, the pineal gland is the main source of plasma melatonin, whereas retinal melatonin seems to represent a local (autocrine and/or paracrine) hormone (Cahill and Besharse, 1995; Falcón et al., 2007a).

Melatonin biosynthesis from serotonin requires the sequential activity of arylalkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87), which converts serotonin to *N*-acetylserotonin, and hydroxyindole-*O*-methyltransferase (HIOMT; EC 2.1.1.4), which transforms *N*-acetylserotonin to melatonin (Klein et al., 1997; Klein, 2007). The nocturnal rise in melatonin production results from an increase in AANAT protein content and activity. This rise is preceded in some species by an increase in *Aanat* gene expression, whereas HIOMT activity and/or expression remains almost constant throughout the light-dark cycle (Falcón, 1999 Falcón et al., 2007b; Vuilleumier et al., 2007).

Teleosts posses two *Aanat* genes; *Aanat1* expressed in the retina (and similar to AANATs of other vertebrates), and *Aanat2* expressed in the pineal with no orthologue in other vertebrate classes (Coon and Klein 2006; Falcón et al., 2007b). Although circadian rhythms in AANAT2 activity and/or expression have been analyzed in different adult fish species (Falcón et al., 2001; Zilberman-Peled et al., 2007), data available on *Aanat2* day-night variations expression during development are rather scarce and most studies have been focused on zebrafish (Gothilf et al., 1999; Vuilleumier et al., 2006). Recently, a study performed in a flatfish, *Scophthalmus maximus*, revealed that contrary to that reported in zebrafish, *Aanat2* did not exhibit daily rhythms at early developmental stages (Vuilleumier et al, 2007). This evidence suggests that at least two strategies

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have been developed during evolution of the teleost lineage regarding the developmental expression of *Aanat2* (Vuilleumier et al, 2007). Nevertheless, the above mentioned study was performed at premetamorphic stages and, to our knowledge, there is no data concerning the daily expression profile of a melatonin-synthesizing system during flatfish metamorphosis. Interestingly, modifications on the melatonin biosynthesizing system occur during amphibian metamorphosis (Wright, 2002; Isorna et al, 2005). In this study, we bring original information on the melatonin biosynthesis enzyme AANAT2 in the flatfish *Solea senegalensis* during early development and metamorphosis. We have cloned the full-length sole AANAT2 and localized *Aanat2* gene expression in developing pineal photoreceptors by *in situ* hybridization. Finally, we also report the daily expression pattern of *Aanat2* at early stages of development and during metamorphosis in this species.

2. Material and methods

2.1. Animals and sampling

2.1.1. Cloning of sole AANAT2

Senegalese sole adult specimens were obtained from CUPIMAR S.L. (San Fernando, Spain) and maintained in the "Laboratorio de Cultivos Marinos" from the University of Cádiz. Animals were anesthetized (phenoxyethanol, SIGMA) and sacrificed during nighttime. Pineals were quickly removed, frozen in liquid nitrogen and stored at −80 °C until used.

2.1.2. Developmental studies

Senegalese sole fertilized eggs were obtained in May 11th 2006 (0 days post-fertilization or dpf) from "IFAPA El Toruño" (Junta de Andalucía, Puerto de Santa María, Spain) and maintained in the "Laboratorio de Cultivos Marinos" (University of Cádiz). Eggs were incubated under natural light environmental conditions (sunrise 07:31 h, sunset 21:22 h) and constant temperature and salinity (19 ± 1 °C and 39 ppt, respectively). Animals were sampled at nine stages of development: before hatching (0 dpf), before the beginning of metamorphosis (2, 4, 6, 9 dpf) and during metamorphosis (pre-, early, middle, and late metamorphosis; 12, 15 19 and 21 dpf, respectively). At each developmental stage, pools of whole-animals were obtained at 14:30 h (ML) and 02:30 h (MD) local time (expressed as Greenwich meridian time +2 h). Pools contained 20–30 specimens at early developmental stages (0–6 dpf), 10–20 specimens at mid developmental stages (9–15 dpf) and 5–10 specimens at late developmental stages (19–21 dpf). We have sampled animals at the same developmental stage measured according to the position in the water column. Samples were frozen in liquid nitrogen and stored at −80 °C until used. All experiments were performed in accordance with the "Principles of Laboratory Animal Care" (NIH published 86–23, revised 1985) and were approved by the Animal Experimentation and Ethics Committee of the University of Cádiz (Spain).

2.2. Cloning of Senegalese sole Aanat2

Total RNA was extracted from eight adult pineal glands using the Trizol® method (Gibco^{BRL}) according to the manufacturer's instructions and it was reverse transcribed using SMART^{TM} RACE cDNA kit (BD Bioscience, Clontech). Two rounds of Touch down PCR were performed for a first partial cloning (see degenerated primers in Table 1). Full-length AANAT2 was obtained by 3′ and 5́-RACE using the same cDNA, internal universal primers (SMART^{TM} RACE cDNA kit) and sole *Aanat2* specific primers designed from the partial sequence first obtained (see Table 1). Amplified products were purified, sub-cloned and sequenced as previously described (Isorna et al., 2006). Sequences obtained were aligned with AANAT sequences from various vertebrate species available in Gene Data Bank.

Table 1
Sequences of the primers employed.

Sole *Aanat2* cloning	
Degenerated primers	
NAT2F1	5́-ATGRCWCMKMAGGTSAGC-3́
NAT2F2	5́-ATCAGYGTGTTYGARATMGARAGAGA-3́
NAT2R1	5́-CCGABBKBGTAYTCCATCTC-3́
NAT2R2	5́-AGGTACTGSARGWARCGCCACA-3́
Specific primers	
SsAANAT2F1	5́-CTTGAGTCAGTGTCCGGAGCTTTCTCTG-3́
SsAANAT2F2	5́-ATCGTCTCTCACAGGAGGCCATGACT-3́
SsAANAT2R1	5́-ATGGCCTCCTGTGAGAGACGATCCTT-3́
SsAANAT2R2	5́-TGACTCAAGAAGTTCAGCACCTCATCCA-3́
Real time PCR	
SsAANAT2PCRqF3	5́-TCAGTGTCCGGAGCTTTCTCTG-3́
SsAANAT2PCRqR3	5́-ATGGCCTCCTGTGAGAGACGAT-3́
Ss*β-actin*PCRqF	5́-GGATCTGCATGCCAACACTG-3́
Ss*β-actin*PCRqR	5́-TCTGCATCCTGTCAGCAATG-3́

2.3. Quantitative real time PCR analysis

Total RNA was extracted from larval pools using "EUROzol" (EuroClone) according to the manufacturer's instructions. Total RNA (1 μg) was retro-transcribed and genomic DNA removed (QuantiTect® Reverse Transcription Kit, Quiagen). Real time gene expression analysis was performed in a Chromo 4™ Four-Color Real-Time System (Biorad), using β-*actin* for normalization (GenBank Accession No. DQ485686). PCR reactions were developed by duplicated in a 25 μl volume using cDNA generated from 1 μg of RNA, iTaq^{TM} SYBR® Green Supermix with ROX (Biorad) and specific primers (0.4 μM, Table 1) designed within the open reading frame of the genes. Calibration curves exhibited efficiencies around 100%. The conditions of PCR reactions were for both genes analyzed (*Aanat2* and β-*actin*): 3 min at 95 °C, 30 s at 95 °C, 30 s at 60 °C and 45 s at 72 °C. Negative controls included replacement of cDNA by water and use of non retro-transcribed total RNA. The ΔΔCt method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression.

2.4. In situ hybridization

Sole larvae from 2, 4 and 21 dpf were obtained at mid-dark, anesthetized in phenoxyethanol and fixed by immersion in 4% paraformaldehyde. Transverse paraffin serial sections (6 μm-thick) were mounted on Tespa-treated (Sigma, 2% Tespa) slides. Sense and anti-sense digoxigenin-labeled riboprobes probes were made using the kit from Roche (Meylan, France) according to the manufacturer's instructions and a sole *Aanat2* cDNA fragment of 306 bp. The hybridization process was as detailed elsewhere (Besseau et al., 2006) with minor modifications. Briefly, the sections were rehydrated and treated with proteinase K (Sigma; 5 μg/ml for 15 min at 37 °C). After post-fixation with 4% paraformaldehyde the sections were hybridized overnight at 50 °C using a probe concentration of 10 μg/ml in hybridization buffer. Digoxigenin was immunodetected using a commercially available kit (anti-DIG FAB diluted 1/500, Roche, Meylan, France). After color development, slides were mounted in Fish Saline:glycerol (1:1). All experiments were replicated using four different animals.

2.5. Immunohistochemical study

Immunohistochemical staining was performed on the same sections processed for *in situ* hybridization using an immunofluorescence method. Before immunostaining, sections were transferred

for 5 min to Coons buffer (0.01 M Veronal, 0.15 M NaCl) containing 0.1–0.2% Triton X-100 (CBT) for 30 min and then saturated in CBT containing 0.5% casein for 30 min. The protocol followed has been previously described by El M'Rabet and cols. (2008). Briefly, sections were incubated overnight with rabbit antibodies against bovine rod opsin (1:500 dilution, CERN-922) and human LW cone opsin (1:500 dilution, CERN-874). The antibodies used in this study have previously been well characterized and used successfully in fish to identify opsins (García-Fernández et al., 1997). Sections were incubated with an antibody against rabbit IgG coupled to fluorescein (1:100 dilution, Jackson ImmunoResearch, West Grove, USA) and mounted with Vectashield (Vector Laboratories, Burlingame, USA). Negative controls were performed by replacing the primary antiserum with buffer or normal non-immune rabbit serum.

2.6. Data analysis

For the alignment of sequences, the CLUSTALW program was employed (http://align.genome.jp/). Sequences obtained in cloning experiments were analyzed using the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). Developmental and daily statistical differences among groups were determined using a two way ANOVA following by a multiple contrast of range test (LSD). As a significant correlation between hour of the day and developmental stage was found, a one way ANOVA was employed followed by the LSD test. All statistical tests were made using the Statgraphics software.

3. Results

The cloning strategy described in Materials and methods produced a 804 bp sequence, from which a translated 208 aa sequence was deduced (Fig. 1). Conserved Motifs A-D, Proteine kinase A (pka) phosphorylation sites and four cysteine residues, characteristic of the superfamily of *N*-acetyltransferases (Dyda et al., 2000; Ganguly et al., 2002; Vuilleumier et al. 2007) are indicated in Fig. 1. Sole AANAT2 deduced protein displayed high identity/homology with other teleost fish as *Scophthalmus maximus* (EF033250.1), *Oryzias latipes* (NP_001098316.1) and *Danio rerio* (NP_571486.1) (91%/95%, 90%/93% and 84%/90%, respectively). By contrast, identity was lower with fish AANAT1 and tetrapod AANATs (66–71%). This was confirmed by the phylogenetic analysis

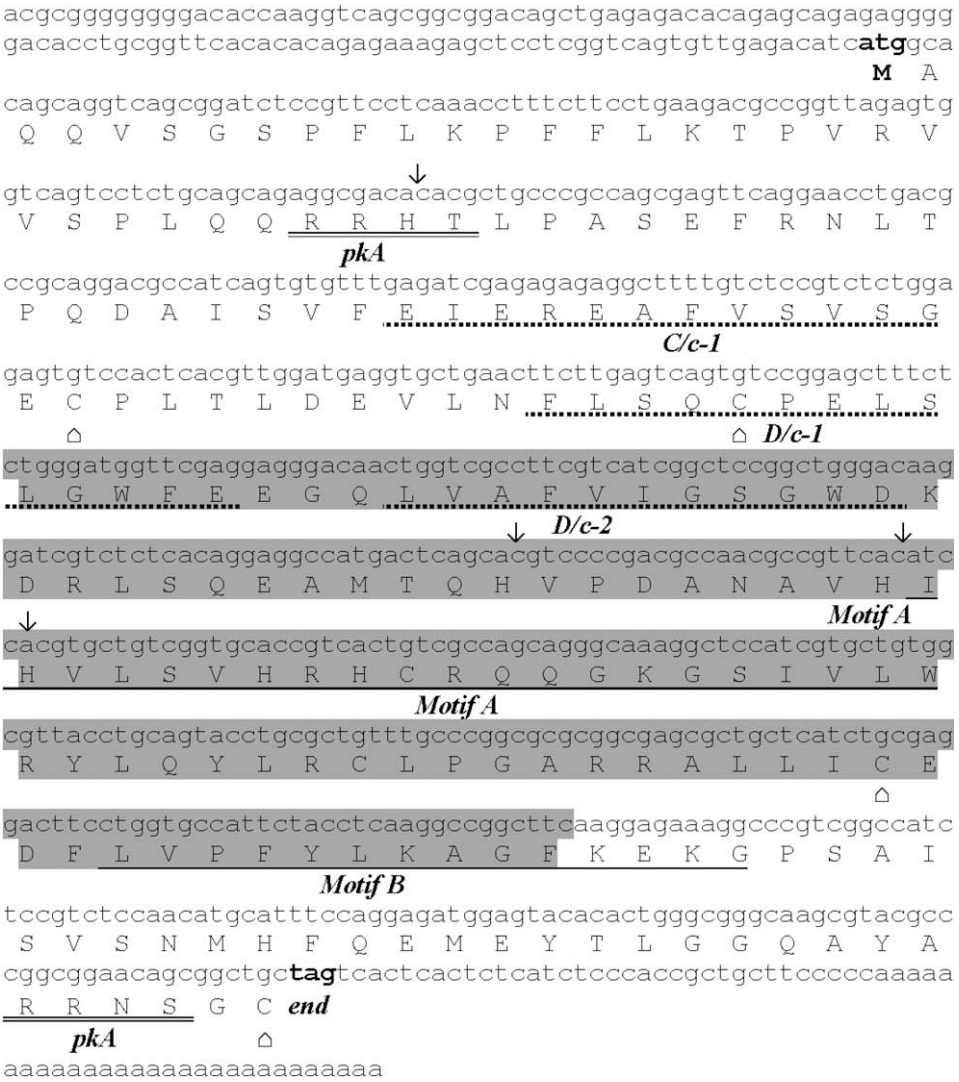

Fig. 1. Full-length nucleotide (804 bp) and deduced amino acid sequence (208 aa) of the Senegalese sole arylalkylamine *N*-acetyltranferase 2. Amino acids are indicated below their respective codons. The start and stop codons are indicated in bold letters. The acetyltransferase domain is shaded while the putative acetyl coenzyme A binding motives and the two phosphorilation site for pkA are indicated by single and doubled underlining, respectively. The three highly conserved regions C/c-1, D/c-1 and D/c-2 found in AANATs from other vertebrates are indicated by dashed underlining. The four highly conserved cystein residues important for disulphide bond formation are indicated by a ⌂ The four conserved histidine residues are indicated by a black arrowhead.

that clearly positioned the sole AANAT2 within the fish AANAT2 branch (Fig. 2).

In order to determine whether the expression of sole *Aanat2* exhibits day-night variations in the developing pineal gland, sole embryos and larvae were maintained under natural photoperiod and sampled at midday and midnight from 0 to day 21 dpf (including pre-, early-, middle- and late-metamorphic stages). Sole *Aanat2* expression increases significantly between 0 and 2–4 dpf (Fig. 3). The sole *Aanat2* mRNA levels remain high at 6 and 9 dpf but a significant 60-fold reduction in expression was seen just before metamorphosis (Fig. 3). Furthermore, we found day-night variation in the expression of sole *Aanat2*, with lower expression during day and higher expression at night (Fig. 3). This daily expression was evident in most stages analyzed but seems to be transiently lost before the onset of metamorphosis (6–12 dpf, Fig. 3).

In situ hybridization studies using a sole *Aanat2* probe on larvae sampled at MD revealed a specific signal in the pineal gland of sole from 2 dpf (Fig. 4A and B). This labeling was also evident at 4 dfp (Fig. 4D and E) and in post-metamorphic stages (Fig. 4G and H).

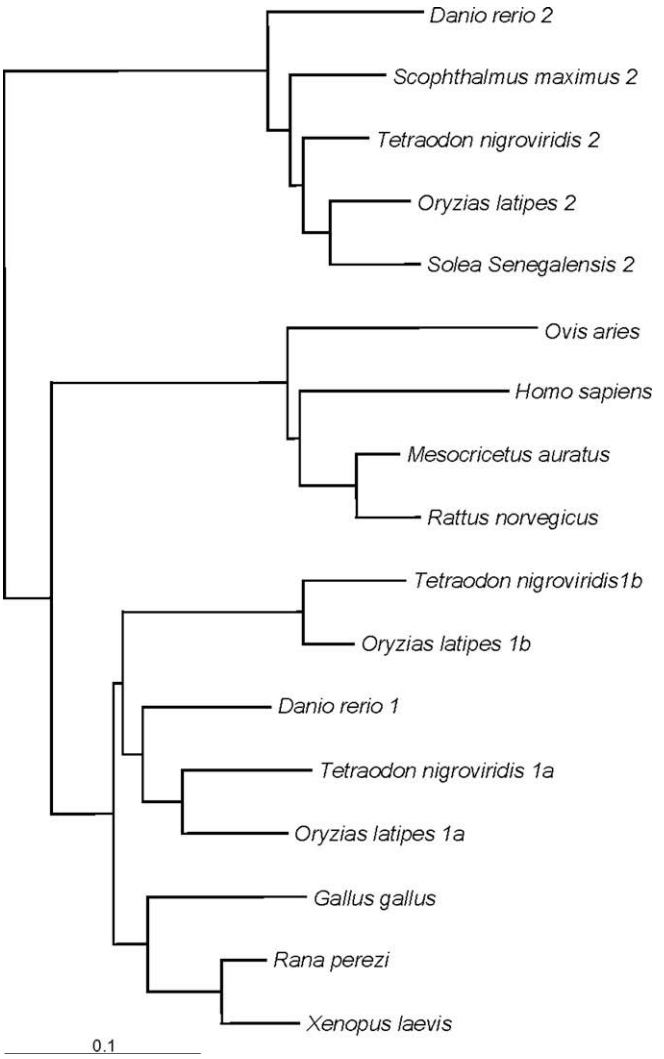

Fig. 2. Phylogenetic trees of AANAT (tetrapod AANATs, fish AANAT1a, AANAT1b and AANAT2). The length of the branches is proportional to the phylogenetic distance. *Gallus gallus* (NP_990489), *Danio rerio* AANAT1 (NP_956998.1) and AANAT2 (NP_571486.1); *Homo sapiens* (NP 001079); *Mesocricetus auratus* (AAD55970); *Oryzias latipes* AANAT 1a (NP_001098302), AANAT1b (NP_001098330) and AANAT 2, (NP_001098316); *Ovies aries* (NP001009461); *Rana perezi* (Isorna et al., 2006); *Rattus norvegicus* (NP 036950); *Scopthtalmus maximus* (EF033250); *Solea senegalensis* (present work); *Tetraodon nigroviridis* AANAT1a and AANAT 1b, (Coon and Klein, 2006); *Xenopus laevis* (AAP57668).

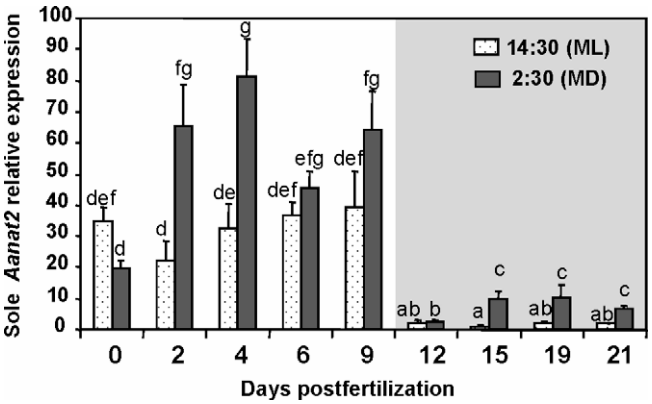

Fig. 3. Expression of *Aanat2* at different stages of the early development and metamorphosis in the Senegalese sole at midday (ML) and midnight (MD). *Aanat2* mRNA was measured by Real Time PCR on pools of animals. Data are shown as the mean ± SEM ($n = 3–4$). The shadow in the graph indicates the metamorphic period. Differences among groups were analyzed by two way ANOVA. As a significant correlation between hour and stage was found ($F = 5.34$, $p = 0.0001$) differences among groups were analyzed by one way ANOVA ($F = 31.33$, $p = 0.0000$) followed by the LSD post-hoc test. There are no statistical differences among groups that share common letters.

No signal was detected with the sense probe in pineal sections (Fig. 4J and K). Immunohistochemical analysis on the hybridized slides using anti-opsin sera showed that the sole *Aanat2* expressing cells corresponded to pineal photoreceptor cells (Fig. 4C,F,I,L).

4. Discussion

In this study we have reported the full-length cloning of sole AANAT2 and the analysis of its developmental expression by real time PCR and *in situ* hybridization. The specific expression of AANAT2 in opsin-immunoreactive pineal cells from sole reinforces the idea that in fish AANAT2 is restricted to pineal photoreceptors (Coon et al., 1999; Vuilleumier et al., 2007; Zilberman-Peled et al., 2007). In turn, fish AANAT 1 and its orthologue AANAT from tetrapods seems to have a more wide distribution in retina (photoreceptors and ganglion cells) and brain (Hamada et al., 1999; Yu et al., 2002; Isorna et al., 2006; Besseau et al., 2006). Recently, AANAT2 has been detected by RT-PCR in retina and peripheral tissues in several fish species (Shi et al., 2004; Fernández-Durán et al. 2007). However, no extra-pineal *Aanat2* expression was detected by *in situ* hybridization in our study, suggesting that, if present, *Aanat2* expression outside the pineal would be much lower than in the pineal gland. Therefore, we can considerer that *Aanat2* expression measured by real time PCR on whole body larvae from sole mainly reflected *Aanat2* pineal expression.

Data obtained from our real time PCR study confirm that the pineal organ develops very early in sole, because *Aanat2* expression was detected as early as at 12 h postfertilization (0 dpf). However, these mRNAs could also have a maternal origin, as described for melatonin receptor mRNAs in eggs and embryos of Japanese quail (Obłap and Olszańska, 2001). Sole *Aanat2* expression increases significantly (3–4 fold) between 0 and 2–4 dpf suggesting a concomitant elevation in melatonin levels. This increase coincides with two important events in sole, hatching occurring at 2 dpf, and the onset of external feeding at 4 dpf. A role for photoperiod and the pineal organ in the timing of hatching has been suggested in the Atlantic halibut (Forsell et al., 1997). Furthermore, the existence of circadian feeding rhythms has been reported in developing sole (Cañavate et al., 2006). In this context, the results obtained in the present study are in agreement with the idea that melatonin could be important in hatching and feeding in developing sole.

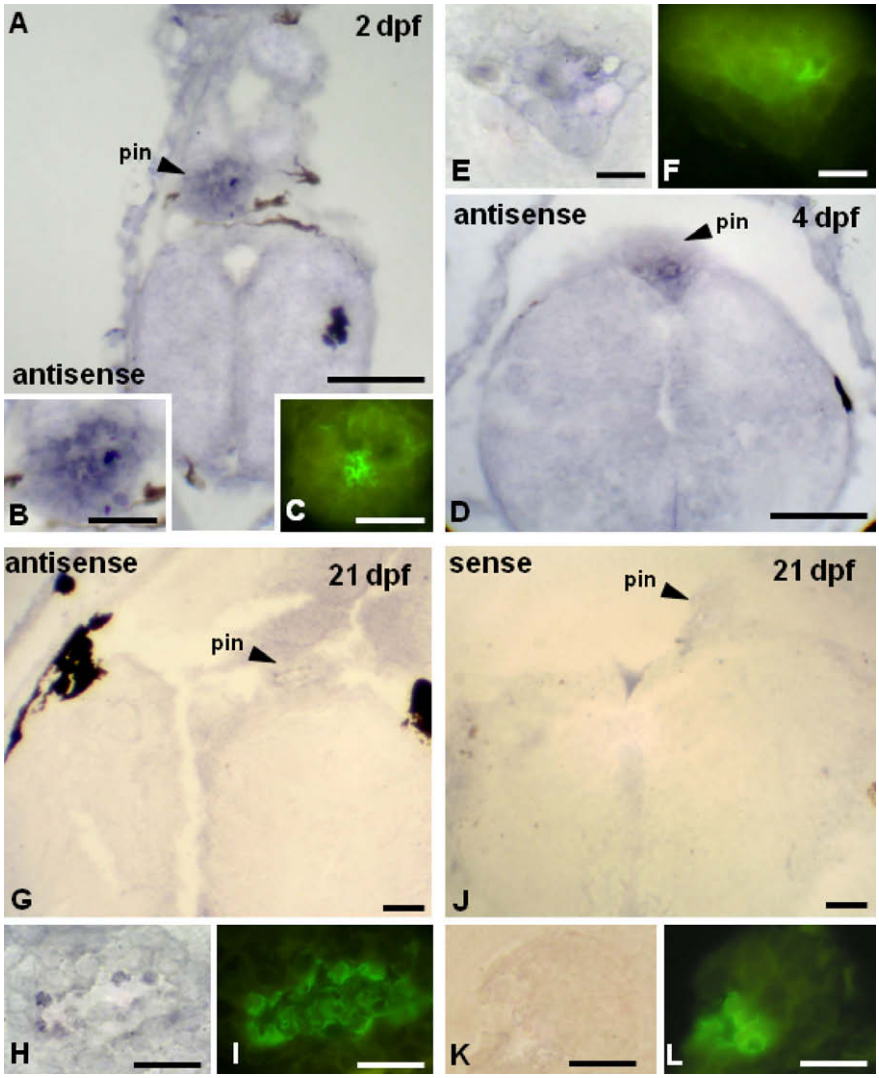

Fig. 4. Identification of *Aanat2*-expressing cells in transverse sections of the pineal organ (pin) from 2 dpf (A, B), 4 dpf (D, E) and 21 dpf (G, H) sole specimens using *Aanat2* antisense riboprobes. Control transverse section hybridized with sense *Aanat2* riboprobe showed no labeling in the pineal (21 dpf, J, K). Immunohistochemical analysis on the previously hybridized sections from 2 dpf (C), 4 dpf (F) and 21 dpf (I, L) sole specimens using cone opsin antibodies showed that *Aanat2*-expressing cells correspond to pineal photoreceptors. Bar scale represents 50 μm in A, D, G, J, 25 μm in B, C, H, I, K, L, and 10 μm in E, F.

One of the most remarkable findings of the present study was the significant down-regulation (60-fold reduction) of *Aanat2* expression just before sole metamorphosis. This change in *Aanat2* expression might implicate dramatic changes in pineal melatonin synthesis and, thus, in sole plasma melatonin levels because the pineal seems to represent the main source of circulating melatonin in most fish species (Falcón et al., 2007b) including sole (Bayarri et al., 2004). This is, to our knowledge, the first report showing a down regulation of *Aanat2* in any species during metamorphosis. Unfortunately, information on melatonin levels is not available in developing flatfishes. Melatonin has been implicated in the control of amphibian metamorphosis, antagonizing the effects of thyroid hormones, which accelerate metamorphic progress (Wright, 2002). As in anurans, thyroid hormones also promote metamorphosis in flatfish (Power et al., 2001; Marchand et al., 2004; Einarsdóttir et al., 2006), and a rise in thyroid hormone levels has also been reported during sole metamorphosis (Klaren et al., 2008; Manchado et al., 2008; unpublished own data). Taken together, these results demonstrate that the levels of pineal AANAT and thyroid hormones exhibit an inverse pattern during sole metamorphosis, which could reflect antagonizing effects of melatoninergic and thyroid systems during this important process, as previously shown in amphibians (Wright, 2002). Interestingly, antagonizing effects of melatonin and thyroid hormones have also been demonstrated in other non-metamorphic fish species (Nayak and Singh, 1987; Kulczykowska et al., 2004).

Finally, we have found daily variation in the expression of sole *Aanat2*, with lower expression during mid-day and higher expression at mid-night. This daily variation was evident from hatching time (2 dpf) to late metamorphosis (21 dpf) although it seems to be transiently lost before the onset of metamorphosis. The pineal organ of sole is already photoreceptive at 2 dpf, whereas retinal photopigments are detected later at 3 dpf (this study; El M'Rabet et al., 2008). These results suggest that the pineal organ of sole is functional and is able to transduce light information at early developmental stages, as in other fish species (Forsell et al., 1997; Kazimi and Cahill, 1999; Vuilleumier et al., 2006). Zebrafish *Aanat2* expression also displayed a conspicuous circadian rhythm as early as 2 dpf (Danilova et al. 2004; Vuilleumier et al., 2006). In contrast, no daily variation in developmental expression of *Aanat2* was observed in the turbot, another flatfish species (Vuilleumier et al., 2007). Based on this evidence, it has been suggested that the molecular mechanisms controlling the development of the pineal melatonin system have been modified during the evolution of tel-

eosts (Vuilleumier et al., 2007). However, the existence of marked differences between sole and turbot, two closely related pleuronectiform species, suggest that fish transcriptional regulation of pineal *Aanat2* during development is species-dependent.

In conclusion, here we report the full-length cDNA sequence of Senegalese sole AANAT2. The specific expression of *Aanat2* in pineal opsin-immunoreactive photoreceptor cells reinforces the idea that AANAT2 is a pineal specific fish enzyme. The rhythmic expression of *Aanat2* as early as 2 dpf and thereafter supports an important role of pineal melatonin in early development in sole. Nocturnal increase of *Aanat2* mRNA levels indicates that at least at the developmental stages studied (from embryo until late metamorphosis) a transcriptional regulation of AANAT takes place in this species. A clear *Aanat2* down-regulation before the onset of sole metamorphosis has been described here for the first time in fish, and demonstrates that, similar to anurans, flatfish melatoninergic and thyroid hormone systems have temporally inversed patterns. Whether this inverse pattern is a result of a direct antagonism between melatonin and thyroid hormones during sole metamorphosis remains to be elucidated.

Acknowledgments

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