JOURNAL OF EXPERIMENTAL ZOOLOGY (MOL DEV EVOL) 312B:231–246 (2009)

Iodothyronine Deiodinases and Thyroid Hormone Receptors Regulation During Flatfish (*Solea senegalensis*) Metamorphosis

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ABSTRACT Thyroid hormone-induced metamorphosis seems to represent an ancestral feature of chrordates (urochordates, cephalochordates and vertebrates), but also of nonchordate animals. Although thyroid hormones and thyroid hormone receptor profiles during metamorphosis have been analyzed in different vertebrate taxa, including fish, developmental expression and activity of type 2 (*dio2*, D2) and type 3 (*dio3*, D3) iodothyronine deiodinases, two key enzymes in anuran metamorphosis, remain unknown in any fish species. The aim of this work was to investigate the development of thyroid hormone system during the metamorphosis of a flatfish species, the Senegalese sole, focusing on the deiodinases developmental profile. We have cloned sole D2 and D3 and analyzed several parameters of thyroid hormones system in pre-, early-, middle-, and late-metamorphic larvae. Both deiodinases contain in their catalytic centers an UGA triplet encoding for a selenocystein (Sec) residue as expected. Left eye migration and rotation in body position were associated with a significant increase in both thyroid hormones and thyroid hormone receptors at the middle–late metamorphic stages. Although *dio2* expression slightly increased during metamorphosis, D2 activity augmentation was much more significant. Sole *dio3* expression declined only slightly, whereas the D3 activity clearly decreased at mid-late metamorphic period. This developmental profile of deiodinases sustained the rise of thyroid hormones levels observed during sole metamorphosis. No clear cut daily rhythms were observed in the parameters analyzed although it seemed that thyroid hormone system was more active during daytime, in particular at late metamorphic stages. These developmental changes point out the importance not only of thyroid hormones and their receptors but also of *dio2* and *dio3* in mediating flatfish metamorphosis, as it has been described in amphibians. *J. Exp. Zool. (Mol. Dev. Evol.) 312B:231–246, 2009*.

Grant sponsor: Spanish MICINN; Grant numbers: AGL2007-66507-C02-01; SAF2006-01319; Grant sponsor: Junta de Andalucía; Grant number: P06-AGR-01939; Grant sponsors: UPMC; CNRS; IFREMER; Grant number: GDR2821; Grant sponsors: Postdoctoral fellow of the Spanish MEC; CIBER Fisiopatología de la Obesidad y Nutrición (CIBERobn).

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Received 21 October 2008; Revised 9 February 2009; Accepted 17 February 2009

Published online 20 March 2009 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.b.21285

How to cite this article: Isorna E, Obregon MJ, Calvo RM, Vázquez R, Pendón C, Falcón J, Muñoz-Cueto JA. 2009. Iodothyronine deiodinases and thyroid hormone receptors regulation during flatfish (*Solea senegalensis*) metamorphosis. J. Exp. Zool. (Mol. Dev. Evol.) 312B:231–246.

The role of thyroid hormones in dramatic developmental changes seems to be an early and conserved characteristic in evolution because these hormones have been involved in the metamorphosis of echinoderm (Heyland et al., 2004), tunicate (Patricolo et al., 2001), amphioxus (Paris et al., 2008), lamprey (Manzon et al., '98, 2001), flatfish (Inui et al., '89; Power et al., 2001) and amphibian (Brown and Cai, 2007) larvae. Therefore, the study of the molecular events associated with metamorphosis in animal groups exhibiting distinct evolutionary trends appears necessary to throw light on this relevant process. As metamorphosis within vertebrates has been much less studied in flatfish that in their anuran counterparts, information about the role of iodothyronine deiodinases in flatfish species is hardly available despite the fact that these enzymes play an essential role in anuran metamorphosis (Brown, 2005; Brown and Cai, 2007).

The transition from larva to juvenile in flatfish is considered as a real metamorphosis that implies a series of regulated processes involving tissue differentiation, biochemical, molecular and physiological changes (Power et al., 2001; Martínez and Bolker, 2003). The most striking manifestation of these processes is the transition from bilaterally symmetric pelagic larvae to asymmetric benthic juveniles, in which both eyes appear on the same, pigmented side of the body (Fernández-Díaz et al., 2001; Power et al., 2001; Martínez and Bolker, 2003). As in anuran amphibians, thyroid hormones drive this physiological process in flatfish (Power et al., 2001; Blanton and Specker, 2007). Endogenous T4 concentrations increase during metamorphosis in the Japanese flounder (*Paralichthys olivaceus*), the summer flounder (*P. dentatus*), the Atlantic halibut (*Hippoglossus hippoglossus*) and the Senegalese sole (*Solea senegalensis*) (Miwa et al., '88; Schreiber and Specker, '98; Einarsdóttir et al., 2006; Klaren et al., 2008). Besides, thyroid hormone or thyroid-stimulating hormone treatments stimulate, whereas exposure to thyroid hormone inhibitors retard flatfish metamorphosis (Miwa et al., '88; Inui et al., '89; Schreiber and Specker, '98; Blanton and Specker, 2007; Manchado et al., 2008). Thyroid hormone actions are mediated through thyroid hormone receptors (TRs), which are members of the nuclear receptor super family, and act as hormone-regulated transcription factors. As well as thyroid hormones, the expression of these receptors increases during metamorphosis in the Japanese flounder, the turbot (*Scophthalmus maximus*), the Atlantic halibut and the Senegalese sole (Yamano and Miwa, '98; Marchand et al., 2004; Galay-Burgos et al., 2008; Manchado et al., 2009). These data are in agreement with the idea that flatfish and anuran metamorphosis are regulated by similar molecular and physiological mechanisms.

Thyroid hormones are activated and inactivated by deiodination, a stepwise removal of iodine from their outer or inner rings performed by iodothyronine deiodinase enzymes in peripheral target tissues (Bianco et al., 2002; Orozco and Valverde, 2005; Blanton and Specker, 2007). Three types of iodothyronine deiodinases, D1, D2 and D3, have been identified in vertebrates, including fish (Bianco et al., 2002; Orozco and Valverde, 2005; Blanton and Specker, 2007; EC numbers 1.97.1.10 and 1.97.1.11). D2 catalyzes the outer ring removal of an iodine residue from T4 giving active T3. D3 can inactivate T4 and T3 by removing an iodine molecule from the inner ring of the iodothyronines, thereby producing reverse T3 (rT3) and 3′,3-T2, respectively. D1 is able to catalyze both outer ring and inner ring deiodinations, but its preferential substrate is rT3 and sulphated iodothyronines (Bianco et al., 2002; Orozco and Valverde, 2005; Blanton and Specker, 2007). D2 and D3, acting at targeted local levels, play an important role in the sequential timing of metamorphic changes in amphibians (Brown, 2005; Brown and Cai, 2007). This study was aimed at getting insight into the role of thyroid hormones, and, more precisely, of deiodinases during metamorphosis of a flatfish. Here, we report the variations of D2 and D3 activities as well as of T3 and T4 levels at four different

stages of the Senegalese sole (*S. senegalensis*) metamorphosis. We also investigated the expression profile of sole *dio2, dio3, TRαA and TRβ,* which had been cloned for this purpose. Finally, as light conditions and photoperiod have been implicated in the modulation of thyroid system and metamorphosis (Gomez et al., '97; Leiner and MacKenzie, 2003; Parmentiern et al., 2004; Cañavate et al., 2006; Watanabe et al., 2007), we have analyzed all these parameters at different times of the day and pooled the values for a daily mean.

MATERIAL AND METHODS

Animals and sampling

Cloning experiments: 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 (Puerto Real, Spain). Animals anesthetized with phenoxyethanol (SIGMA, St Louis, MO) were sacrificed during daytime and brains were quickly removed, frozen in liquid nitrogen and stored at −80°C until used. Animals were treated in agreement with the European Union regulation concerning the protection of experimental animals.

Developmental studies: Senegalese sole fertilized eggs were obtained in May 11, 2006 (0 days post-fertilization or 0 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 at a density of 100 eggs/L in 600 L cylinder conical tanks with gentle aeration at natural light environmental conditions (sunrise 07:31 hr, sunset 21:22 hr). Water, which was pumped from a salt well without need of additional filtering, had a stabilized temperature and salinity of 19 ± 1°C and 39 ppt, respectively. After hatching, larvae were transferred to an 800 L tank at a density of 40 individuals/L. Larvae were fed rotifers (*Brachionus plicatilis*) enriched with *Isochrysis galbana* from 4 to 10 dpf. The density of rotifers was gradually increased from 5 to 10 rotifers/mL. Larvae were fed *Artemia franciscana* metanauplii from 9 dpf with an initial density of 0.1 *Artemia*/mL. This density was gradually increased to 10 *Artemia*/mL at the end of the rearing period. Food was administered at noon during all the experimental procedure. Animals were sampled at four stages of development: pre-metamorphic stage (12 dpf), early metamorphosis (15 dpf), middle metamorphosis (19 dpf) and late metamorphosis (21 dpf). At each developmental stage, several pools of animals were obtained at four different points of the day (local time expressed as Greenwich meridian time +2 hr): 14:30, 19:30, 02:30 and 07:30 hr, which correspond to Zeitgeber Time (ZT) 7, 12, 19 and 24, respectively. The purpose of sampling at different times of the day was to ensure that developmental differences were not hidden by putative daily differences. Pools contained 10–20 specimens at pre- and early metamorphic stages (12–15 dpf) and 5–10 specimens at middle–late developmental stages (19–21 dpf). To follow the progress of the metamorphic process, larvae were photographed under microscope at each sampling day. As the onset of metamorphosis is not age but size-determined, and development within a tank or batch of fish can vary widely (Fernández-Díaz et al., 2001; Einarsdóttir et al., 2006), we have sampled animals at the same developmental stage measured as their position in the water column. Therefore, the majority of pooled animals had developed as indicated in Figure 1. Samples were frozen in liquid nitrogen and stored at −80°C until used.

Thyroid hormones determinations

High specific activity [^{125}I]-T4, [^{131}I]-T4 and [^{125}I]-T3 ($\approx$3000 μCi/μg) were synthesized in our laboratory using T3 and 3,5-T2 as substrates, as previously described (Morreale de Escobar et al., '85). These radiolabeled hormones were used for the determination of D2 activity and for the highly sensitive T4 and T3 radioimmunoassays (RIAs). For D3 activity [^{125}I]-T3 from NEN (Perkin-Elmer Life & Analytical Sciences, Boston, MA) was used.

Thyroid hormone levels were determined using highly sensitive and specific RIAs as previously described (Morreale de Escobar et al., '85) with minor modifications (Calvo et al., 2002). If necessary, samples from the same developmental stage were pooled. Pools of larvae were homogenized in ice in 5 volumes of buffer (0.32 M Sucrose, 10 mM Hepes, pH 7.0) and a portion was separated to measure deiodinases activities. [^{131}I]-T4 and [^{125}I]-T3 were added to each sample as internal tracers for recovery calculations. Part of these homogenates were extracted twice with methanol and further purified on AG 1×2 resin columns (Bio-Rad Laboratories, Inc., Richmond, CA). The iodothyronines were eluted with 70% acetic acid, evaporated to dryness and extensively counted for the calculation of individual recoveries. RIA buffer was then added, and the iodothyronine contents were determined in duplicate. Blank tubes (with

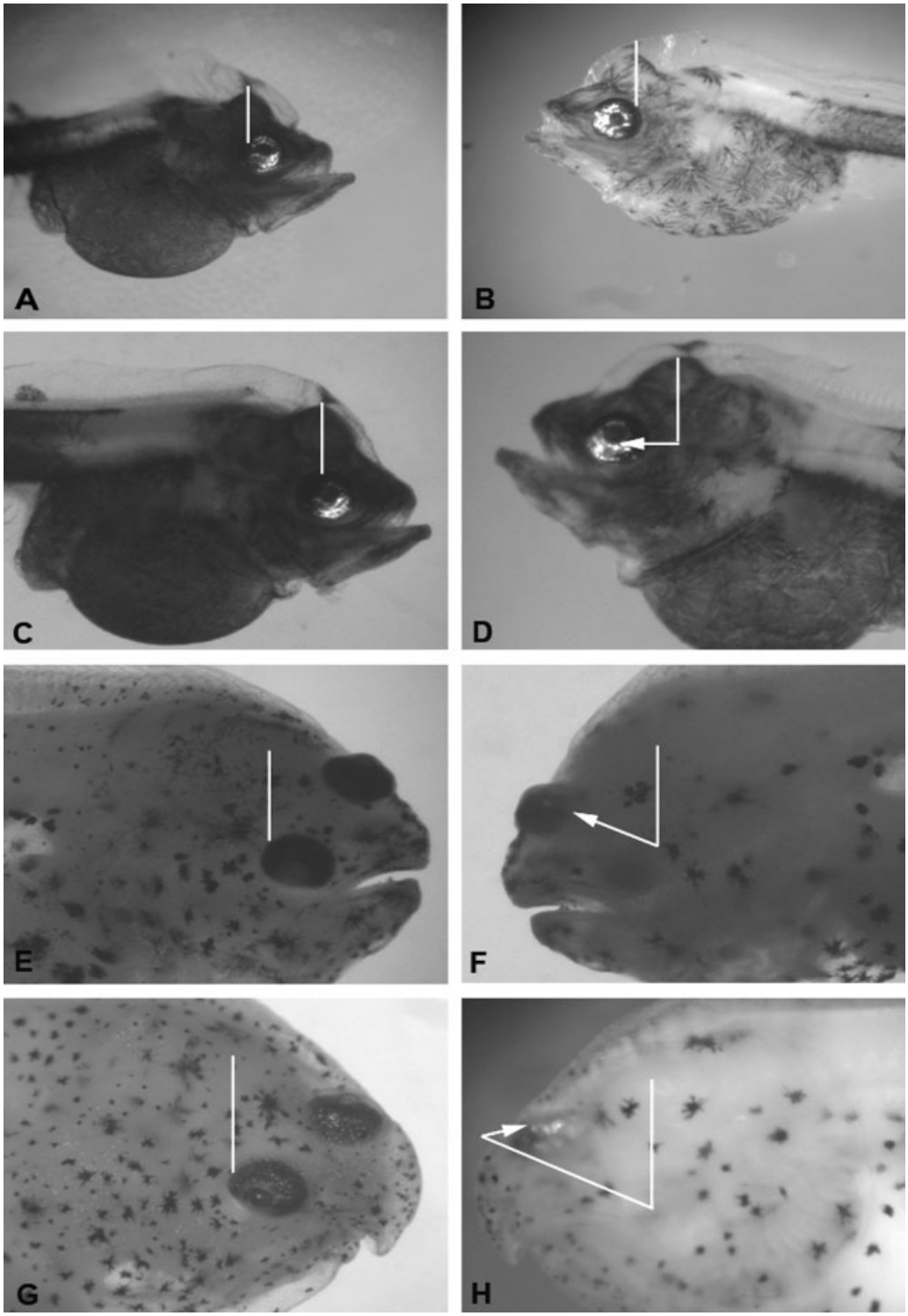

Fig. 1. Metamorphosis of Senegalese sole. Animals were maintained as indicated in the section ''Material and Methods.'' Specimens from days 12 (**A**, **B**), 15 (**C**, **D**), 19 (**E**, **F**) and 21 (**G**, **H**) post-fertilization are represented from the top to the bottom. The right side of animals (which face upward at the end of the metamorphosis) is shown in the left column. The left side of the animals (facing the bottom at the end of the metamorphosis) is shown in the right column. Left eye migration and rotation in body position can be observed across development. The dashed lines represent left eye migration in relation to the position of the right eye. Magnification: 18× .

no sample) were run in parallel during the whole process. The limits of detection of the assay were 2.5 pg for T4 and 0.75 pg for T3. Concentrations were calculated using the amounts of T4 and T3 found in the RIAs, the recovery of the tracers added and the protein content of the samples extracted. T3 concentrations at 12 dpf were very close to the blank values and were considered near the limits of detection.

Cloning of sole thyroid hormone receptors and type 2 (dio2) and type 3 (dio3) deiodinases

Total RNA was extracted from two adult brains using the Trizol® method (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions, and mRNA was purified from total RNA (150 μg) using oligo(deoxythymidine) beads (DYNAL®; Biotech, Oslo, Norway). The mRNA was reverse transcribed using SMART™ RACE cDNA kit (BD Bioscience, Clontech, Palo Alto, CA). This cDNA was used as template in the polymerase chain reaction (PCR) for the partial cloning of the four genes (sole *TR*α, *TR*β, *dio2* and *dio3*) and 3′RACE experiments using Clontech Advantage™ (BD Bioscience, Clontech, Mountain View, CA) polymerase. Degenerated primers (see Table 1) were designed from known thyroid hormone receptors and deiodinase sequences available from the public databases. Amplified products were purified (Nucleo Spin®, Machery-Nagel, Hoerdt, France) and subcloned in PGEM-T EASY vector (Promega, Charbonnières, France) following commercially available protocols. The vector containing the amplified insert was electroporated into bacteria from which several positive clones were obtained and sequenced.

The 3′-untranslated region (3′-UTR) sequence of Senegalese sole *TR*α, *TR*β, *dio2* and *dio3* were obtained by 3′-RACE after construction of a cDNA library using the SMART™ RACE cDNA kit Luc (BD Bioscience, Clontech, Palo Alto, CA) and specific primers designed in the fragments of specific sequences cloned (Table 1). The amplified products were subcloned, amplified and sequenced as indicated above.

The 5′-UTR sequence of *dio3* was obtained using another strategy. Total sole brain RNA (2.8 μg)

TABLE 1. Primer sequences used for cloning sole type 2 and type 3 deiodinases and thyroid hormone receptors

1A	
*dio*2F1	5′-ATYTGCCYGKYTTCTTCTCCAACTG-3′
*dio*2R2	5′-GCIAYGTTRGCRTTRTTRTCCATGC-3′
*dio*2F2	5′-TSCGSTBCRTHTGGAACAGCTT-3′
*dio*2R3	5′-GGKTGWGCCTCRTCRATGTAGACCA-3′
*dio*3F1	5′-TSCTSYTKCCYCGBTTCCT-3′
*dio*3R1	5′-GTAKCCCTCYGGMCCCCKGCCCCCCTGGTA-3′
*dio*3F2	5′-AYGRICARAARYTGGACTTYYTCAA-3′
dio3R2	5′-TAVGGSGCRTCYGWGCTSACCCAGC-3′
TRαF4	5′-CTGTTCMWGGRTAYRTYCCCA-3′
TRR2	5′-TCRAASACYTCBAGGAAGAG-3′
TRαF7	5′-STGGAARCAGAARCGCAA-3′
TRαR3	5′-ACACTCCACCTTCATGTG-3′
TRβF3	5′-CATKCARMATGGGTACAT-3′
TRR2	5′-TCRAASACYTCBAGGAAGAG-3′
TRβF6	5′-GCHCARGGCAACCACTGGAA-3′
TRβR4	5′-CGCAGGTCYGTCACYTTCAT-3′
1B	
ssdio2F1	5′-ACCAAACTCTAAGGTGGTGGTGGTG-3′
ssdio2F2	5′-ATTTTGAGTCATTAGACCGCCCTCTG-3′
ssdio3F1	5′-GATCCTGGACTGCATGAAAGGGAAG-3′
ssdio3F3	5′-ACTTTGGCAGCTGCTCCTGACCGCCATTCAT-3′
ssdio3R1	5′-CGGATGCGCCTCCTCGATATATACAACTAA-3′
ssdio3R3	5′-ATGAATGGCGGTCAGGAGCAGCTGCCAAAGT-3′
ssdio3R4	5′-TCGCTTCCCTTTCATGCAGTCC-3′
ssTRαF1	5′-AGTCTGGCTCAGTTCAACCTAGATGA-3′
ssTRαF2	5′-AAGCTGCTGATGAAGGTAACAGACCT-3′
ssTRβF1	5′-GTGGACATAGAGGCCTTCAGTCAGTTTAC-3′
ssTRβF2	5′-GCTGTCCTCCTTTAACTTGGACGACTC-3′

was retro-transcribed at 45°C using the specific primer ss *dio3*R1 (Table 1) and the "Reflection Kit" (Active Motif, Carlsbad, CA). After treating with ribonuclease H (Invitrogen, Prat de Llobregat, Spain), cDNA was purified (Centri Sep columns, Princeton Separations, Freehold, NJ) and a poly-A tail was added in the 3′ hydroxyl terminus (Terminal deoxynucleotidyl Transferase, Amersham, Biosciences, Uppsala, Sweden). This cDNA was employed as template in 5′RACE PCR experiments using an "AmpliTaq Gold®" polymerase (Applied Biosystems, Carlsbad, CA), *Not*I-oligodT$_{(@)}$ and specific *dio3* primers from sole (Table 1). The amplified products were subcloned, amplified and sequenced as indicated above. To ensure the identification, the sequences obtained were aligned with deiodinase DNA sequences from various vertebrate species available in Gene Data Bank.

Quantitative real time PCR analysis

Total RNA was extracted individually from larval pools using "EUROzol" (EuroClone, Milan, Italy) according to the manufacturer's instructions. Total RNA (1 μg) was retro-transcribed using a kit that eliminates genomic DNA contaminations (QuantiTect® Reverse Transcription Kit, Quiagen, Madrid, Spain). Real time gene expression analysis was performed in a Chromo 4TM Four-Color real time System (Biorad). PCR reactions were developed in a 25 μL volume using cDNA generated from 1 μg of RNA, iTaq™ SYBR® Green Supermix with ROX (Biorad, Alcobendas, Spain) and specific primers (0.4 μM, Table 2). Standard curves were generated with serial dilutions of cDNA transcribed from 1 μg of total RNA. All calibration curves exhibited slopes close to −3.32 and efficiencies around 100%. The conditions of PCR reactions were almost similar for the five genes analyzed: 3 min at 95°C, 30 sec at 95°C, 45 sec at 56°C (60°C for TRα) and 45 sec at 72°C. The number of cycles was 30 for β *actin* and 36–37 for, *dio2, dio3*, TRα and TRβ. Duplicate samples of each gene were analyzed in the same reaction. PCR products obtained were run in agarose gels and sequenced to ensure the specificity of the amplification. Besides, melting curves were analyzed for each sample, in order to make certain that only a single sequence was amplified. β-*actin* was used for normalization (GenBank accession number DQ485686). Negative controls included replacement of cDNA by water and the use of nonretro-transcribed total RNA. The ΔΔCt method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression.

TABLE 2. Primers used in real time quantitative PCR experiments

ssdio2Fq	5′-GGGACGAATGCCATCTTCTG-3′
ssdio2Rq	5′-GGTGGCTGAGCCAAAGTTG-3′
ssdio3Fq	5′-TCATCAGGGATGAGAGGGTG-3′
ssdio3Rq	5′-CCTCAGCCCGGATATTCTGT-3′
ssTRαFq	5′-ACTACATCAACCATCGCAAGC-3′
ssTRαRq	5′-CGATCATTCGCAGGTCTGTT-3′
ssTRβFq	5′-TGGAGCAACATTCCTCTGGT-3′
ssTRβRq	5′-TTGTTGTGGCCTTTTCACCT-3′
ssβactinFq	5′-GGATCTGCATGCCAACACTG-3′
ssβactinRq	5′-TCTGCATCCTGTCAGCAATG-3′

Outer ring deiodination activity (D2 activity)

D2 activity was measured in the same homogenates as those used to quantify thyroid hormones concentrations, after dilution to 1:10 in buffer+ DTT, to get 10 mM DTT. Before each assay [^{125}I]-T4 was purified by paper electrophoresis to separate the contaminating iodine. The amount of iodine in the blanks was always lower than 0.5–1% of the total radioactivity. D2 activity was determined as described previously (Ruiz de Oña et al., '88; Obregón et al., '91) using 100.000 cpm [^{125}I]-T4, 2 nM T4, 1 μM T3, 20 mM DTT and 1 mM propylthiouracil in 0.1 M potassium phosphate buffer (pH 7.0), 50–100 μg protein/100 μL total volume during 1 hr at 24°C. The ^{125}Iodine released was separated on Dowex 50W-X2 columns equilibrated in 10% acetic acid. The protein content was determined after precipitation of the homogenates with 10% trichloro-acetic acid to avoid interferences from DTT in the colorimetric reaction. Results were expressed in fmols/hr/mg protein.

Inner ring deiodination activity (D3 activity)

D3 activity was measured in the same homogenates used to quantify thyroid hormones (1:5), further diluted about 1:10 in buffer+10 mM DTT, to have 30–50 μg protein per assay tube. D3 activity was determined as previously described (Hernandez et al., 2006) using 200.000 cpm [^{125}I]-T3, containing 2 nM T3, 20 mM DTT and 1 mM propylthiouracil in 0.1 M potassium phosphate buffer (pH 7.0), an aliquot of the homogenates containing 30–50 μg protein/50 μL total volume during 1 hr at 37°C. The [^{125}I]-3,3′-diiodothyronine produced was separated from [^{125}I]-T3 by paper chromatography as described (Bellabarba et al., '68) and the T3 and 3,3′-T2 spots identified by autoradiography, counted and the results expressed as %

of 3′-3-T2 produced from T3 in fmols/hr/mg protein. The amount of iodine in the blanks was about 2% of the total radioactivity.

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/). The existence of putative SECIS elements in the 3′-UTR sequences of sole *dio2* and *dio3* were studied using the SECISearch 2.19 software (http://genome.unl.edu/SECISearch.html).

Developmental or daily statistical differences among groups were determined using one-way ANOVA following by a multiple contrast of range test (LSD). If necessary, values were transformed (logarithmic transformation) to get a normal distribution and homogeneity of variances (only for *dio3* expression). All statistical tests were made using the Statgraphics software.

RESULTS

Morphological changes during sole development

Under our rearing conditions pre-metamorphic 12 days post-fertilization (dpf) animals swim on the top of the water column (Fig. 1A and B). Left eye migration started at 15 dpf, and the larvae initiated migration down in the water column (early metamorphosis, Fig. 1C and D). The rotation in body position was clear at 19 dpf. At this stage the left eye appeared at the level of the dorsal line and the animals settled at the down of the tanks (middle metamorphosis, Fig. 1E and F). At 21 dpf both eyes were positioned in the same (right) side of the body, which then faced upwards (the left side facing the bottom), and the larvae were clearly benthic (late metamorphosis, Fig. 1G and H). Other modifications affected the mouth, opercular orifices, fins and pigmentation (Fig. 1).

Cloning of Senegalese sole dio2 and dio3

The cloning strategy used allowed us to obtain a 711 bp sequence of the Senegalese sole *dio2*, which has a small 3′-UTR with no selenocystein insertion sequence (SECIS) structure. The deduced 205 aa translated sequence (GenBank accession number AM902723) displays 83, 78 and 74% of identity with *Fundulus heteroclitus* (AAB39651), *Oncorhynchus mykiss* (AAL25653) and *Danio rerio* (NP997954) D2 proteins, respectively. The full-length *dio3* sequence is 1,638 bp in length (GenBank accession number AM902722). The deduced 268 aa protein sequence also displays high identity/similarity with D3 cloned from other fishes (89/95% with *Oreochromis niloticus* [CAA71997.1], and 67/80%, with *Neoceratodus forsteri* [AAR05175.1], respectively). The phylogenetic tree built after a comparative analysis of sequences further confirmed that the two sequences isolated were representative of *dio2* and *dio3* deiodinases (Fig. 2).

The characteristic TGA triplet codifying for a selenocystein is present at the expected position

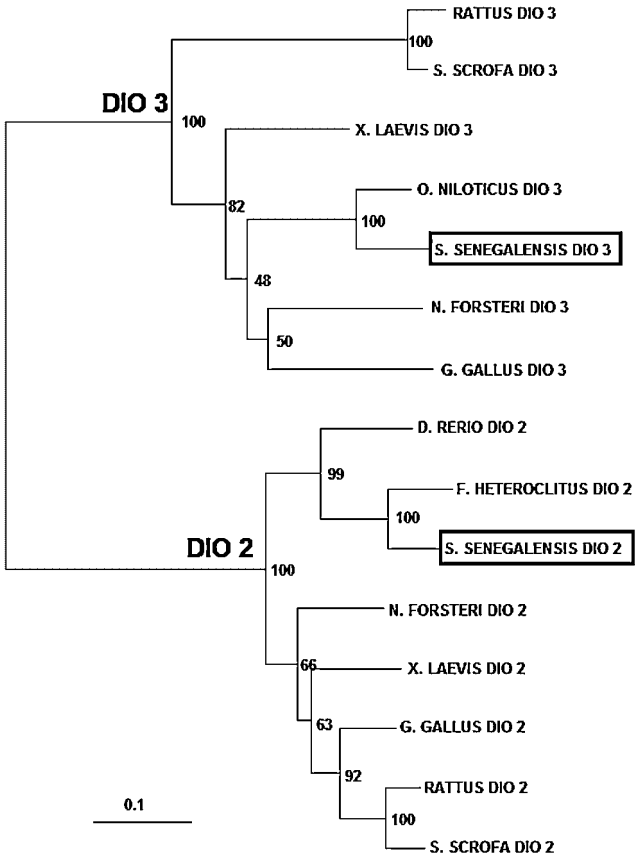

Fig. 2. Phylogenetic unrooted tree showing the interrelationships of the different iodothyronine deiodinases sequences. Each of the sole cloned deiodinase fits into one category closely linked to those of the other *dio2* and *dio3* available sequences. Sequences used are published in the GenBank: *D. rerio* D2 (BC059608); *F. heteroclitus* D2 (U70869); *G. gallus* D2 (NM_204114) and D3 (NM_001122648); *N. forsteri* D2 (AF327438) and D3 (AY339982); *O. niloticus* D3 (Y11111); *R. rattus* D2 (AB011068) and D3 (U24282); *S. scrofa* D2 (AY533207) and D3 (AY533208); *S. senegalensis* (present data) D2 (AM902723) and D3 (AM902722); *X. laevis* D2 (AF354707) and D3 (BC106400).

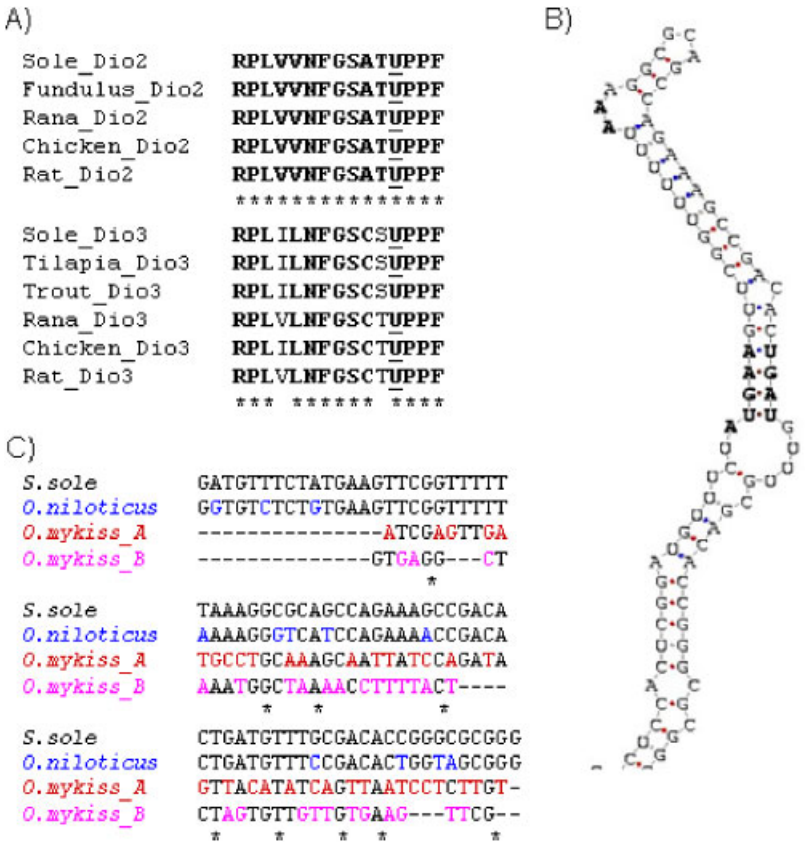

Fig. 3. (**A**) Catalytic center of D2 and D3 deiodinases in several species. Amino acid sequence identity of the active catalytic centers of the deduced amino acid sequences of the D2 and D3 deiodinases of several representative vertebrate species (sole: present work; tilapia, frog, chicken, rat: Bianco et al., 2002; trout: Bres et al., 2006). Common amino acids are indicated in bold letters. Sec residue is underlined. (**B**) Hypothetic SECIS structure of the sole *dio3* (SECISearch 2.19 software available in http://genome.unl.edu/SECISearch.html). Dots indicate non-Watson–Crick interactions. (**C**) Aligment of *dio3* SECIS elements of three fish species: *S. sole* (present work); *O. niloticus* (Y11111); *O. mykiss* (Bres et al., 2006). Nucleotide identity of the different sequences with sole *dio3* is indicated by the same color as the name of the sequence. The asterisks indicate common nucleotides for the four sequences.

when compared to *dio2* and *dio3* sequences from other vertebrates (Fig. 3A). Sole D2 catalytic center has a 100% identity with the consensus sequence deduced from rat, chicken, frog and *Fundulus* D2 (Fig. 3A). The active catalytic center of D3 displays 100% identity among fish species and 87–93% identity with other vertebrate species (Fig. 3A). The length of the sole 3′-UTR sequence of *dio3* is 710 bp and it possesses a putative SECIS sequence that presents high identity with tilapia *dio3* and a lower identity with trout SECIS structures (Fig. 3B and C).

Cloning of thyroid hormone receptors

The same strategy and the same cDNA employed for *dio2* and *dio3* cloning were used to obtain partial sequences of thyroid hormone receptor α (*TR*α) and thyroid hormone receptor β (*TR*β) (data not shown). During the progress of this study several sequences of *S. senegalensis* TRα and TRβ have been published by another group (Manchado et al., 2009). In this article, the authors described the existence of two TRα genes (TRαA and TRαB) and one TRβ gene, as well as several splice isoforms of TRαB and TRβ forms that represent truncated thyroid hormone receptors. The partial sequences of TRα and TRβ cloned in our study were 732 and 912 bp in length, and have 100% of identity in the ORF with TRαA (GenBank accession number AB366000) and TRβ (GenBank accession number AB366001) available sequences, respectively. However there are some differences in the final part of the 3′-UTR between the TRs sequences cloned in our laboratory and the sole TRs previously published by Manchado

et al. (2009). Therefore, we have called our sequences ssTRαA2 and ssTRβ2.

Thyroid hormone levels

Mean T4 levels were low in pre- and early-metamorphic larvae (12 and 15 dpf), and increased significantly in middle and late metamorphic stages (19 and 21 dpf) (Fig. 4A). A significant decrease in T4 levels was observed between 19 and 21 dpf (Fig. 4A).

T3 was undetectable at 12 dpf; levels increased from 15 to 19 dpf and decreased slightly thereafter (Fig. 4B). T3 values were four fold lower than those measured for T4 although they followed parallel developmental patterns (Fig. 4B, 9).

Expression of thyroid hormone receptors

The forward and reverse primers used in this study to analyze TRαA2 expression were present in the ORF of TRαA but not in TRαB sequence cloned by Manchado et al. (2009), whereas primers used to quantify TRβ expression were located in 3′-UTR and were specific from this sequence (see Table 1). These facts, together with sequencing of PCR products and analysis of melting curves, support that we are determining the expression of single TRαA and TRβ sequences. Thyroid hormone receptor expression increased around 3.5 fold (TRαA2) or 7 fold (TRβ2) from pre-metamorphosis (12 dpf) to metamorphic climax (19 dpf). At this stage, both TRαA2 and TRβ2 presented a peak (Fig. 5A and B) coinciding with the highest T4 and

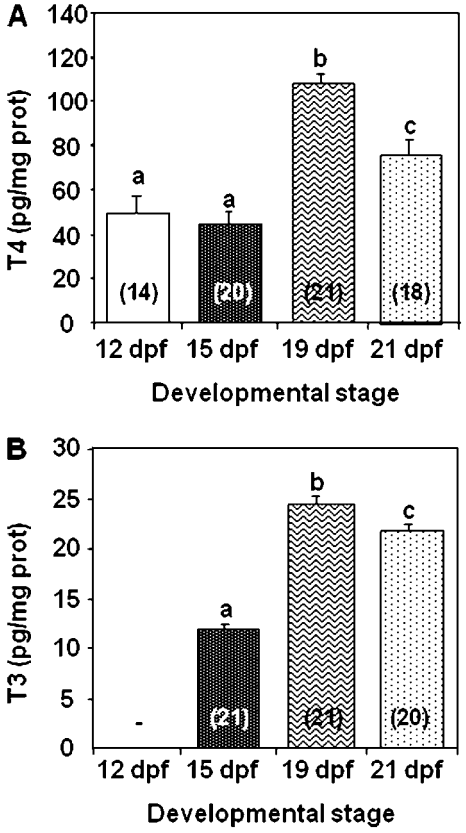

Fig. 4. T4 (**A**) and T3 (**B**) concentrations at different stages of the metamorphosis in the Senegalese sole. Thyroid hormones were measured by radioimmunoassay on pools of animals. Data are shown as the mean ± SEM (n = 14–21; the number inside parenthesis indicates the sample size for each group). T3 levels at 12 dpf were below the limit of detection of the assay. Differences among groups at different stages were analyzed by a one-way ANOVA followed by the LSD post hoc test (A: $F = 25.393$, $P < 0.0001$ for T4; B: $F = 88.71$, $P < 0.0001$ for T3). There are no statistical differences among groups that share common letters.

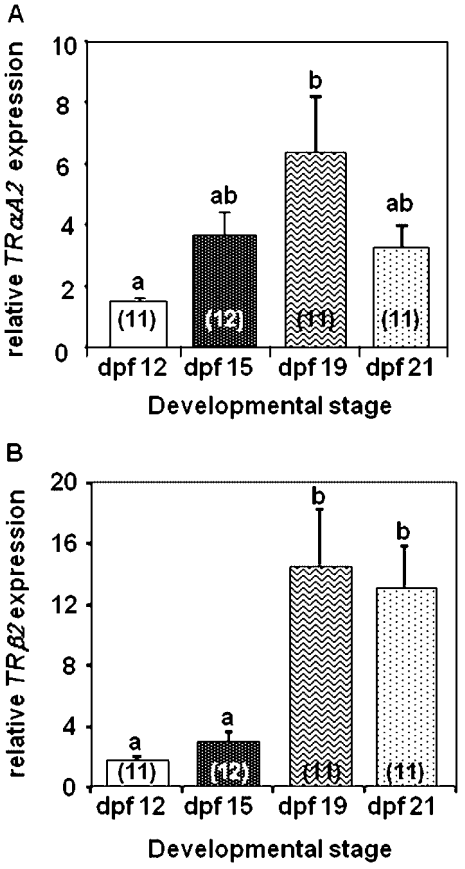

Fig. 5. *TR*αA2 (**A**) and *TR*β2 (**B**) expression at different stages of the metamorphosis in the Senegalese sole. The mRNA abundance was measured by real time PCR on pools of animals. Data are shown as the mean ± SEM (n = 11–12; the number inside parenthesis indicates the sample size for each group). Developmental differences were analyzed by one-way ANOVA (A: $F = 4.845$, $P < 0.01$; B: $F = 11.96$, $P < 0.001$) followed by the LSD post hoc test. There are no statistical differences among groups that share common letters.

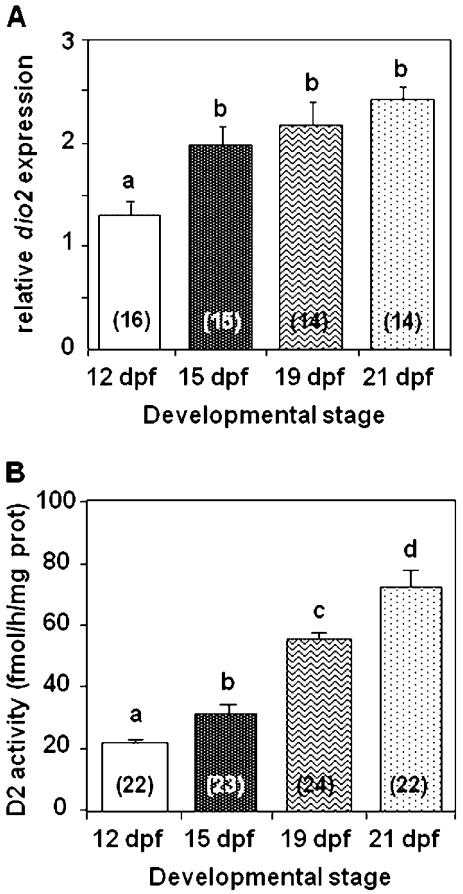

Fig. 6. (**A**) *Dio2* expression at different stages of the metamorphosis in the Senegalese sole. The mRNA abundance was measured by real time PCR on pools of animals. (**B**) D2 activity at different stages of the metamorphosis in the Senegalese sole. Enzymatic activity was measured using ^{125}I-T4 as substrate on pools of animals. Data are shown as the mean ± SEM (A, $n = 14$–16 and B, $n = 22$–24; the number inside parenthesis indicates the sample size for each group). Differences among groups were analyzed by one-way ANOVA followed by the LSD post hoc test (A: $F = 8.891$, $P < 0.001$; B: $F = 53.949$, $P < 0.0001$). There are no statistical differences among groups that share common letters.

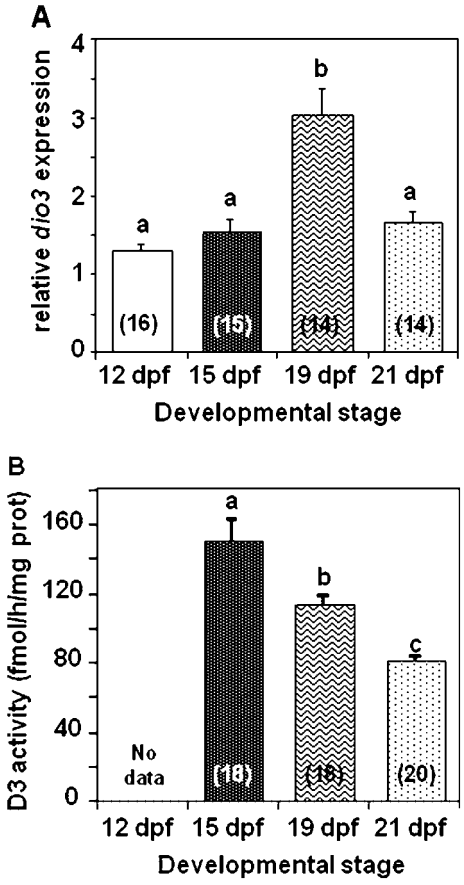

Fig. 7. (**A**) *Dio3* expression at different stages of the metamorphosis in the Senegalese sole. The mRNA abundance was measured by real time PCR on pools of animals. (**B**) D3 activity (IRD) activity at different stages of the metamorphosis in the Senegalese sole. Enzymatic activity was measured using [^{125}I]-T3 as substrate on pools of animals (see Material and Methods). Data are shown as the mean ± SEM (A, $n = 14$–16 and B, $n = 18$–20; the number inside parenthesis indicates the sample size for each group). Differences among groups were analyzed by one-way ANOVA (A: $F = 15.926$, $P < 0.0001$; B: $F = 13.92$, $P < 0.0001$) followed by the LSD post hoc test. There are no statistical differences among groups that share common letters.

T3 levels. However, though TRαA2 abundance declined at 21 dpf (Fig. 5A), TRβ2 transcript remained elevated during late metamorphosis (Fig. 5B).

Dio2 mRNA expression and D2 ORD activity during sole metamorphosis

Mean *dio2* mRNA levels were statistically lower at 12 dpf than at 15 dpf, and remained high at the other stages investigated (Fig. 6A). The elevation in *dio2* expression correlated with D2 activity during sole metamorphosis, although D2 activity increase was more pronounced at 19 and 21 dpf than at early stages (Fig. 6B). Thus, *dio2* expression exhibited a two-fold increase from pre-metamorphosis to late metamorphosis, whereas D2 activity rises around 3.5 times (Fig. 6).

Dio3 expression and D3 activity (D3 IRD) activity during sole metamorphosis

Mean *dio3* expression was higher at metamorphic climax (19 dpf) than at the other stages investigated indicating that there is a transient increase and a subsequent decrease in expression along metamorphosis (Fig. 7A). D3 activity decreased significantly from 15 to 21 dpf. At late metamorphosis the D3 activity was only a half of what it was at early metamorphosis (Fig. 7B).

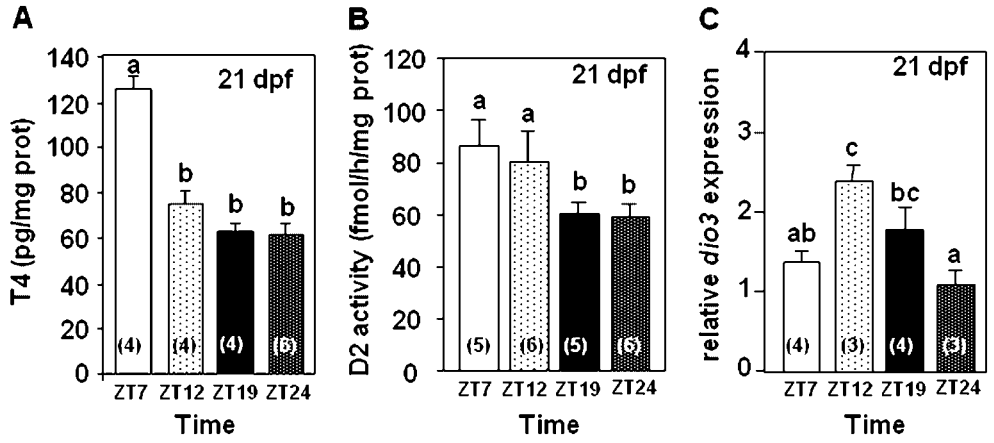

Fig. 8. T4 concentrations (**A**), D2 activity (**B**) and *dio3* expression (**C**) in late metamorphic (21 dpf) Senegalese sole specimens at different zeitgeber times. Data are shown as the mean ± SEM (A, $n = 4–6$; B, $n = 5–6$; C, $n = 3–4$; the number inside parenthesis indicates the sample size for each group). Differences among groups were analyzed by one-way ANOVA (A: $F = 4.193$, $P < 0.001$; B: $F = 5.056$, $P < 0.001$; C: $F = 7.44$, $P < 0.01$) followed by the LSD post hoc test. There are no statistical differences among groups that share common letters.

Daily variations in thyroid hormone system and deiodinases during sole metamorphosis

As it is indicated in material and methods, the thyroid hormone system and deiodinase expression and activity from sole were analyzed at different times of the day. The different parameters analyzed did not show consistent daily variations at most developmental stages analyzed. However, significant daily variations for some parameters (T4, D2 activity, *dio3* expression) were observed at late metamorphosis (21 dpf, Fig. 8). Late metamorphic larvae display T4 levels twice higher at midday (ZT7) than at nighttime (ZT19, ZT24) (Fig. 8A). D2 activity was also higher at ZT7 and ZT12 (daytime) than at ZT19 and ZT24 (nighttime) (Fig. 8B). In turn, *dio3* expression increased significantly from ZT7 to ZT12 (afternoon) and then decreased from ZT12 to ZT24 (Fig. 8C). No significant daily differences were detected at late metamorphosis for the other parameters analyzed (data not shown).

DISCUSSION

In this study, we have reported the cloning of sole type 2 and type 3 deiodinases and provided original information on their developmental expression and activity, together with concomitant changes in other parameters of the thyroid hormone system. This developmental pattern during sole metamorphosis agrees with that described in amphibians (Brown and Cai, 2007), suggesting that not only thyroid hormones levels, but also thyroid hormone receptor and deiodinase profiles have shared characteristics during flatfish and amphibian metamorphosis.

Dio2 and dio3 sequences

The analysis of the cloned sequences and their comparison with other available sequences reveal that they encode sole D2 and D3 proteins, respectively (Sambroni et al., 2001; Orozco et al., 2002; Sutija et al., 2004; Bres et al., 2006). Both deduced amino acid sequences include a selenocystein (Sec) residue in their respective catalytic cores, a characteristic feature of vertebrates' deiodinases (Bianco et al., 2002; Blanton and Specker, 2007). The sequence of sole *dio3* also includes a Sec insertion sequence (SECIS) in the 3′-UTR. A eukaryotic SECIS element present in the 3′-UTR of selenoproteins (including iodothyronine deiodinases) is the signal that recodes the in-frame UGA from a STOP to a Sec codon (Fagegaltier et al., 2000; Bianco et al., 2002). Our results suggest that sole expresses only one *dio3* splice variant as is the case in tilapia (Sanders et al., '99), but in contrast with the situation in trout (Bres et al., 2006). The *dio2* sequence cloned here contains a stretch of 28 adenosines, but no upstream polyadenylation signal, which could be an indication that this is not a true poly(A) tail; i.e., the 3′-UTR region from sole *dio2* is incomplete. This would explain the lack of a SECIS structure in the sole sequence. The cloning of *dio2* has always been a difficult matter because this

family of genes possesses an extended 3′-UTR (up to 7.5 Kb in length) including the SECIS structure (Bianco et al., 2002; Orozco et al., 2002). Alternatively, the sequenced cloned here might be a splice variant, as occurs in mammals (Bianco et al., 2002). In the lungfish *N. forsteri*, D2 mRNAs of different sizes are expressed in a tissue-specific manner, including a brain isoform lacking the SECIS structure (Sutija et al., 2003).

Thyroid hormones and thyroid hormone receptors during sole metamorphosis

Consistent data evidence the crucial role of thyroid hormones in modulating flatfish development and metamorphosis (Miwa et al., '88; Inui et al., '89; Schreiber and Specker, '98; Power et al., 2001; Blanton and Specker, 2007; Klaren et al., 2008). In the Senegalese sole, the first thyroid follicles were evident at 4–5 days after hatching and these follicles increased in size and number during the metamorphosis concomitantly with an augmentation in the intensity of T4 and T3 immunoreactivity (Ortiz-Delgado et al., 2006; Klaren et al., 2008). Endogenous thyroid hormone levels during flatfish metamorphosis have been measured only in a few species. The onset of metamorphosis is marked by an abrupt increase in T4 levels and the final metamorphic stages are accompanied by a reduction in the measurable T4 (Miwa et al., '88; Schreiber and Specker, '98; Hotta et al., 2001; Gavlik et al., 2002: Einarsdóttir et al., 2006; Klaren et al., 2008; Manchado et al., 2008). Furthermore, the larval T3 content is significantly lower and the amplitude of T3 variation during development is much smaller than that of T4 (Hotta et al., 2001; Einarsdóttir et al., 2006; Klaren et al., 2008). Interestingly, a very similar developmental profile of thyroid hormone levels has been observed in this work in sole.

Besides the thyroid hormone increase, an up-regulation of thyroid hormone receptors occurs in the flatfish species studied until now (Yamano and Miwa, '98; Marchand et al., 2004; Galay-Burgos et al., 2008) and also in the sole (Manchado et al., 2009; present results, Figs. 5 and 9). According to the study recently published by Manchado et al. (2009) in sole, in our work the metamorphic increase in the expression of TRβ is more pronounced than that of TRαA (7-fold increase versus 3.5-fold increase). Although there are differences in the amplitude of the peak, it seems that in these four fish species the TRα (the form

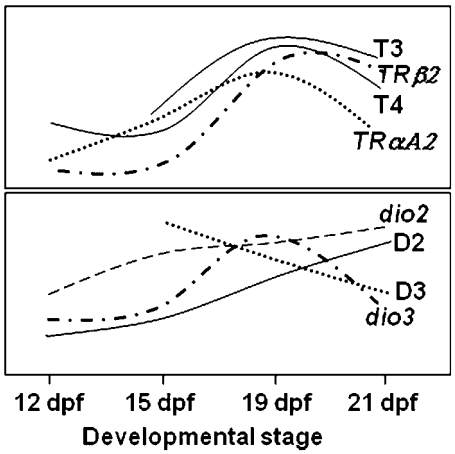

Fig. 9. Development of thyroid hormone system during the sole metamorphosis. This figure summarizes all data obtained in this work, in order to remark the parallelism among the increase in thyroid hormone levels, *TR*αA2, *TR*β2 and *dio2* expression and D2 activity in the sole metamorphic climax as well as the decrease in D3 activity, *dio3* and *TR*αA2 expression at late metamorphosis.

TRαA in the Japanese flounder, the Atlantic halibut and the Senegalese sole) expression increases at the metamorphic climax and decreases thereafter (Yamano and Miwa, '98; Marchand et al., 2004; Galay-Burgos et al., 2008 and present results in the sole). Nevertheless, data reported on TRβ expression during metamorphosis differ among species. Thus, in Japanese flounder (Yamano and Miwa, '98) and sole (Manchado et al., 2009; this study), TRβ expression increases during metamorphic climax and remains high later; in the turbot, TRβ does not increase until post-metamorphic juvenile stages (Marchand et al., 2004); and in the Atlantic halibut TRβ form presents a peak (higher than those of TRα form) during the metamorphic climax, decreasing thereafter (Galay-Burgos et al., 2008).

Some authors have suggested that the functional differences between the receptor types may be a reflection of differences in their expression profiles (Galay-Burgos et al., 2008). However, thyroid hormone receptor regulation and functions are probably much more complex because more than one TRα and one TRβ forms exist in the same species (Nelson and Habibi, 2006) and there are at least four TR forms, TrαA, TrαB, TRβ and TRβ2, in the sole (Manchado et al., 2009, present work). Besides, alternative splicing variants that result in truncated receptors are also present in several mammalian and fish species including the sole (Marchand et al., 2001; Nelson and Habibi, 2008;

Manchado et al., 2009). In the sole a truncated TRβ receptor (TRβv) is also up-regulated during metamorphosis (Manchado et al., 2009). Evidence from the literature shows that the mechanisms of action of TR and biochemical action of given target genes of TRα and TRβ are different (see review from Nelson and Habibi, 2008). All this TR complex system probably provides a fine-tune regulation of cellular thyroid hormone responses. In conclusion, a conserved thyroid hormone content and thyroid hormone receptor increase (TRα and/or TRβ) at metamorphic climax appear as a shared characteristic between flatfish and amphibian development (Brown and Cai, 2007; Galay-Burgos, 2008; present results) Interestingly, iodothyronines can also induce metamorphosis in the cephalochordate amphioxus by binding to a receptor homologous to vertebrate thyroid hormone receptors (Paris et al., 2008). In contrast, thyroid hormones levels decrease as lamprey metamorphosis progresses, and this reduction appears necessary for the completion of metamorphosis because thyroid hormones block the initiation of this event (Manzon et al., '98, 2001).

Iodothyronine deiodinases during sole metamorphosis

If previous studies mainly focused on the variations in T3 and T4 levels during fish metamorphosis, little attention has been paid to the enzymes of the thyroid hormone metabolism. We report here that *dio2* mRNA expression as well as D2 activity increased during metamorphosis. This fact contrasts with previous observations in turbot indicating *dio2* expression did not change significantly during metamorphosis (Marchand et al., 2004). We find a positive correlation between the increases in *dio2* mRNA expression and D2 activity (see Fig. 9), which further supports the assumption that the outer ring deiodination activity that we measured here did correspond to a true D2 activity. In this sense, an elevation of T3/T4 ratio, indicative of an increase in outer ring deiodination, has also been described recently during sole metamorphosis (Klaren et al., 2008). The elevation in *dio2* expression and activity in sole during early and middle metamorphic stages occurs in parallel with a rise in thyroid hormones levels and thyroid hormone receptor expression (see Fig. 9). This is in agreement with the parallel increases in D2 and T4 observed in the developing rat fetal brain (Ruiz de Oña et al., '88). However, these results are in contrast with previous data that show a T4 down-regulation of *dio2* mRNA and activity in vertebrates including adult fish (Orozco et al., 2002; Orozco and Valverde, 2005). No such down regulation was observed here perhaps because thyroid hormones levels where not high enough or because this pathway is active in adult vertebrates only, not in larvae. Indeed, thyroid hormone treatments down regulated D2 activity in the sea lamprey (*Petromyzon marinus*) and *dio2* mRNA expression in zebrafish (*D. rerio*), only after metamorphosis or hatching, respectively, not before (Manzon et al., '98; Walpita et al., 2007).

Dio3 has been revealed as a direct thyroid hormone-target gene during the metamorphosis in *Xenopus* and *Rana* (Becker et al., '97; Brown, 2005). Our results suggest that *dio3* could also be a thyroid hormone-target gene in Senegalese sole because its expression is positively correlated with thyroid hormones levels and TRαA expression along metamorphosis. Thus, *dio3* expression was up-regulated at the middle metamorphic stage (19 dpf) and down-regulated at late metamorphic period (21 dpf), coinciding with a significant rise and decrease in T4 levels and TRαA expression (see Fig. 9). It should be interesting to determine if sole *dio3* promoter exhibits thyroid hormone response elements that could regulate its expression, as it has been described for human type 1 iodothyronine deiodinase gene (Zhang et al., '98). D3 activity does not follow *dio3* developmental expression profile. The highest D3 activity was found at the beginning of the metamorphosis (15 dpf) whereas *dio3* expression peaked at the metamorphic climax (19 dpf) suggesting that post-transcriptional mechanisms modify D3 activity at mid-late metamorphosis. The decline in *dio3* expression and D3 activity together with the maintaining of *dio2* expression and the increase in D2 activity at mid-late metamorphic stages could explain the rise of thyroid hormones levels observed during sole metamorphosis.

It should be noted that a similar pattern, i.e., a sharp drop in D3 activity and a rapid rise in both D2 and TRβ has been described at the metamorphic climax in the tail of amphibians (Brown and Cai, 2007). However, an inverse pattern has been reported for lamprey intestinal deiodinase activities, so that D2 predominates in early metamorphosis but D3 predominates in mid- and late metamorphosis, probably contributing to the characteristic depression of plasma T4 and T3 levels during spontaneous metamorphosis (Eales et al., 2000). Taken together, these results suggest that

the endogenous concentration of thyroid hormones, the amounts of the thyroid hormone receptors and the activities of deiodinases that synthesize and inactivate thyroid hormones appear shared characteristics that play important roles in the timing of metamorphic events in both amphibians and flatfishes, whereas the regulation of this event in lampreys would be a derived condition.

Daily cycles of thyroid hormones, D2 and D3 during metamorphosis

Although the existence of daily rhythms of thyroid hormones has been reported in adult fish, no data are available concerning the onset of these rhythms during development. Moreover, there is no information to date on putative daily rhythms of fish deiodinase activity and/or expression neither in adult nor developing fish. Therefore, our study represents the first approach to these aspects in fish, which are of particular interest in metamorphic species.

In teleosts, there is evidence for daily rhythms of plasma T4, with higher levels during the photophase, whereas plasma T3 levels exhibit little or no daily variations (Gomez et al., '97; Leiner and MacKenzie, 2003). Moreover, a photoperiodic control of deiodinases has been recently reported in several adult avian and mammalian species (Watanabe et al., 2007). In this study, we have found concomitant higher T4 levels and D2 activity during daytime at the end of the metamorphosis but not before, suggesting that thyroid hormones rhythmicity is a late/post-metamorphic characteristic in sole. In turn, *dio3* expression also exhibited daily changes in sole during metamorphosis but its daily profile does not follow D3 activity variations. The bad correlation between *dio3* expression and D3 activity at different day times could also be indicating the existence of post-transcriptional mechanisms that regulates daily D3 activity. Similar discrepancies have already been reported for other enzymes like the retinal arylalkylamine-*N*-acetyltransferase 1 (AANAT1), in which daily mRNA expression also appears out of phase in relation to AANAT1 protein abundance and activity, emphasizing that changes in mRNA are not entirely reliable predictors of changes in protein content and activity (Besseau et al., 2006).

Our results also suggest that thyroid hormone system is more active during daytime in developing sole, at least at late metamorphosis, as it has been described in adult fish (Gomez et al., '97; Leiner and MacKenzie, 2003; Ebbesson et al., 2008). Further experiments appear necessary to demonstrate the existence of daily circadian rhythms in these parameters using different lighting conditions (constant light, constant dark, time shift) and to determine if these putative daily rhythms are light-entrained and/or clock-controlled, and if other zeitgebers as feeding time could also be important for rhythm synchronization. Studies performed in *S. senegalensis* suggest the existence of a daily feeding rhythm during development that can also be modified by manipulating light conditions (Cañavate et al., 2006). However, feeding time alone seems not responsible of diel T4 and D2 activity rhythms observed in our study because experimental animals have been fed once per day (at noon) at every developmental stage but daily rhythms of T4 and D2 were only detected at particular metamorphic stages. Alternatively, post-prandial T4 increase could represent a post-metamorphic characteristic in the Senegalese sole.

SUMMARY

We have cloned *dio2* and *dio3* cDNAs in Senegalese sole. These iodothyronine deiodinases exhibit active catalytic centers that are highly conserved through vertebrate phylogeny. Furthermore, we have described, for the first time in a metamorphic fish, concomitant changes in thyroid hormones levels, thyroid hormone receptors, *dio2* and *dio3* activity and expression along this important process. Our results have shown significant changes in T4 and T3 levels, thyroid hormone receptor expression, *dio2* expression and D2 activity, which appear up-regulated during metamorphosis as well as in *dio3* expression and D3 activity, which decrease at late metamorphosis. These developmental changes point out the importance of *dio2* and *dio3* in mediating the thyroid hormone effects in flatfish metamorphosis, as it has been described in amphibians. As deiodinases play an important role as local modulators of thyroid hormone actions, in situ hybridization studies will provide additional information of great interest to better understand flatfish metamorphosis.

ACKNOWLEDGMENTS

The authors thank Dr. J. Pedro Cañavate and IFAPA ''El Toruño'' (Consejería de Innovación,

Ciencia y Empresa, Junta de Andalucía, Puerto de Santa María) for the generous gift of fertilized sole embryos. We also thank all staff from the ''Planta de Cultivos Marinos'' (University of Cádiz) for the maintaining of animals used in developmental studies.

Grant sponsor: Spanish MICINN (AGL2007-66507-C02-01 to J.A.M.C. and SAF2006-01319 to M.J.O.); grant sponsor: Junta de Andalucía (P06-AGR-01939 to J.A.M.C.); grant sponsor: UPMC, CNRS and IFREMER (GDR2821 to J.F.); grant sponsor: postdoctoral fellow of the Spanish MEC to E.I.; grant sponsor: ''CIBER Fisiopatología de la Obesidad y Nutrición'' (CIBERobn) is an initiative of ISCIII (Instituto de Salud Carlos III), Spain.

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