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Molecular characterization, gene expression and transcriptional regulation of thyroid hormone receptors in Senegalese sole

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ABSTRACT

Thyroid hormones (THs) play a key role in larval development, growth and metamorphosis in flatfish. Their genomic effects are mediated by thyroid hormone receptors (TRs). In this study, cDNAs encoding for TRαA, TRαB, and TRβ have been sequenced in Senegalese sole (*Solea senegalensis*). Main domains and conserved motifs were identified. Also, a truncated TRαB isoform (referred to as TRαBtr) and a spliced TRβ variant (referred to as TRβv) were detected. A phylogenetic analysis grouped both TRα and TRβ genes into two separate clusters with their fish and mammalian counterparts. Expression profiles during larval development and in juvenile tissues were analyzed using a real-time PCR approach. In juvenile fish, TRαA, TRαB, TRβv, and TRβ showed distinct transcript levels in tissues. During metamorphosis, only TRβv and TRβ modified their mRNA levels in a similar way to the T4 contents. To evaluate the possible regulation of TRs by their cognate ligand T4 during sole metamorphosis, larvae were exposed to the goitrogen thiourea (TU). TRβ transcripts decreased significantly at 11 and 15 days after treatment. Moreover, adding exogenous T4 hormone to TU-treated larvae restored the steady-state levels or even increased TRβ and TRβv mRNAs with respect to the untreated control. Overall, these results demonstrate that TRβ transcription is up-regulated by THs playing a major role during metamorphosis in Senegalese sole.

1. Introduction

Thyroid hormones (THs) play a key role in fish growth, development and metamorphosis (Power et al., 2001). THs exert their actions either by binding to thyroid hormone receptors (TRs) (genomic pathway) or through cellular signal transduction systems and cell surface receptors (non-genomic pathway), some of which are also mediated via the TRs (reviewed in Davis et al. (2008); Oetting and Yen (2007)). TRs belong to the subfamily I of nuclear hormone receptors (NRs) (Germain et al., 2006; Gronemeyer et al., 2004). As most NRs, TRs possess a modular structure composed of an N-terminal A/B domain, a conserved C domain (DNA binding domain, DBD), a D domain (hinge region) and a moderately conserved E domain (ligand binding domain, LBD). Moreover, a TR spliced-isoform containing an F domain has been recently reported (Takayama et al., 2008). The C region harbors the DBD core, which is composed of two zinc finger motifs and targets the receptor to specific thyroid response elements (TRE). The LBD possesses a dimerization surface and a ligand-dependent transactivation function, the AF-2 motif located in its C-terminal part (Germain et al., 2006; Olefsky, 2001). TRs can bind DNA as monomers, homodimers, or, preferentially, heterodimers with retinoid X receptors (RXRs), since heterodimerization strongly increases binding to the TRE and transcriptional activity (Ikeda et al., 1994; Mangelsdorf and Evans, 1995). Although these heterodimers have been traditionally considered as non-permissive ones (with RXR as silent partner), in some particular cellular environments RXR agonists can also bind and activate transcription (Castillo et al., 2004; Li et al., 2004; Shulman et al., 2004).

Vertebrate possess two principal TR isoforms termed TRα and TRβ. Teleost genomes are the result of three rounds of large scale gene duplications since the genomes of ancestral invertebrate chordates. Thus, different genes encoding the TRα and TRβ isoforms can be found in fish. Two genes encoding for TRα, referred to as TRαA and TRαB (Galay-Burgos et al., 2008; Harada et al., 2008; Kawakami et al., 2008; 2003b; Marchand et al., 2001; Yamano et al., 1994) and two TRβ genes in *Conger myriaster* (Kawakami et al., 2003a) have been reported. This complexity is even higher if we consider the multiple TR isoforms as a result of alternative splicing, affecting mainly the hinge region (Galay-Burgos et al., 2008; Harada et al., 2008; Kawakami et al., 2007; 2003a; Marchand et al., 2001; Yamano and Inui, 1995). All these genes and spliced variants can play different functions and hence it is important to establish their tissue-specific and larval development patterns.

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Metamorphosis is a critical stage in flatfish (order Pleuronectiformes) development. During metamorphosis, larvae shift from planktonic to a benthic mode of life, involving drastic changes in morphology and physiology. Although flatfish species clearly differ in the time of commencement and in the duration of metamorphosis, in all species studied to date, the involvement of THs as a key mediator of this process is universal (Manchado et al., 2008a; Miwa et al., 1988; Solbakken et al., 1999). Some studies in Pleuronectiformes have focused on the role of different TRs during metamorphosis. In *Paralichthys olivaceus* and *Psetta maxima*, TRαA and TRβ mRNA transcript levels increase during the metamorphic climax although the former decreases in postclimax (similarly to T4 levels) whereas the latter increases to reach its highest levels (Marchand et al., 2004; Yamano and Inui, 1995). In *Hippoglossus hippoglossus*, a peak in the expression of TRαA (although not statistically significant) and TRβ at metamorphic climax (stage 9) was also reported. However, neither of these studies examined the possible regulation of TRs by their cognate ligands (THs).

Senegalese sole (*Solea senegalensis*) is a flatfish that undergoes metamorphosis very early during larval development, between 12 and 19 days after hatching (DAH) (Fernández-Díaz et al., 2001; Manchado et al., 2008a). In a recent study, we showed that thiourea (TU) treatments blocked eye migration and resulted in an increase of thyrotropin and thyroglubin mRNA levels (Manchado et al., 2008a). These data supported the involvement of THs on Senegalese sole metamorphosis as well as a negative feedback regulation at a transcriptional level of these hypothalamic–pituitary–thyroid axis genes. Since THs exert their actions by binding to TRs, it is necessary to identify the set of TRs and to study their regulation in *S. senegalensis* in order to achieve a better understanding of TU-mediated responses. This present work aimed at characterizing TRs in *S. senegalensis*. Gene expression studies were also carried out to assess tissue-specific and developmental expression patterns. Drug treatments were employed to determine a possible regulation by THs.

2. Material and methods

2.1. Fish sampling and experimental treatments

Senegalese sole juveniles and larvae were obtained from the facilities of IFAPA (Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica) Centro *El Toruño* (El Puerto Santa María, Cádiz, Spain). Juvenile weight and larval culture conditions were similar to those reported in Manchado et al. (2008b).

Thiourea (TU) and T4 treatments were carried out as described in Manchado et al. (2008a). Briefly, in the first experiment, larvae were initially stocked at a density of 100 individual L^{-1} in 200 L round tanks. TU (30 ppm; 394 μM) was added to the tanks 7 days after hatching (DAH) and water was kept stagnant for 24 h. Twenty percent of the water was then exchanged daily maintaining constant the TU concentration. Three pools of larvae were collected before treatment (7 DAH) and at 11 (18 DAH) and 15 (22 DAH) days after treatment, washed with DEPC water, frozen directly in liquid nitrogen and stored at −80 °C until analysis. A second tank with the same characteristics was used to rear sole larvae that remained as the untreated control.

In the rescue experiment, TU (30 ppm; 394 μM) was added on day 4 to ensure lack of endogenously synthesized T4 at the commencement of exogenous T4 treatment. At day 7, untreated control larvae were transferred to a pair of 16 L cylinder tubes at an initial density of 45 larvae L^{-1}. TU-treated larvae were transferred to four tubes, two of which were supplemented with 100 ppb T4 (0.112 μM). After this new exposure, 20% water was exchanged every other day maintaining constant TU and TU+T4 concentrations. Pools of larvae ($n = 3$) were collected 8 and 13 days after the commencement of T4 treatment, washed with DEPC water, frozen in liquid nitrogen, and stored at −80 °C until analysis. The effects of drug treatments on metamorphosis progression are described in Manchado et al. (2008a).

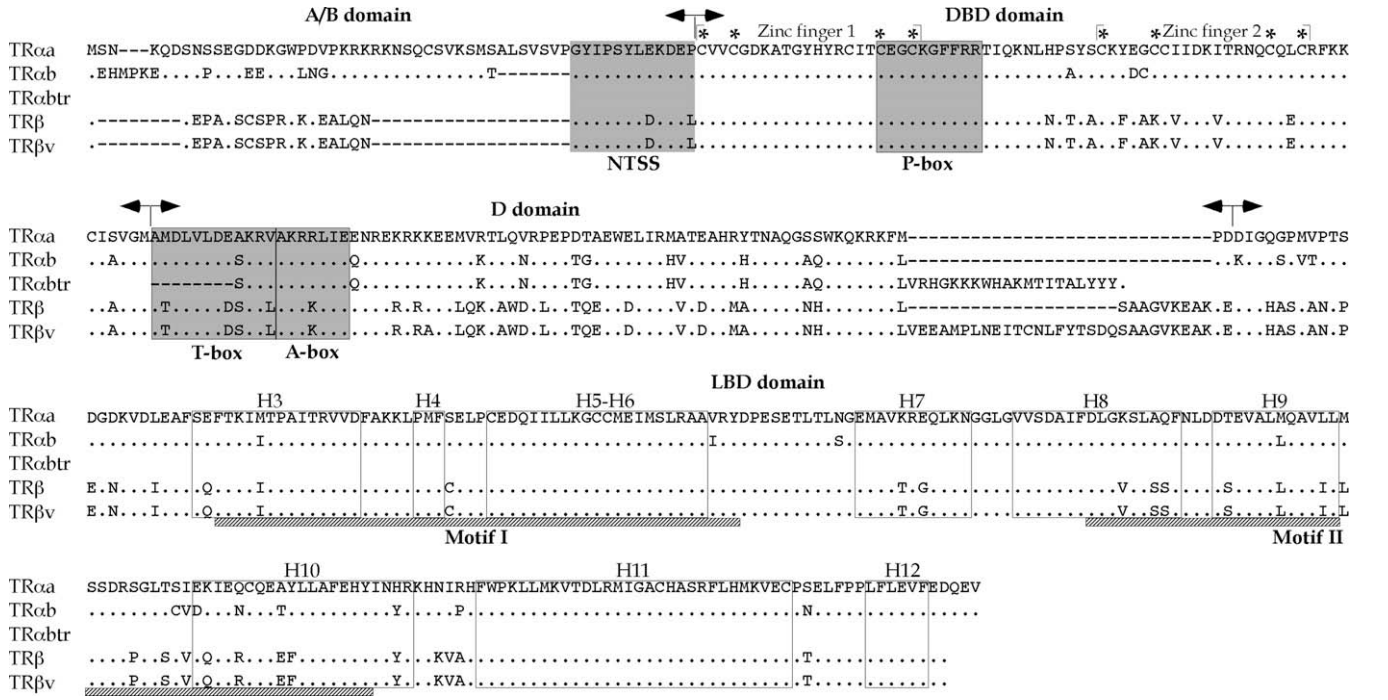

Fig. 1. Alignment of TR amino acid sequences from *S. senegalensis*. Dots indicate identity, and hyphens represent indels. The A/B, DBD, D and LBD domains are separated by arrows. Two conserved zinc fingers are between brackets. "∗" stands for the functionally important cysteine residues in zinc fingers. The 12 highly conserved helices are boxed and the characteristic motif I and II in the LBD domain are indicated by a bar.

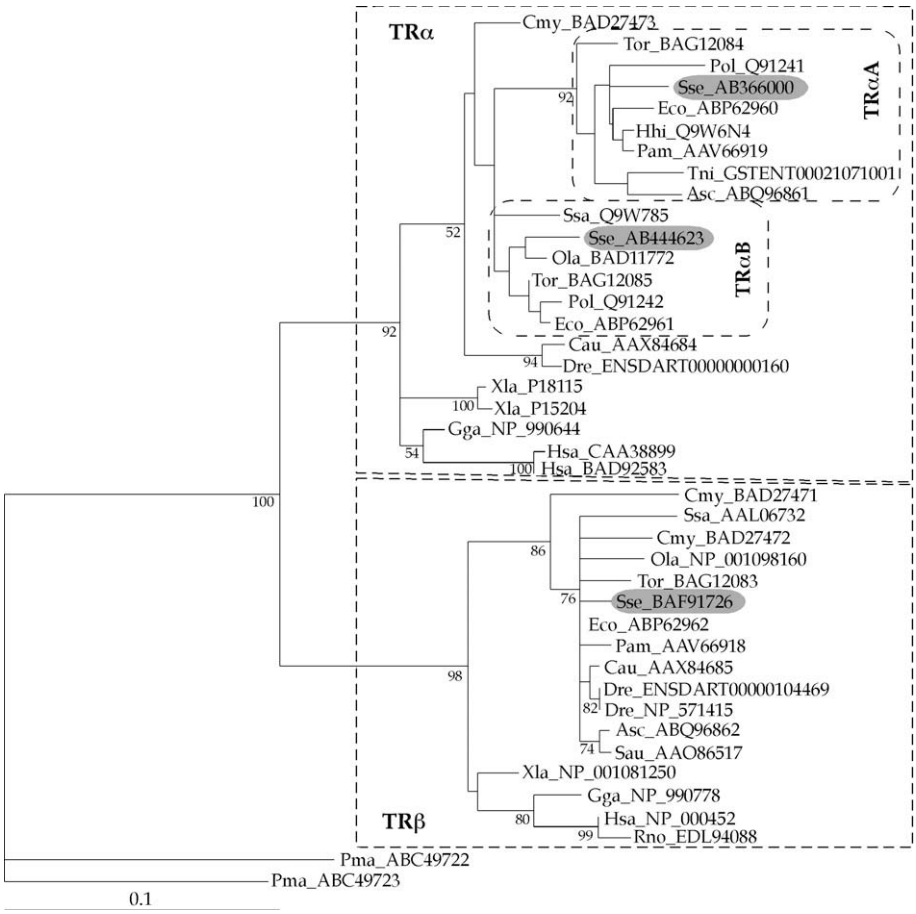

Fig. 2. Phylogenetic relationships among TRs from a wide range of vertebrates using the maximum likelihood method. TRs from *Petromyzon marinus* (Pma) were used as outgroups to root tree. Only bootstrap values higher than 50% are indicated on each branch. The scale for branch length (0.1 substitutions/site) is shown below the tree. Abbreviations are: *Homo sapiens* (Hsa), *Rattus norvegicus* (Rno), *Gallus gallus* (Gga), *Xenopus laevis* (Xla), *Acanthopagrus schlegelii* (Asc), *Carassius auratus* (Cau), *Conger myriaster* (Cmy), *Danio rerio* (Dre), *Epinephelus coioides* (Eco), *Hippoglossus hippoglossus* (Hhi), *Oryzias latipes* (Ola), *Paralichthys olivaceus* (Pol), *Pseudopleuronectes americanus* (Pam), *Tetraodon nigroviridis* (Tni), *Salmo salar* (Ssa), *Sparus aurata* (Sau), *Solea senegalensis* (Sse), *Thunnus orientalis* (Tor). Accession number for each sequence is indicated.

Table 1
Primers used in this study. Fragment size and gene target are indicated for those used for real-time PCR. F and R denote forward and reverse primer, respectively.

Target	Primers		Fragment size (bp)
	Primer pair name	Sequence (5′→3′)	
TRβ(global expression)	SseTRbF1	CTGGAGCCCACGCAGGAGGA	57
	SseTRbR2	CATGTGGGCATCAGTCACCATACGG	
TRβ	SseTRbF3	AAACAGAAGCGGAAGTTCCTGAGTGCAG	100
	SseTRbR4	CTTTGTTTCCTTCAGGTGTGTTTGCCATC	
TRβv	SseTRbvF	CCTGGTCGAGGAAGCAATGCCTCTT	85
	SseTRbvR	TTCCTTCACCCCCGCTGCACT	
TRαa	SseTRaaF	CGCAAGTTCATGCCGGATGATATCG	117
	SseTRaaR	TGTGATGGCGGGTGTCATTATCTTGGT	
TRαb	SseTRabF	GCGCTGTTGCAGGCCGTGCT	140
	SseTRabR	AAGTGGGGAATGTTGTGCTTGCGGTA	
TRαbtr	SseTRabtrF	GTGGCACGCAAAAATGACAATTACAGCACT	151
	SseTRabtrR	GTATTTTTCTCTGGTCATGCTGCGTTTTGG	
	TRA1	GGCTACCACTACCGCTGCATCACCTG	
	TRA2	CATGTGGAGGAAGCGGCTGGC	
	TRab3	ACCATTCAAAAGAACCTCCACCCCAGC	
	TRab4	GAATTTGCGCTTCTGTTTCCACTGTGC	
	SseTRab1	AACCAGTGCCAGCTATGCCGCTTC	
	SseTRab2	GTTCTGCTCAATCAGCCGCCGTTT	
	TRB1	AGAACCGGGAGCGCCGTCGGAA	
	TRB2	GGGGTGATAATTTTTGTAAACTGACTGAAGG	

2.2. Cloning and molecular characterization of TRs

For TRαA and TRαB receptors cloning cDNA from premetamorphic larvae was amplified using the primer pairs TRA1/TRA2 and TRab3/TRab4, respectively. Two fragments of 981 and 336 bp were obtained for TRαA and TRαB, respectively. To achieve full-length cDNAs, 5′ and 3′ ends were amplified from larval cDNA libraries (Manchado et al., 2007b) using universal primers (T3 and T7) and the specific reverse (SseTRA2 and SseTRab2 for TRαA and TRαB, respectively) and forward (SseTRA1 and SseTRab1 for TRαA and TRαB, respectively) primers. For TRβ cloning, the strategy was similar to that described for TRαs. A fragment of 269 bp was amplified using the primers TRB1 and TRB2 and full cDNA was obtained from cDNA libraries using universal and specific SseTRB1 and SseTRB2 primers.

Alignments of sequences were carried out and identities calculated using the MegAlign *v*7.2.1 program from the LASERGENE software suite (DNASTAR). For phylogenetic analysis, only the DBD and LBD domains of TRs from mammals and fish (see Fig. 2) were used. Sequences were retrieved from GenBank/EMBL/DDBJ. Moreover, an additional search was conducted in the www.ensembl.org database for *Danio rerio*, *Oryzias latipes,* and *Tetraodon nigroviridis*. The bestfit model of sequence evolution (JTT+I+G) was determined using the ProtTest version 1.3 (Abascal et al., 2005). Phylogenetic analysis was carried out using the PHYLIP package (Felsenstein, 1989) as follows: a bootstrap analysis was carried

out using SEQBOOT (1000 replicates), and data were then analyzed by maximum likelihood (PHYML; 1000 replicates), which generated 1000 trees. The consensus phylogenetic tree was subsequently obtained (CONSENSE). Trees were drawn using the TreeView program *v*1.6.2 (Page, 1996).

2.3. RNA isolation and gene expression analysis

Homogenization of larvae ($n = 3$ independent pools/sampling) and juvenile tissues ($n = 3$ specimens) was carried out in the Fastprep FG120 instrument (Bio101) using Lysing Matrix D (Q-BioGene) for 40 s at speed setting 6. Total RNA was isolated from 50 mg of *S. senegalensis* tissues or pools of larvae using the RNeasy Mini Kit (Qiagen). For skeletal muscle, heart and skin the RNeasy Fibrous Tissue Mini Kit (Qiagen) was utilized. All RNA isolation procedures were carried out in accordance with the manufacturer's protocol. In all cases, total RNA was treated twice with DNase I using the RNase-Free DNase kit (Qiagen) for 30 min. RNA sample quality was checked on an agarose gel, and quantification was spectrophotometrically accomplished. Total RNA (1 μg) from each sample was reverse-transcribed using the iScript™ cDNA Synthesis kit (Bio-Rad) in a 20 μl volume. Real-time PCR analysis was carried out using an iCycler (Bio-Rad). Reactions were done in a 25 μl volume containing cDNA generated from 10 ng of original RNA template (2 μl of a 1/10 cDNA dilution), 300 nM each of specific forward and reverse primers (Table 1), and 12.5 μL of iQ™ SYBR Green Supermix (Bio-Rad). Matching oligonucleotide primers were designed using the Oligo *v*6.89 software (Medprobe). The amplification protocol used was as follows: initial denaturation and enzyme activation for 7 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, and 70 °C for 30 s. Each assay was done in duplicate. For normalization of cDNA loading, all samples were run in parallel with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH-2*) or 18S rRNA for larval development and juvenile tissues, respectively (Infante et al., 2008b; Manchado et al., 2007a). Relative mRNA expression was determined using the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). Day 2 after hatching larvae and liver were used as calibrators for larval development and tissue analyses, respectively. Untreated larvae at 7 DAH and at 15 DAH were used as a calibrator in the TU and rescue experiment, respectively. Comparisons between groups were made by one-way analysis of variance, followed by a Tukey test for identification of the statistically distinct groups. Significant differences were accepted for $P < 0.05$.

3. Results

3.1. Molecular characterization of TRα and TRβ cDNAs

TRαA encoded a transcript of 2799 nucleotides (nt) [Accession No. AB366000] that contained a short 5′-untranslated region (103 nt) followed by an open reading frame (ORF) of 414 codons. The 3′-untranslated region was 1454 nt long and included a canonical polyadenylation signal (AATAAA, 2773–2778) and a short oligo-A tail. For TRαB, a partial fragment of 1952 nt was identified [Accession No. AB444623]. The ORF comprised 409 codons, and it was preceded by a 334-nt 5′-untranslated region. In addition, two truncated TRαB cDNAs [Accession No. AB444624 and AB444625] differing in 3′ UTR length were identified. The TRβ cDNA possessed an ORF of 1188 nt (Accession No. AB366001). The 5′ and 3′-untranslated region were 63 and 373 nt long, respectively. Moreover, a TRβ spliced variant, referred to as TRβv, containing a 60-bp insertion was also identified (Accession No. AB444626).

Sequence identities between TRα and TRβcDNAs from *S. senegalensis* and a range of vertebrates are depicted in Table 2. Identities between TR fish orthologs were in the range 91.5–97.2% whereas for TR paralogs oscillated between 75.6–90.9%. A value of 89.7% was observed between TRαA and TRαB from *S. senegalensis* and 77.3 and 78.2% between TRβ and TRαA and TRαB, respectively.

Alignment of predicted polypeptide sequences revealed the modular structure characteristic of the NR superfamily with the N-terminal A/B domain, a conserved DBD, a hinge region (D domain), and a LBD (E domain) (Fig. 1). The two zinc fingers and the regulatory elements P-box as well as the T-box and A-box in the C-terminal extension (CTE) of the DBD were conserved. Although A/B domain was clearly divergent among TRs, the N-terminal signature sequence (NTSS) was well conserved. In the LBD domain, the consensus motif I (spanning helix 3–6) and motif II (from the middle of helix 8 to the middle of helix 10) and the putative AF2 activation domain core (helix 12) were also identified.

A phylogenetic tree constructed by the maximum likelihood method from a multiple sequence alignment of predicted polypeptides Senegalese sole TR, and a wide range of vertebrate counterparts (Fig. 2), showed that Senegalese sole TRα and TRβ grouped into two highly consistent and separate clades (92 and 98 bootstrap for TRα and TRβ clades, respectively). Within specific TRα and TRβ clade, teleosts clustered as a paraphyletic group with respect to their tetrapod counterparts. TRαA and TRαB grouped separately although only TRαA clade was supported by a significant bootstrap value (92).

3.2. Expression levels of TRα and TRβ genes in tissues

Steady-state levels of TR transcripts were quantitated in liver, spleen, brain, gills, intestine, stomach, head kidney, heart, skeletal muscle, and skin from Senegalese sole juveniles (average weight = 23.2 g) (Fig. 3). TRαA, TRαB and TRβ were discovered in all tissues although significant differences in mRNA abundance were detected. TRαA and TRαB showed the highest mRNA levels in brain. TRαA transcripts were also high in head kidney and TRαB in gills and heart. In contrast, these genes showed low expression levels in skeletal muscle. TRβ was expressed ubiquitously with the lowest expression level also in skeletal muscle. The spliced variant TRβv exhibited a distinct expression pattern with the highest

Table 2
Percentage of amino acid sequence identity among Senegalese sole (Sse) TRs and several other vertebrate TRs as calculated using MegAlign. In Sse, identity between TRαA and TRβ is shown. For TRαB, identities with respect to TRβ and TRαA, respectively are separated by "/". TRβ: Pol (*P. olivaceus* ;D45245), Eco (*E. coioides*; ABP62962), Tor (*T. orientalis*; BAG12083), Xla (*X. laevis*; NP_001081250), Hsa (*H. sapiens*; NP_000452); THRαA: Pol1 (Q91241), Eco1 (ABP62960), Tor1 (BAG12084); THRαB: Pol2 (Q91242), Eco2 (ABP62961), Tor2 (BAG12085); TRα: Xla1 (P15204), Hsa1 (CAA38899).

	Sse	TRβ					TRα							
		Pol	Eco	Tor	Xla	Hsa	Pol1	Eco1	Tor1	Pol2	Eco2	Tor2	Xla1	Hsa1
TRβ		97.0	97.2	95.4	86.3	85.0	77.3	78.4	80.9	79.8	78.7	80.5	78.7	79.1
TRαA	77.3	77.9	78.1	78.4	76.9	75.6	94.9	95.4	94.6	86.9	85.6	90.3	85.6	83.2
TRαB	78.2/89.7	78.9	78.9	78.4	77.4	75.6	88.8	89.5	90.9	93.3	91.5	97.0	86.5	84.7

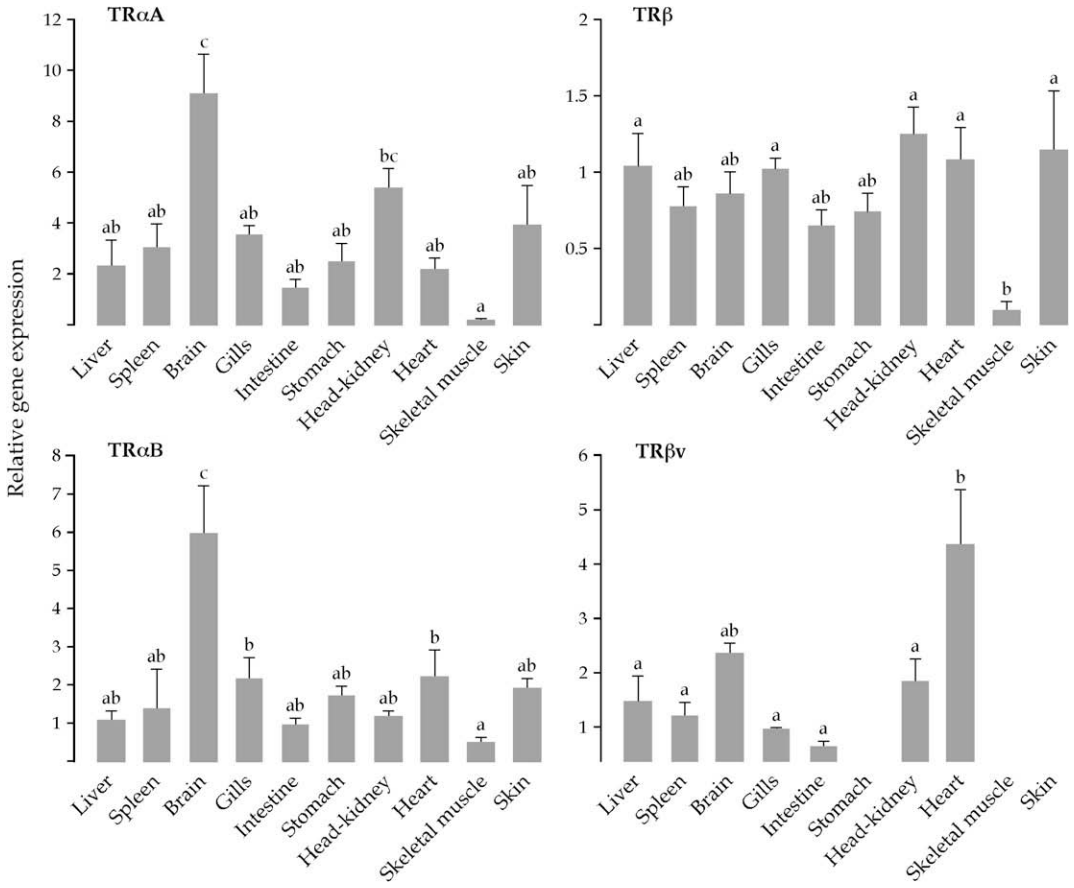

Fig. 3. Relative expression levels of TR genes in different tissues from Senegalese sole. Data were expressed as the mean fold difference (mean ± SEM, $n = 3$) from the calibrator group (liver). Values with the same superscript are not significantly different ($P > 0.05$).

mRNA levels in heart and brain and undetectable levels in stomach, skeletal muscle and skin.

3.3. Expression levels and regulation during larval development

Expression profiles of TR genes during larval development (2–22 DAH) were also determined. TRαA and TRαB were expressed at a constant level during whole larval development (Fig. 4). Only a small significant peak was observed for TRαA at 9 DAH. In any case, the expression levels did not correlate with T4 contents. TRαBtr showed a similar pattern with no significant changes during development or metamorphosis. In contrast, TRβ exhibited an expression profile similar to that for T4 levels. TRβ transcripts increased progressively from 9 DAH to metamorphic climax (16 DAH when T4 contents were maximum) keeping high until the completion of metamorphosis. TRβv expression pattern was similar to TRβ with a peak at metamorphic climax. The simultaneous amplification of both TRβ and TRβv mRNAs (TRβ global) showed the same temporal expression profile with similar increasing values.

To investigate the possible regulation of TRβ by THs during metamorphosis, 7 DAH larvae were exposed to the goitrogen TU. TU-treated larvae showed 5.5 and 5.9 lower TRβ mRNA levels ($P < 0.05$) than controls at both 11 and 15 days after treatment, respectively (Fig. 5). To confirm these data, a rescue assay to determine the ability of T4 to revert the TU effect was carried out. TU-treated larvae showed lower TRβ transcripts (although at 20 DAH the value was not statistically significant) than untreated controls. Interestingly, TU+T4 treated larvae exhibited higher TRβ and TRβv transcript levels than untreated and TU-treated larvae 8 days after T4 treatment (Fig. 6). In contrast, no significant change was detected later at 13 days after T4 treatment. TRαA did not show significant differences under either of the treatments whereas TRαB only increased slightly at 20 DAH with TU.

4. Discussion

TRs play a central role in the genomic regulatory cascade of THs. In the present study, we have identified two TRα and one TRβ cDNAs in the Senegalese sole. Also, a truncated TRαB isoform and a spliced TRβ variant were detected. Both TRα and TRβ receptors possessed the typical modular organization of the NR superfamily. All of them had a conserved DBD domain with two zinc fingers and a LBD domain (except the truncated isoform) containing the typical 12 helices (Marchand et al., 2001). The TR-specific NTSS motif in chordates [GYIPYL(D/E)KE(P/Q/L)] (Wu et al., 2007) was also identified in the variable A/B domain. The P-box determining DNA binding specificity interacting (Nelson et al., 1994; 1995), and the T-box and A-box regions that contribute to dimerization and DNA binding stabilization, respectively, (Muñoz and Bernal, 1997; Rastinejad et al., 1995) were well conserved. The LBD domain was absent only in the truncated TRαBtr isoform. This truncated isoform is similar to that reported in *Carassius auratus* (Nelson and Habibi, 2006). Although these authors suggested that it could be encoded by a different gene, the identification of a "GT" at the commencement of the divergent region with respect to TRαB in both species indicates that it could really represent an intron sequence. Although additional analyses are required to confirm this hypoth-

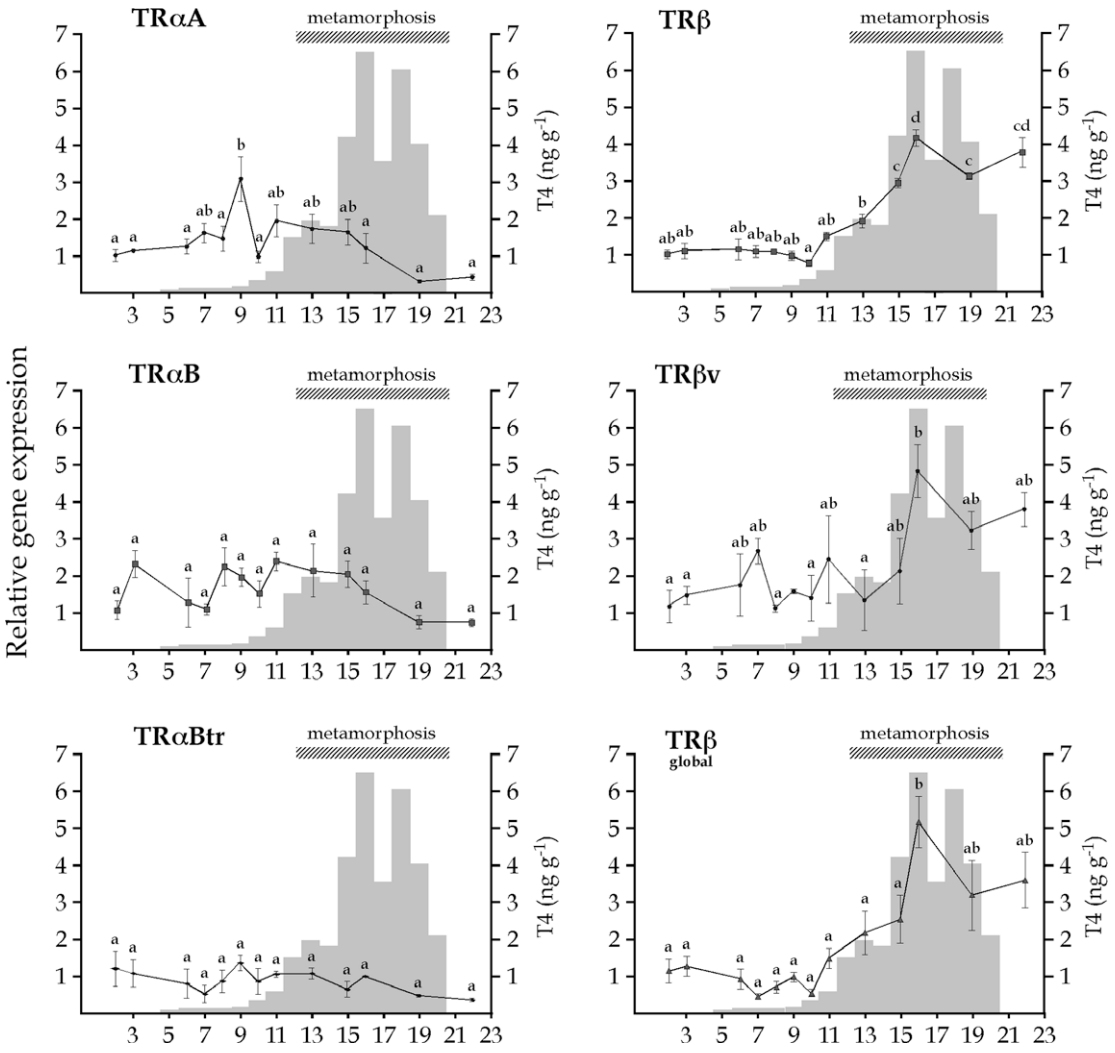

Fig. 4. Relative expression levels of TR genes during larval development in Senegalese sole. "TRβ global" refers to the simultaneous quantitation of TRβ and TRβv levels. Expression values were normalized to those of *GAPDH-2*. Data were expressed as the mean fold difference (mean ± SEM, $n = 3$) from the calibrator group (2 DAH). Values with the same superscript are not significantly different ($P > 0.05$). The interval for the metamorphosis process is indicated with a bar. T4 levels have been taken from Manchado et al. (2008a).

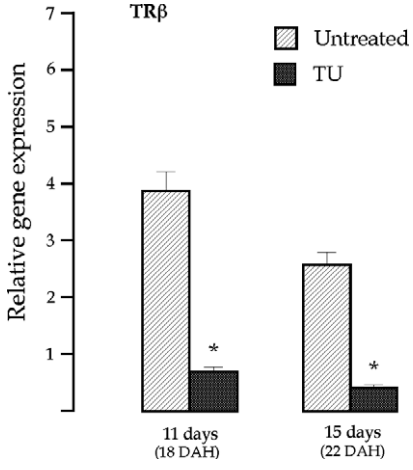

Fig. 5. Relative TRβ expression levels in untreated and TU-treated larvae. Expression values were normalized to those of *GAPDH-2*. Data were expressed as the mean fold difference (mean ± SEM, $n = 3$) from the calibrator (untreated control 7 DAH). Days after TU treatment (394 μM) are indicated. Fish age is between brackets. Values with asterisk are significantly different ($P > 0.05$) from the corresponding value for the untreated control.

esis, this truncated isoform could modulate T3 action by inhibiting the trans-activation of T3-responsive genes by the other TR forms as suggested in mammals (Lazar et al., 1989).

Phylogenetic analysis revealed that both TRα and TRβ receptors clustered with their teleost counterparts. Both TRα types (TRαA and TRαB) grouped into two separate clades indicating that they have probably been retained during evolution after the 3R duplication acquiring different roles due to neofunctionalization and subfunctionalization processes. In contrast, only one TRβ gene was identified in teleosts except in *C. myriaster* (Kawakami et al., 2003b), probably reflecting a specific-lineage duplication instead of the consequence of a whole-genome duplication. However, alternative splicing of TRβ transcripts seems to be a common feature in fish (Galay-Burgos et al., 2008; Kawakami et al., 2007; 2003a; Marchand et al., 2001; Yamano and Inui, 1995). In this study, we have identified a TRβv that contains a 60-bp insertion strongly conserved in other flatfish such as *H. hippoglossus* (Galay-Burgos et al., 2008), *P. olivaceus* (Yamano and Inui, 1995) and *Pseudopleuronectes americanus* (Accession No. AY794222). This insertion could represent a canonical 'GT. . .AG' spliceosomal intron (Simpson et al., 2002). Analysis of the TRs genomic structure would assist in a better comprehension of processing and posttranscrip-

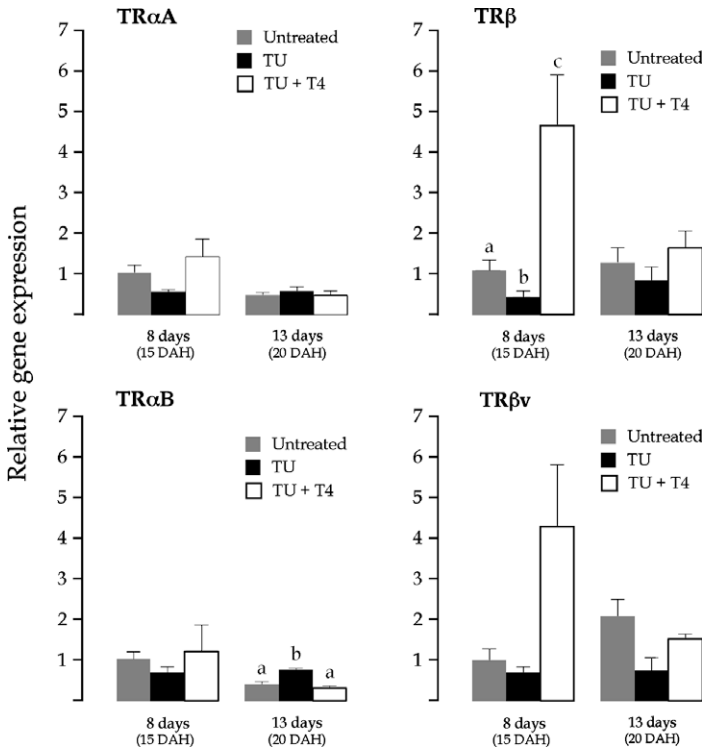

Fig. 6. Relative TR expression levels in untreated (grey), TU-treated (black) and TU + T4-treated (white) larvae. TU (394 μM) was added at 4 DAH and T4 (0.112 μM) at 7 DAH. Expression values were normalized to those of *GAPDH-2*. Data were expressed as the mean fold difference (mean ± SEM, $n = 3$) from the calibrator (untreated control 8 days after T4 treatment). Fish age is indicated between brackets. Values with the same superscript are not significantly different ($P > 0.05$) from the corresponding value for the untreated control.

tional regulation mechanisms occurring in this type of NRs in Senegalese sole.

Senegalese sole TR genes were expressed ubiquitously in tissues although transcript levels clearly differed among them. TRβv mRNAs also showed distinct abundances in tissues with the highest levels in heart and brain (Fig. 3). Ubiquitous expression of TRs has been widely described in fish. In adult *Sparus aurata*, TRβ transcripts showed similar levels in all tissues (skeletal muscle, heart, intestine, brain, kidney, liver, and gill) (Nowell et al., 2001). In *C. myriaster*, cTRβ1 receptor was expressed ubiquitously with the highest abundance in brain, pituitary, ovary, and kidney whereas cTRβ2 was located specifically in brain and pituitary gland (Kawakami et al., 2003a). In *H. hippoglossus*, TRβ and a spliced variant could be detected in all tissues although the former possessed reduced mRNA levels in heart and white muscle and the latter high transcript levels in brain, heart, and muscle (Galay-Burgos et al., 2008). In *C. auratus*, TRαA, TRαB, TRβ and a truncated TRβ isoform were widely expressed, although at different level, among tissues. Expression patterns were similar in males and females although transcript levels fluctuated significantly in some tissues depending on the reproductive season (Nelson and Habibi, 2006). All these data indicate that TRs represent a complex family of regulatory proteins whose expression can be modulated by the environment and in a tissue-specific manner providing a fine-tune regulation of the cellular TH responses.

Metamorphosis is a critical developmental stage mediated by THs in flatfish. The function of TRs as transducers of genomic TH responses has converted these NRs in targets to clarify the molecular mechanisms that govern metamorphosis. In Senegalese sole, only TRβ and the TRβv transcript levels increased during metamorphosis, resembling clearly the profile of T4 content (Fig. 4). Similar results were reported in *H. hippoglossus* with the TRβ gene exhibiting an expression peak at climax (stage 9) (Galay-Burgos et al., 2008). In contrast, neither TRαB nor TRαBtr mRNA levels changed significantly and TRαA only showed a statistically significant peak at 9 DAH. These data agree with those reported for halibut where TRαb was found to be expressed at similar levels throughout the metamorphic stages and TRαa transcripts increased although not statistically significant at climax (Galay-Burgos et al., 2008). The importance of TRβ in metamorphosis has also been reported in *Xenopus* where TRβ, and not TRα, expression correlates with the metamorphosis climax and is regulated by THs (Yaoita and Brown, 1990). Different results were reported for *P. olivaceus* and *P. maxima*. Although TRβ transcripts increased at climax and remained high until completion of metamorphosis, only TRα showed an expression profile similar to T4 contents with a peak at climax (Marchand et al., 2004; Yamano and Miwa, 1998). Further investigation is required to elucidate the precise role of each TR gene, their spliced variants as well as their regulatory interactions to coordinate metamorphosis in flatfish. Also, a comparative cross-species analysis between Pleuronectiformes will contribute to clarify the gain and loss of functions of TR orthologs during evolution and their role to coordinate such drastic morphological and physiological changes.

Exogenous treatments with TU have proven useful to study TH-mediated gene expression changes of factors involved in the hypothalamic–pituitary–thyroid axis, glycolytic pathway, translational apparatus, cell structure and defense in *S. senegalensis* (Infante et al., 2008a; 2007; Manchado et al., 2007a; 2008a,b). Our results demonstrate that only TRβ is positively regulated by their cognate ligand during metamorphosis (Figs. 5 and 6). No data about TH-dependence of TRs in flatfish is available although in zebrafish larvae an up-regulation of both TRα1 and TRβ1 by THs has been described (Liu et al., 2000). This differential regulation of TRs represents a valuable tool for the organism to undergo distinct cel-

lular responses under stress, environmental challenges, metabolic requirements or physiological homeostasis. Nevertheless, the most intriguing result is the time-dependent differences in TRβ responses to T4 treatments (Fig. 6). Whereas exogenous T4 induced TRβ mRNAs to a higher level than untreated larvae at 15 DAH (when the untreated larvae close to climax), no statistically significant difference was detected at 20 DAH (completion of metamorphosis). We can hypothesize that THs activate the transcription of TRβ that in turn would activate downstream-regulated genes to promote metamorphosis. Moreover, this TRβ induction might be mediated by TRα avoiding an overexpression of TRβ gene and modulating responses after the climax. However, the involvement of other factors such as the retinoic acid, a known regulator of larval development in fish, through the RXR receptors, as well as a post-transcriptional regulation by phosphorylation of TRs are hypothesis that deserve to be investigated.

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