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The thyroid gland and thyroid hormones in Senegalese sole (*Solea senegalensis*) during early development and metamorphosis

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Abstract

We here describe the ontogeny and morphology of the thyroid gland in Senegalese sole (*Solea senegalensis*), and correlate these with whole body concentrations of thyroid hormones during early development and metamorphosis. Under our rearing conditions at 19.5 °C, most larvae entered metamorphosis in stage 1 at 15 days post-hatching (dph), and completed metamorphosis in stage 4 at 25 dph. The onset of metamorphosis coincided with surges in whole body T4 and T3 concentrations. Crossmon's trichrome stain colored the lumen of follicular structures brightly red, and this co-localized with a T4-immunoreactivity. Thyroid follicles were first observed in stage 0 pre-metamorphic larvae at 5 dph of age, and were detected exclusively in the subpharyngeal region, surrounding the ventral aorta. Increases in whole body thyroid hormone levels coincided with a 2½-fold increase in the total thyroidal colloid area in stage 1 larvae (aged 15 dph) compared to stage 0 larvae (12 dph). This was preceded by an approximately 40%-increase in the follicles' epithelial cell height in stage 0 larvae at 12 dph compared to larvae at 5 dph, and by an increase in the whole body T3/T4 ratio, indicative of an increase in outer ring deiodination. We conclude that in *S. senegalensis* there is a clear chronology in the activation of the thyroid gland that starts in early pre-metamorphic larvae.

Keywords: *Solea senegalensis*; Metamorphosis; Thyroid hormones; Thyroid gland; Ontogeny

1. Introduction

Metamorphosis is a crucial developmental phase in flatfish species. The transformation from a symmetric pelagic larva to an asymmetric benthic juvenile most conspicuously involves eye migration and craniofacial remodelling. Other transformations are recalibration of vision, changes in skin pigmentation and scale patterns, body shape, digestive tract, and feeding behavior. Thyroid hormone is a key endocrine factor in metamorphosis. Indeed, elevated whole body thyroxine (T4) levels during pre-metamorphosis and metamorphic climax were measured in flatfish species (Einarsdóttir et al., 2006; Miwa et al., 1988; Schreiber and Specker, 1998). Also, the mRNA expression of thyroid hormone receptor subtypes peaks during climax and post-climax of developing Japanese flounder, *Paralichthys olivaceus* (Yamano and Miwa, 1998), coinciding with whole body T4 concentrations that reach maximum values during the same metamorphic stages in this species (Tagawa et al., 1990a). In non-flatfish teleosts, thyroid hormones also affect pervasive transformations, *e.g.,* by inducing precocious metamorphosis and smoltification, accelerating yolk absorption, and inducing larval somatic growth (de Jesus and Hirano, 1992; de Jesus et al., 1998; Miwa and Inui, 1985; Tagawa and Hirano, 1987). Furthermore, treatment with T4 or T3 rescues zebrafish (*Danio rerio*) and

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Mozambique tilapia (*Oreochromis mossambicus*) larvae from developmental arrest induced by thyrostatics (Brown, 1997; Reddy and Lam, 1992b).

Metamorphosis is often associated with the acquisition of a new habitat. In an ecological context, it presents a period of increased vulnerability (Osse and Van den Boogaart, 1997). Indeed, year-class strength of feral flatfish populations is greatly determined by mortality operating during pre-juvenile stages (van der Veer and Nash, 2001). In aquaculture, differences in timing of the metamorphic stages expose slower growing larvae to larger, potentially cannibalistic, settled juveniles. Moreover, defective metamorphosis can result in maldevelopment and malpigmentation of juveniles and adults. All these factors form a major obstacle in the successful aquacultural exploitation of flatfish (Power et al., 2001).

Exogenous thyroid hormones, administered by simple immersion protocols to gravid females, larvae, or pre-metamorphic juveniles, enhance the maturation of oocytes, improve larval survival, synchronize metamorphosis, and produce uniform cohorts in flatfish (Gavlik et al., 2002; Solbakken et al., 1999; Stickney and Liu, 1999; Subburaju et al., 1998; Tanaka et al., 1995; Yoo et al., 2000). However, in some flatfish and non-flatfish species, thyroid hormone treatment of gravid females and larvae was found to have negative effects, *viz*. increased prevalence of skeletal abnormalities in larval offspring and growth retardation in developing larvae (Huang et al., 1998; Mylonas et al., 1994; Reddy and Lam, 1992a). The effects of thyroid hormones seem to be dependent on the dose, the developmental stage at which they are administered, and/or the biological species (Huang et al., 1998; Power et al., 2001). The manipulation of the thyroid hormone status to improve broodstock quality clearly has intricacies that are, as yet, poorly understood.

The Senegalese sole (*Solea senegalensis*) is a species with great economic potential in the North African Atlantic and in the Iberian countries, where it is cultured at a commercial scale (Dinis et al., 1999; Imsland et al., 2003). *S. senegalensis* is a euryhaline marine teleost flatfish with a wide distribution in littoral and estuarine environments of the Eastern Atlantic from the Gulf of Biscay to the coasts of Senegal (Cabral and Costa, 1999; Desoutter, 1990). The natural spawning season is from February to the end of May, and a second, shorter period from October to early November (see García-López et al. (2006) for a summary on the reproductive biology of *S. senegalensis*). Spawning is influenced by ambient temperature, with the optimum temperature in the range of 15–21 °C (Anguis and Cañavate, 2005).

Thus far, there are very few reports on the endocrine aspects of Senegalese sole metamorphosis. Ortiz Delgado et al. (2006) described only thyroid gland morphology in *S. senegalensis*, and the putative involvement of insulin-like growth factors in *S. senegalensis* metamorphosis was investigated by Funes et al. (2006). We here describe the ontogeny and morphology of the thyroid gland in *S. senegalensis*, and correlate these with whole body concentrations of thyroid hormones during early development and metamorphosis. This study aims to gain more insight in the changes in the thyroid system of *S. senegalensis* during critical early developmental events.

2. Materials and methods

2.1. Animals

Fertilized *S. senegalensis* eggs were obtained from Centro IFAPA "Agua del Pino" (Huelva, Spain) (Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía) on 12 May 2005. Eggs were transported to laboratories of the Instituto de Ciencias Marinas de Andalucía, CSIC (Puerto Real, Cádiz, Spain) 2 days before hatching. Eggs, larvae and juveniles were kept at 19.5 °C throughout. Newly hatched larvae (4 dph) were transferred to three 300-l tanks containing seawater with a salinity of 33 ppt. Larvae were kept at an initial density of 60–70 larvae per litre. A continuous water flow of 0.3–0.6 l/min and a 12 h light:12 h dark photoperiod were maintained during culture. At an age of 3 dph, larvae were fed on rotifers (*Brachionus plicatilis* O.F. Müller 1786), strain S-1 (size range: 120–300 μm), at a prey density of 5–10 individuals/ml; and on freshly hatched *Artemia* nauplii from 6 dph onwards. Microalgae (*Nannochloropsis gaditana*) at a final concentration of 3×10^5 cells/ml, were also added to the rearing tanks from first feeding. From the three tanks samples consisting of 50–111 larvae were taken on the time points indicated in the figures. To characterize the metamorphic progress, larvae were staged using the staging scheme in Table 1 that is based on a scheme developed by Fernández-Díaz et al. (2001). Frequency distributions were constructed from which the maximal frequency (%) of developmental stages 1, 2 and 3, and the larval age (dph) at which they were observed were derived. For the purpose of histology and immunocytochemistry, samples were briefly (5 s) rinsed with distilled water and fixed in Bouin-Hollande's fixative for 24 h at room temperature. Samples were stored in 70% ethanol at room temperature until further analysis. To determine whole body total thyroid hormone concentrations, larvae were rinsed with distilled water, adhering water was removed with a tissue, and larvae were pooled to obtain a total sample weight of 0.5–1 g. Samples were frozen in liquid nitrogen and stored at −80 °C until further analysis. Our experimental protocols complied with the Guidelines of the European Union Council (86/609/EU) and of the University of Cádiz (Spain) for the use of laboratory animals.

2.2. Histology

Larvae were dehydrated in a graded ethanol series and toluene, and embedded in paraffin. Ten-micrometer sections were collected on glass microscope slides coated with poly-L-lysine, and allowed to settle overnight at 45 °C. After deparaffination in xylene and rehydration in a graded ethanol series, sections were stained with a modification of Crossmon's trichrome tissue stain (Crossmon, 1937): 0.2 g Light Green (Light Green SF yellowish, from Chroma-Gesellschaft, Stuttgart, Germany), 0.25 g Orange G (Searle Diagnostic, High Wycombe, UK) and 0.3 g acid fuchsin (Fuchsin S, from Chroma-Gesellschaft) were dissolved in 50 ml distilled water at 80 °C. The solution was cooled to room temperature, and 1 g of phosphotungstic acid hydrate was added, followed by 2 ml glacial ethanoic acid and 100 ml absolute ethanol. The final solution was filtered and stored. Crossmon's trichrome stain was followed by a conventional haematoxylin counter-stain. Images were captured using a Leica DM-RBE light microscope and a Leica DC500 digital camera. The red-stained colloid of single follicles in the digitalized images was manually selected and quantified using the MetaMorph software from Molecular Devices Corp. (Downingtown, PA, USA). Serial sections of whole larvae were analyzed for the number of colloid-containing thyroid follicles, and total colloid area. Care was taken to count only once those follicles that appeared in subsequent serial sections.

Table 1
External features of *Solea senegalensis* larvae raised at 19.5 °C

Metamorphic stage	External features
0.1	Pelagic, bilaterally symmetric larvae Vertical swimming plane Mouth closed, no skin pigmentation Body length approximately 2.5 mm
0.2	Pelagic, bilaterally symmetric larvae Yolk sac is largely absorbed Mouth open, eyes and skin pigmentation appear Body length approximately 4 mm
0.3	Similar in shape to larvae in stage 0.3 Body length > 4 mm More abundant skin pigmentation
1	Pelagic, asymmetric larvae First appearance of migration of the left eye, left eye still ventral of the dorso-medial line
2	Left eye touches dorso-medial line, left eye visible from the right-ocular side First appearance of dorsal fin ray absorption First appearance of lateral flattening of the body and change of swimming plane
3	Left eye has crossed the dorso-medial line
4	Benthic, asymmetric larva Left eye has reached its final position, both eyes are now positioned on the right side of the body Orbital arch is clearly visible Laterally flattened body shape

2.3. Immunocytochemistry

A rabbit thyroxine-specific antibody was obtained from ICN Biomedicals (Zoetermeer, The Netherlands). Sections were deparaffinated in xylene and hydrated in a graded ethanol series and distilled water, pre-incubated for 15 min in 3% H_2O_2 to abolish endogenous peroxidase activity, and rinsed in distilled water and Tris-buffer (pH 7.8), respectively. Then, sections were incubated overnight at room temperature with the primary antibody (anti-T4 diluted 1:5000 in Tris-buffered saline). Sections were washed in Tris-buffer and incubated with the secondary antibody goat-anti-rabbit IgG (dilution 1:200) for 1 h at room temperature. Afterwards, slides were rinsed again in Tris-buffer (3 × 5 min) and incubated in a 1:50 diluted peroxidase–anti-peroxidase solution in TBS for 45 min at room temperature. Slides were rinsed in Tris-buffer and incubated for 10–15 min in a diaminobenzidine solution containing 0.04% $NiSO_4$ and 0.2% H_2O_2 to detect primary antibody binding. Finally, slides were rinsed in distilled water, dehydrated and mounted with Entellan®.

2.4. Thyroid hormone extraction and analysis

Larval samples were dried at 60 °C to constant dry weight. Thyroid hormones were extracted as described by Tagawa and Hirano (1987). Samples (0.02–0.05 g larval dry weight) were homogenized in 2.6 ml ice-cold 99:1 (vol./vol.) methanol:ammonia containing 1 mM of the iodothyronine deiodinase inhibitor 6-*n*-propyl-2-thiouracil (PTU). Homogenate and extraction media were thoroughly mixed for 10 min at 4 °C, and then centrifuged at 2000*g* (15 min, 4 °C). This procedure was repeated twice, supernatants were pooled and lyophilized. The residue was resuspended in 875 µl of a 6:1 vol./vol. mixture of chloroform and 99:1 methanol:ammonia including 1 mM PTU, and 125 µl barbital buffer (50 mM sodium barbitone in distilled water, at pH 8.6). Samples were mixed for 10 min at room temperature. The upper phase was aspirated and lyophilized at 45 °C. Residues were redissolved in 60 µl barbital buffer containing 0.1% bovine serum albumin. Aliquots of 25 and 50 µl were taken for T4 and T3 analysis, respectively. Total T4 (tT4) and T3 (tT3) concentrations were measured in duplicate with a competitive ELISA (Human Gesellschaft für Biochemica und Diagnostica GmbH, Wiesbaden, Germany) according to the manufacturer's instructions. Calibrators were prepared in the same barbital buffer matrix as the samples. A logit-log plot produced linear calibration curves from which total thyroid hormone levels in the samples were calculated by least squares linear regression. The intra-assay and inter-assay coefficients of variation for the tT4 and tT3 ELISA reported by the manufacturer are 4.2% and 3.3%, and 2.6% and 2.4%, respectively. The reported cross reactivity of the ovine anti-T4 antibody to D-T4 is 98% (the reactivity to L-T4 is set at 100% as a reference), and to L-T3 and D-T3 is 3% and 1.5%, respectively. The reported cross reactivity of the ovine anti-T3 antibody to L-T4 is <0.02% (setting the reactivity to L-T3 at 100%). Cross reactivities of both antibodies to diiodothyronine, diiodotyrosine and iodotyrosine are <0.01%. The reported sensitivities of the tT4 and tT3 ELISA are 4 ng/ml T4 and 0.05 ng/ml T3, respectively.

2.5. Statistics

Differences between two groups were tested using Student's *t*-test (two-tailed) for unpaired data. Morphometric data were analyzed by one-way analysis of variance (ANOVA) using time (dph) as a source of variation. Tukey–Kramer's multiple comparisons test was used as a post-test, when appropriate. Statistical significance was accepted at $P < 0.05$, and is indicated with the following symbols: $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$.

3. Results

Fig. 1 shows a typical frequency distribution of the developmental stages during *S. senegalensis* metamorphosis. Stages 0–4 follow chronologically, but larvae do not develop synchronically. Judged from the width of the frequency distributions, the transit of larvae through a developmental stage takes 4–6 days to complete. Under our rearing conditions, larvae from developmental stages 1, 2,

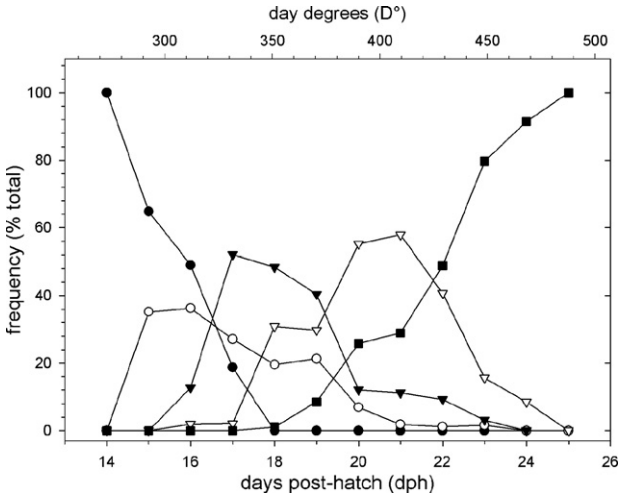

Fig. 1. Frequency distribution of the metamorphic stages of *S. senegalensis* larvae as a function of larval age (dph). Samples contained 50–111 larvae. The distribution is representative for two different batches of larvae raised in four different tanks. Day degrees (D°, top *x*-axis) are calculated as the product of days-post-hatch and the constant temperature (19.5 °C) at which the larvae were raised. Symbols: (●) stage 0; (○) stage 1; (▼) stage 2; (▽) stage 3; (■) stage 4.

Table 2
Maximal frequency (%) of developmental stages 1, 2 and 3 of *Solea senegalensis* larvae and the larval age (dph) at which it is observed

Metamorphic stage	Maximal frequency (%)	Larval age (dph)
1	52 ± 7	16 ± 0.6
2	48 ± 7	18 ± 0.6
3	58 ± 20	20 ± 0

Means ± SD from 3 samples consisting of 50–111 larvae each are shown. (Maximal frequencies of the initial stage 0 and final stage 4 are, implicitly, 100%.)

3 and 4 are simultaneously present in considerable numbers between 18 and 21 dph. Table 2 shows the maximum frequencies of stages 1, 2 and 3 during sole metamorphosis. The mean larval ages at which the maximum frequencies appear show little variation, as indicated by the coefficients of variation ⩽3%. In contrast, the maximum frequencies of stages 1, 2 and 3 are approximately 50% and have coefficients of variation ranging from 12% to 28%, again indicative for asynchronous development of larvae within the same batch. Few abnormalities were observed during metamorphosis; per sample, 0–2.5% of the larvae displayed incorrect eye migration, abnormal pigmentation, or malabsorption of the dorsal fin rays.

Thyroid hormones are detectable in eggs with pre-hatch *S. senegalensis* larvae at concentrations of 1.5 ± 0.4 ng T4/g and 0.3 ± 0.09 pg T3/g wet weight (13 ± 3 ng T4/g, 2.5 ± 0.8 pg T3/g dry weight). Whole body total T4 levels in *S. senegalensis* larvae are low in the early stages of development, sharply increase at 9 dph, *i.e.* when all larvae are in stage 0, and reach a maximum at 15 dph (Fig. 2). The fourfold increase in T4 levels in 2-dph larvae compared to pre-hatch larvae is statistically significant ($P = 0.0003$). At 17 dph, total T4 levels have decreased by 53% and remain stable at this level throughout the sampling period. Whole body total T3 concentrations are three orders of magnitude lower than those of total T4 (Fig. 2). Except from the increase from 25 to 40 dph, the changes in total T3 levels exhibit a profile similar to that of total T4. The profiles of total whole body T4 and T3 levels normalized for wet body weight and dry body weight are virtually identical, indicating that the fractional body water content is stable and does not confound hormone concentration measurements.

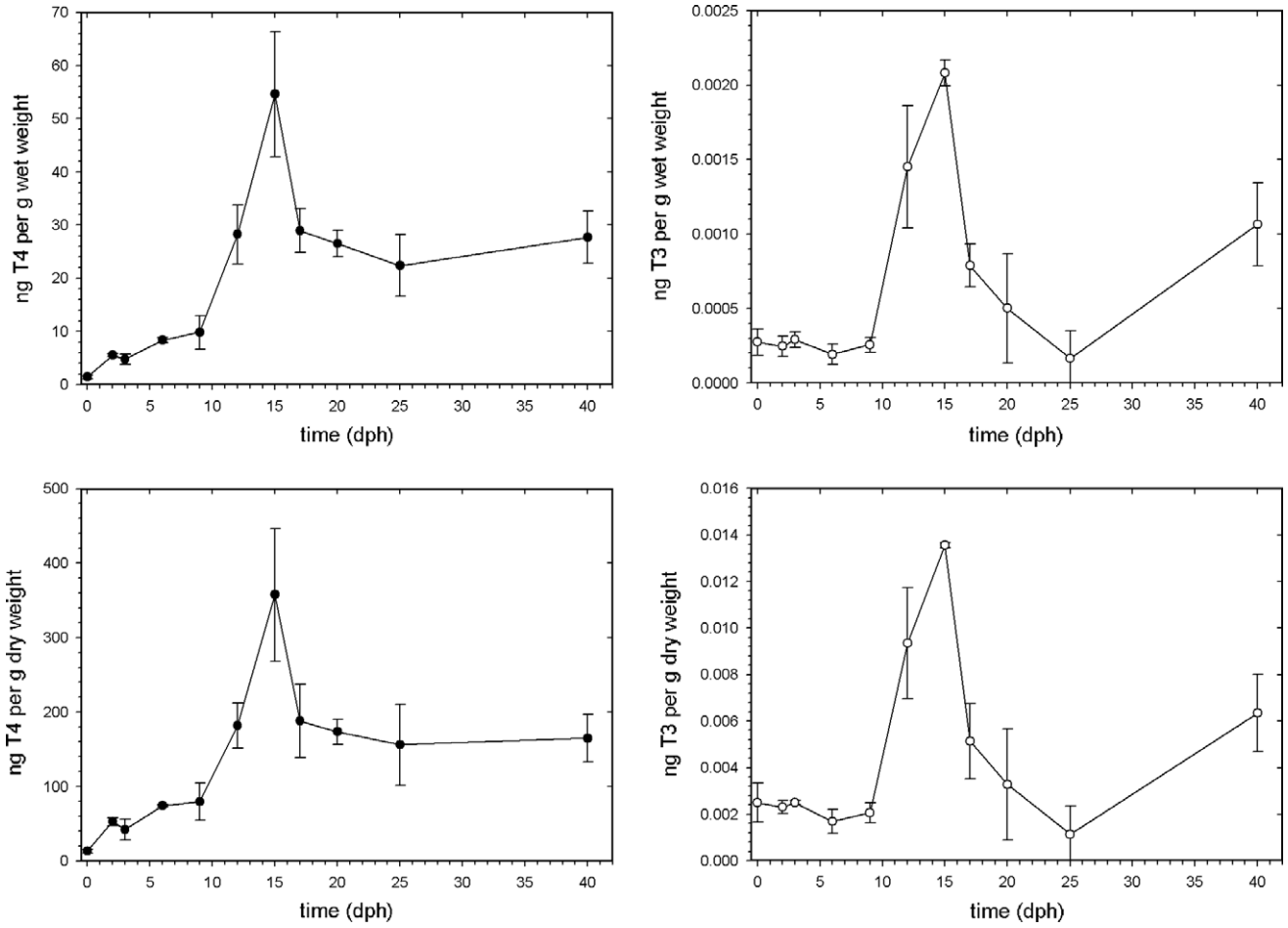

Fig. 2. Whole body total T4 (●) and total T3 (○) concentrations, expressed per gram wet body weight (top panels) and per gram dry body weight (bottom panels), in *S. senegalensis* during larval development. Larvae included in the ⩽12 dph-samples are pre-metamorphic and were verified to be in stage 0. Similarly, larvae at 15, 17, 20 and ⩾25 dph represent metamorphic stages 1, 2, 3, and 4, respectively. Each point represents the average of three pooled samples comprising 0.5–1 g wet weight larval biomass. Data are expressed as means ± SD.

The molar ratio of T3 to T4 is highest in pre-hatch larvae, and decreases in hatched larvae (Fig. 3). Between 9 and 15 dph, the T3/T4 ratio rises to a peak value but is still well lower than in pre-hatch larvae. The twofold increase in the molar ratio observed at 12 dph compared to 6-dph larvae is statistically significant ($P = 0.046$). In 40-dph juveniles the T3/T4 ratio had significantly increased fivefold compared to 25-dph animals ($P = 0.023$).

Fig. 4 shows that the bright-red stain of the lumen of follicular structures obtained with Crossmon's trichrome co-localizes with a specific T4-immunoreactivity, and we therefore identify these structures as colloid-containing thyroid follicles. Thyroid follicles were first observed in some, but not all, larvae at 5 dph of age (Fig. 5). Thyroid tissue in *S. senegalensis* larvae was exclusively located in the subpharyngeal region, surrounding the ventral aorta. Peak levels of whole body T4 and T3 concentrations, measured at 15 dph, coincide with statistically significant increases of the total area of colloid in the thyroid follicles at this time point (Fig. 6). Thyrocyte cell height had increased significantly by 37% in pre-metamorphic stage 0 larvae aging 12 dph compared to larvae at 5 dph, and this increase was maintained throughout metamorphosis (Table 3).

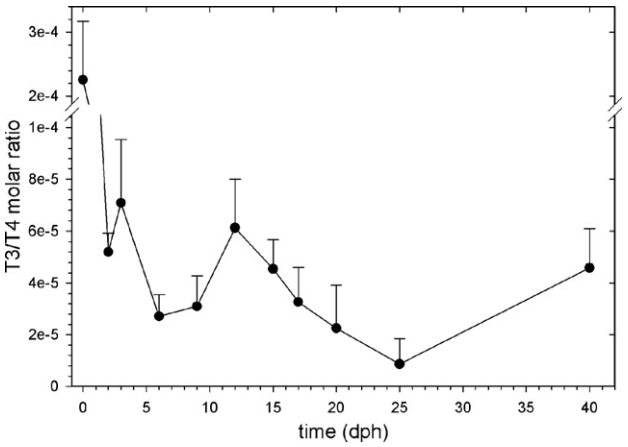

Fig. 3. Molar ratios of whole body total T3 to total T4 concentrations (expressed in mole per gram dry body weight) in *S. senegalensis* during larval development. Ratios are calculated from the data in Fig. 2, and presented as means + SD.

4. Discussion

The onset of metamorphosis in *S. senegalensis*, under our rearing conditions, was clearly marked by the transition of larvae from stage 0 to stage 1 at 15 dph. This event coincided with surges in whole body T4 and T3 concentrations, as it does in other flatfish (de Jesus et al., 1991; Einarsdóttir et al., 2006; Miwa et al., 1988) and non-flatfish species (Crane et al., 2004; de Jesus et al., 1998; Deane and Woo, 2003; Szisch et al., 2005). The increases in whole body thyroid hormone levels coincided, again, with a 2½-fold increase in the total thyroidal colloid area in stage 1 larvae (aged 15 dph) compared to pre-metamorphic stage 0 larvae (12 dph), indicative for a proliferation of thyroid

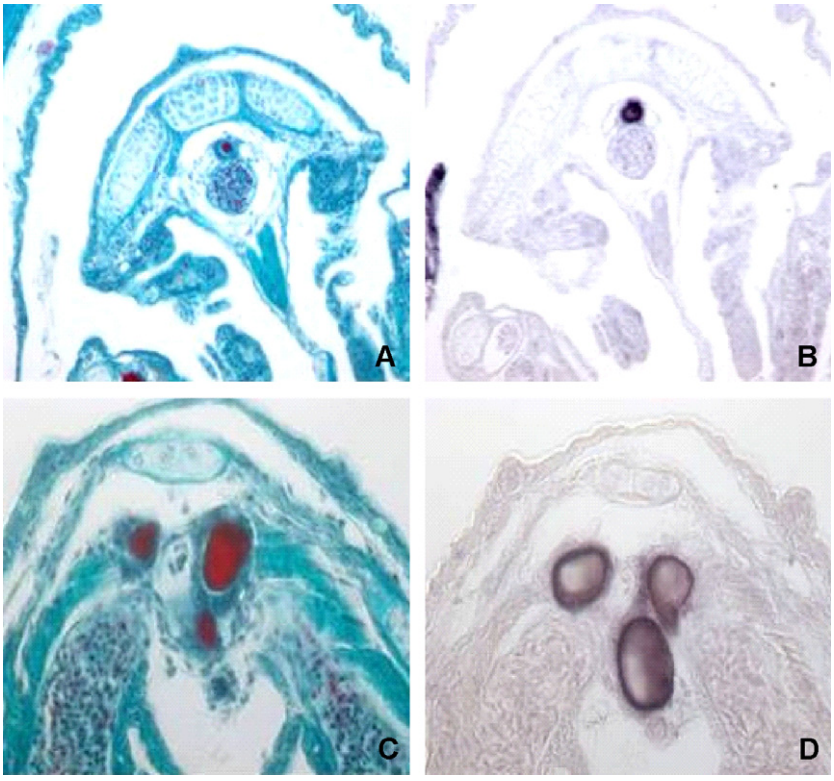

Fig. 4. Serial transversal sections (section thickness: 10 μm) of the subpharyngeal region of *S. senegalensis* larvae (metamorphic stage 4; larval age 24 dph), stained with Crossmon's trichrome (A and C) or anti-T4 (B and D).

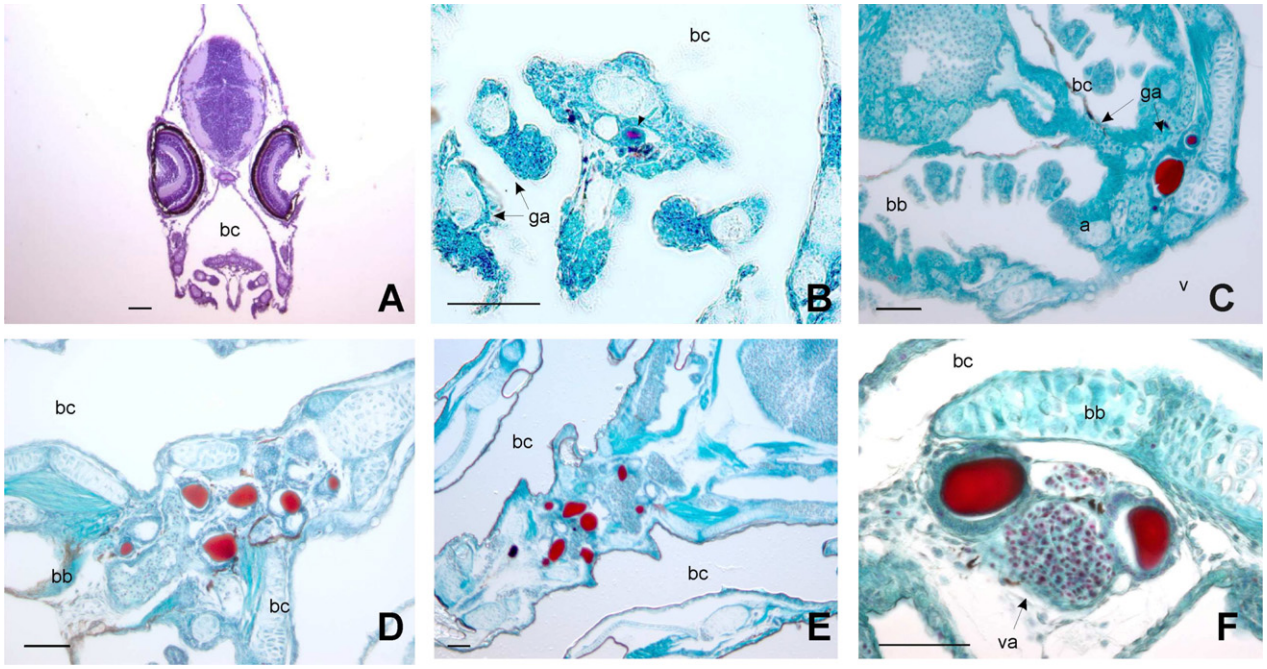

Fig. 5. Haematoxylin/eosin (A) and Crossmon's trichrome (B–F) staining on transversal and sagittal sections of *Solea senegalensis* larvae. (A) Transversal section of a pre-metamorphic larva at 5 dph showing from dorsal to ventral: brain, left and right eye, buccal/pharyngeal cavity and gills. (B) Transversal section through the subpharyngeal region of a larva at 5 dph showing a brightly stained follicle (arrow head). (C–E) Frontal sections through the subpharyngeal region of a larva in metamorphic stage 1 at age 15 dph, stage 2 at age 18 dph, and stage 3 at 21 dph, respectively. (F) Transversal section through the subpharyngeal region of a larva in stage 4 at 24 dph. Scale bar represents 100 μm. Abbreviations: a, atrium; bb, basibranchial bone; bc, buccal cavity; ga, gill arches; v, ventricle; va, ventral aorta.

tissue. This was preceded by an approximately 40%-increase in the follicles' epithelial cell height and whole body T3/T4 ratio in stage 0 larvae at 12 dph. In addition, this coincides with a maximum in whole body energy content observed by Parra and Yúfera (2001) in *Solea* larvae of this age. Thus, there is a clear chronology in the metamorphosis-associated activation of the thyroid gland that already starts in pre-metamorphic larvae. This warrants the investigation of components of the thyroid system, other than variations in thyroid hormone levels, that are activated during early post-embryonic development.

The activation of the thyroid gland in vertebrates is controlled by the hypothalamus–pituitary–thyroid (HPT) axis. Indeed, the hypertrophy of thyrocytes that we observed can be interpreted to be indicative for a sustained stimulation by TSH from the pituitary pars distalis (Collins and Capen, 1980). Evidence for the functional involvement of the pituitary gland in flatfish metamorphosis was obtained in Japanese flounder (*P. olivaceus*) larvae, where treatment with bovine TSH induced an increase in whole body T4 levels and accelerated metamorphosis (Inui et al., 1989). In *S. senegalensis*, the pituitary gland is already visible in pre-metamorphic larvae at an age of 1 dph (Piñuela et al., 2004), but it is unclear if thyrotrope cells have already developed at that time. In Atlantic halibut (*Hippoglossus hippoglossus*), pituitary thyrotropes appear relatively late, in first-feeding larvae, compared to growth hormone-, prolactin- and somatolactin-producing cells that are already detectable in the yolk sac stage (Einarsdóttir et al., 2006). In flatfish species other than *S. senegalensis*, pituitary thyrotropes appear at the onset of metamorphosis or increase in number during pre-metamorphic stages (Einarsdóttir et al., 2006; Miwa and Inui, 1987). Moreover, the inferior lobes of the hypothalamus appear as late as 3 dph in *S. senegalensis*, and diencephalic regions continue to develop well into the post-metamorphic stages (Piñuela et al., 2004). It remains to be established whether a functional HPT axis is present, and what thyrotropic factor induces the early activation of the thyroid gland during pre-metamorphic stages of *S. senegalensis*.

We interpret the increases in the molar T3/T4 ratio, observed in stage 0 larvae at 12-dph and in 40-dph stage 4 juveniles, to be indicative for an upregulation of a peripheral 5′-deiodinase activity. Indeed, 5′-deiodinase types 1 and 2 are expressed differentially temporally during post-embryonic development in turbot (*Scophthalmus maximus*), zebrafish and rainbow trout (*Oncorhynchus mykiss*) (Marchand et al., 2004; Orozco et al., 2003; Walpita et al., 2007), indicating their involvement in metamorphosis. The conversion, by 5′-deiodination, of T4 to the potently bioactive thyroid hormone T3 is an important, if not the most important metabolic pathway of thyroid hormones. Compared to amphibians and agnathans, the role of deiodinases in the metamorphosis of flatfish is very poorly studied. We are currently characterizing *S. senegalensis* 5′-deiodinase types 1 and 2 by polymerase chain reaction (PCR) and

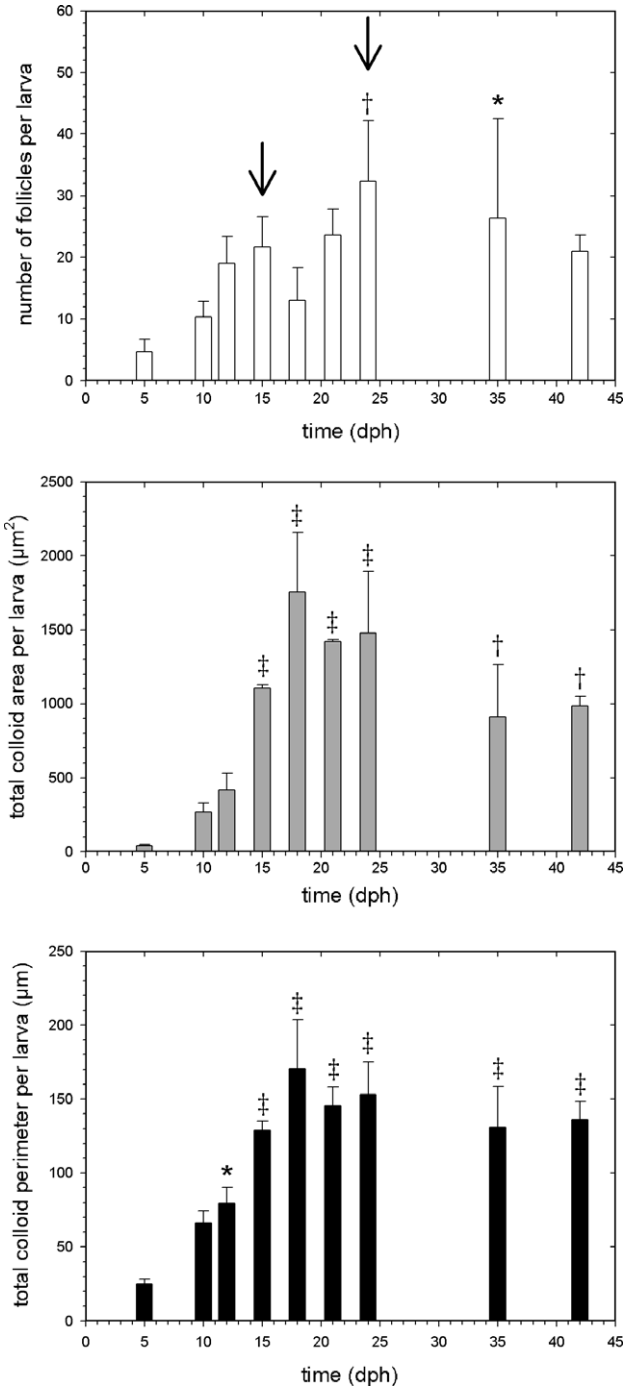

Fig. 6. Morphological parameters of the thyroid gland of *S. senegalensis* during larval development. The total number of thyroid follicles per larva (top panel), and the total area (μm^2) of the follicular colloid per larva (bottom panel) are shown. Data are presented as the average of all follicles in three larvae per time point and expressed as means ± SD. Arrows indicate start and end of metamorphosis at 15 and 24 dph, respectively. Larvae included in the 5-, 10- and 12 dph-samples are pre-metamorphic and were verified to be in stage 0. Similarly, larvae at 15, 18, 21 and ⩾24 dph represent metamorphic stages 1, 2, 3, and 4, respectively. Using time (dph) as a source of variation, analysis of variance of the number of thyroid follicles per larva, and the total colloid area per larva yielded the following *F*- and *P*-values, respectively: $F = 4.198$, $P = 0.0055$; $F = 19.105$, $P < 0.0001$. Statistically significant differences with respect to the first time point (5 dph) are indicated by the symbols: $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$.

Table 3
Epithelial cell heights (μm) of thyrocytes in *Solea senegalensis* larvae

Larval age (dph)	Epithelial cell height (μm)	*P*
5	5.4 ± 1.4	—
12	7.4 ± 0.3	<0.05
15	7.8 ± 1.5	<0.05
18	7.5 ± 0.9	NS
24	7.9 ± 0.9	<0.01
42	6.8 ± 2.5	NS

Data represent measurements of 3 × 3 thyrocytes in 3 thyroid follicles per larva in a group of 3. Larvae included in the 5- and 12 dph-samples are pre-metamorphic and were verified to be in stage 0. Larvae at 15, 18 and ⩾24 dph represent metamorphic stages 1, 2, and 4, respectively. Using time (dph) as a source of variation, analysis of variance of epithelial cell height resulted in $F = 3.723$, and $P = 0.0063$. The probabilities (*P*) of the differences with respect to the first time point (5 dph) are indicated. (NS: not significant.)

nucleotide sequencing, and intend to establish their tissue distribution and ontogeny.

Crossmon's trichrome stain satisfactorily stained the colloid in the lumen of the thyroid follicle brightly red, which facilitated the identification of thyroid follicles and computer-assisted analysis of digitalized images. The staining of the colloid is similar to that obtained with Cleveland-Wolfe's trichrome stain in Atlantic halibut (Einarsdóttir et al., 2006). Most likely, the anionic azo dye Orange G, the common ingredient in both trichrome stains, binds to acidophilic groups, probably basic amino acid side chains of colloidal proteins. We observed the first thyroid follicle at 5 dph, which time point is roughly in agreement with the findings of Ortiz Delgado et al. (2006) who detected follicular T4 immunoreactivity in *S. senegalensis* larvae 6 dph of age. Remarkably, we have counted approximately four times the number of thyroid follicles in developing sole larvae as these authors do using a haematoxylin–eosin stain. Interestingly, the trichrome-stained colloid in the first follicles of these early *S. senegalensis* larvae colors more pink than red, and this was also observed in Atlantic halibut yolk sac larvae (Einarsdóttir et al., 2006). For sure this indicates a biochemical property of the early thyroidal colloid, perhaps of thyroglobulin in particular, that is different in older larvae. Whether this also reflects differences in, *e.g.*, colloidal protein density or thyroid hormonogenesis between early and late larvae remains speculative.

Peripheral components of the thyroid system, *i.e.* thyroid hormone receptors and iodothyronine deiodinases, are already detectable, albeit by their mRNA expression, in embryonic life stages as early as blastulation of gilthead seabream (*Sparus auratus*), zebrafish and Japanese flounder (Liu et al., 2000; Nowell et al., 2001; Power et al., 2001; Walpita et al., 2007; Yamano and Miwa, 1998). In many teleostean species, the ligands and substrates for these receptors and enzymes are already present in fertilized eggs, in concentrations ranging from 50 to 100 fmol to more than 20 pmol T4 or T3 per gram (Mylonas et al., 1994; Power et al., 2001; Tagawa et al., 1990b). Embryonic thyroid hormone levels change markedly during development:

by the time of hatching, T4 and T3 concentrations can have decreased to undetectable levels as they do in two Sparidae species (Tagawa et al., 1990b). Conversely, they may have increased as dramatically as 27-fold for T4 levels in pre-hatch chinook salmon (*Oncorhynchus tshawytscha*) eggs (Greenblatt et al., 1989), indicating an active role of teleostean embryos in whole body thyroid hormone economy. Indeed, early and developing teleostean embryos can load radio-iodide and synthesize thyroid hormones (Brown, 1997; Elsalini and Rohr, 2003; Greenblatt et al., 1989). Interestingly, T4 immunoreactivity has been observed in non-follicular cells before thyroid follicles have appeared in early (80 h post-fertilization) zebrafish larvae (Wendl et al., 2002). Clearly, the developing teleostean embryo expends, produces, and metabolizes thyroid hormones. This can explain the low, but apparently physiologically relevant T3 concentrations, that were two orders of magnitude lower than the lowest concentration reported in literature, that we measured in *S. senegalensis* pre-hatch larvae.

Larvae from the same batch of fertilized eggs develop asynchronically, and this represents a major aquaculture problem as smaller larvae are exposed to potentially cannibalistic larger juveniles. We have not classified larvae by age only, but by both developmental stage and age. The use of a standardized method of sampling has been suggested by Sæle et al. (2004) to reduce data variability between larval samples from within and between batches. Indeed, under our rearing conditions, larvae from stages 1, 2, 3 and 4 are simultaneously present in considerable numbers between time points 18–21 dph. Judged from the width of the frequency distributions shown in Fig. 2, the transit of a cohort through a developmental stage takes 4–6 days to complete. We derive similar results from a study by Fernández-Díaz et al. (2001). Depending on the diet provided, these authors observed that *S. senegalensis* larvae entered metamorphosis between 9 and 15 dph, but showed equally as protracted transit times as in our study. Our larvae came from the same batch of fertilized eggs, and were exposed to the same water quality, temperature, photoperiod and feeding regime. This strongly suggests that individual internal factors, and not environmental factors, primarily underlie asynchronous larval development. We intend to investigate what distinguishes the thyroid system of an early metamorphosing larva from that in a larva that enters metamorphosis relatively late. This should yield further insights in the plasticity of the thyroid system, *i.e.* the HPT-axis, during sole metamorphosis, and can be the basis for a well-designed protocol for thyroid hormone treatment in the improvement of flatfish broodstock.

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