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Pituitary–thyroid axis development during the larval–juvenile transition in the pejerrey *Odontesthes bonariensis*

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The morphological development of the thyroid gland of pejerrey *Odontesthes bonariensis* during larval–juvenile transition was studied and related to whole-body concentrations of thyroxine (T_4) and tri-iodothyronine (T_3). A complementary (c)DNA fragment of the thyroid-stimulating hormone β-subunit (*tshb*) was sequenced and transcript levels quantified during this period. Follicles with eosinophilic and T_4-immunoreactive colloids were detected at hatching together with *tshb* transcript levels and whole-body concentrations of T_4 and T_3 hormones. Thyroid follicles were located in the subpharyngeal region associated with the ventral aorta below the hyoid bone. Follicle structure switched from the rounded form at hatching to oval in juveniles. Significant increase of follicle number per larva, mean colloidal area and total colloidal area was observed throughout development with maximum values at the end of the larval–juvenile transition. A significant decrease of *tshb* expression together with a significant increase in T_4 and T_3 whole-body concentrations was observed prior to achieving the juvenile phenotype. These results are in accordance with a negative feedback regulation of *tshb* expression by thyroid hormones and a possible association between thyroid hormone levels and the acceleration of metabolic processes necessary to complete metamorphosis.

Key words: metamorphosis; *Odontesthes bonariensis*; thyroid follicle; thyroid hormones; thyroid-stimulating hormone.

INTRODUCTION

Pejerrey *Odontesthes bonariensis* (Valenciennes 1835), is a species of regional socioeconomic importance due to sport fishing and aquaculture (Somoza *et al.,* 2008). This fish has emerged as a biological model to study the influence of external factors on sex determination and the gonadal differentiation process (Fernandino *et al.,* 2013). In addition, the relatively high sensitivity of this species to pollutants (Carriquiriborde & Ronco, 2006; Gárriz *et al.,* 2015), together with its availability for aquaculture research

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(Miranda *et al.,* 2006; Miranda & Somoza, 2009), has recently promoted its inclusion as acute toxicity testing species in Argentina (IRAM, 2007).

In spite of the significant advances made on rearing techniques of this species (Miranda *et al.,* 2006; Colautti *et al.,* 2010; Lichtenstein *et al.,* 2010; Sciara *et al.,* 2011; Gómez-Requeni *et al.,* 2012; Chalde *et al.,* 2014, 2016), the asynchronous development and low survival of *O. bonariensis* larvae remain a major constraint (Chalde *et al.,* 2011). Several morphological and physiological changes have been described during the first week after hatching. Fin fold absorption, caudal fin formation, body-shape transformation and sexual determination–differentiation processes were some main events leading the acquisition of the juvenile morphology (Strüssmann *et al.,* 1996; Ito *et al.,* 2005; Chalde *et al.,* 2011). The normal morphological transformation of *O. bonariensis* during larval–juvenile transition was described by Chalde *et al.* (2011). These authors defined the juvenile phenotype when the absorption of the fin fold is complete and the caudal fin acquires its definitive homocercal structure. Moreover, the end of this transition occurs when the fish attains >20·1 mm in total length (Chalde *et al.,* 2011).

Several morphological, physiological and ecological changes are regulated by thyroid hormones (TH) during larval–juvenile transition in fishes (McMenamin & Parichy, 2013). The thyroid hormones thyroxine (T_4) and tri-iodothyronine (T_3) are involved in a wide variety of processes that control growth, development, differentiation and metabolism in vertebrates (Hulbert, 2000; Power *et al.,* 2001; Blanton & Specker, 2007; McMenamin & Parichy, 2013). The functional units of thyroid gland in vertebrates are the follicles formed by epithelial cells surrounding a lumen full of colloid containing the T_4 precursor (Hulbert, 2000). While the thyroid follicles in tetrapods are encapsulated by connective tissue, however, in teleost fishes they are present as isolated follicles (Ortiz Delgado *et al.,* 2006; Geven *et al.,* 2007; Kawakami *et al.,* 2008; Klaren *et al.,* 2008; Hsu *et al.,* 2014). Regarding THs pathway in vertebrates, it is known that the production of T_4 by the thyroid gland is positively regulated by the thyroid-stimulating hormone (Tsh), a glycoprotein secreted by the pituitary gland (Szkudlinski *et al.,* 2002). T_4 is released from thyroid follicles and metabolized in extra thyroidal tissues into a more potent form T_3. This peripheral transformation is mediated by the action of the type 2 outer ring deiodinase enzyme (Darras & Van Herck, 2013).

Since thyroid action during larval–juvenile transition of *O. bonariensis* is unknown, the aim of this study was to describe the early development of the pituitary–thyroid axis, establishing baseline information on normal development. This information can be related to behavioural, morphological and physiological changes during this period.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN AND ANIMAL SAMPLING

Fertilized eggs were obtained in October from spontaneous spawning of a 6 year old *O. bonariensis* brood-stock at the IIB-INTECH aquaculture facilities (14L:10D photoperiod, temperature 17–18° C and salinity 1·5). Two groups of 1000 newly hatched larvae were kept in 130-l fibreglass tanks with flow-through water (one exchange every 4 h) under 12L:12D photoperiod and 1·5 salinity. Larvae were kept at 16·8 ± 0·4° C (mean ± S.D.), similar to the water temperature during the main hatched period in the wild (Elisio *et al.,* 2014, 2015). Water temperature was recorded every hour using waterproof electronic data-loggers (www.ibuttonlink.com). The

TABLE I. Total length (L_T) and days post hatching (dph) corresponding to early developmental stages of *Odontesthes bonariensis* (modified from Chalde *et al.,* 2011)

Developmental stages	Morphological features	L_T range (mm)	dph
A	Hatching	6·5–7·6	0
B	Notochord flexion and rays segmented	9·1–10·4	7–21
C	Caudal-fin rays completed and first dorsal-fin rays appeared	11·1–12·6	14–28
D	Forked homocercal caudal fin	13·9–15·0	21–35
E	Caudal-fin rays bifurcated	18·4–19·4	28–49
F	Fin-fold absorption and juvenile phenotype acquired	20·1–23·0	42–56

larvae were fed daily with *Artemia* nauplii until week 6 after hatching and thereafter supplemented with artificial fish food (www.shulet.com).

Fish were sampled weekly, early in the morning, from hatching to week 8, covering the entire period of larval–juvenile transition. Developmental stages (Table I) of 10 fish at hatching and 25 fish per week from there onward were assigned by length and anatomical features following the classification suggested by Chalde *et al.* (2011).

In order to facilitate the measurement of total length (L_T), fish were photographed using a dissecting microscope (Nikon SMZ800; www.nikon.com) connected to a digital camera and measured to the nearest 0·001 mm using digital images and the Image-pro plus 4.5 software (www.mediacy.com/imageproplus). Body mass (M_B) was measured to the nearest decimal milligram with an analytical balance. The L_T–M_B relationship was described following the equation proposed by Keys (1928): $\log_{10}M_B = b\log_{10}L_T + \log_{10}a$. Fulton's (1904) condition factor (K) was calculated in accordance with: $K = 1000M_B{L_T}^{-3}$, where M_B is body mass given in mg and L_T is total length given in mm. K was used as an indicator of body shape and the changes of this were related to the developmental stages.

The fish were euthanized by inmersion in benzocaine solution (ethyl p-aminobenzoate) and handled in accordance with the local regulation by the Institutional Committee for the Care and Use of Laboratory Animals of National University of San Martín (www.unsam.edu.ar/secretarias/investigacion/cicuae.asp).

HISTOLOGICAL AND MORPHOMETRIC ANALYSIS OF THYROID GLAND

The heads of five larvae per week were fixed in Bouin's solution for 16 h at 4° C, dehydrated and embedded in paraffin. Sagittal sections of 6 µm thickness were stained with haematoxylin and eosin and examined under microscope (Nikon Eclipse E200; www.nikon.com). All sections with thyroid follicles were photographed and analysed using the Image-Pro 4.5 software (www.mediacy.com/imageproplus). The presence of vacuoles in the periphery of the colloid was considered to be indicative of an actively secreting follicle. All follicles were counted and the area, length and width of six colloids randomly selected were measured on the photographs where the larger area was observed. The mean colloidal area per larva and the total colloidal area per larva were calculated from these data. The shape of the follicle was described according to Ponton (2006), where a higher aspect ratio (R_A) indicates a more elongated follicle: $R_A = L_{Tmax}{W_{max}}^{-1}$, where W_{max} is the maximum width.

For the immunostaining, sagittal sections (thickness 6 µm) of the heads of three newly hatched larvae were mounted on slides treated with 3-aminopropyltriethoxysilane (SigmaAldrich; www.sigmaaldrich.com). The sections were deparaffinized, hydrated in a series of alcohols and blocked with phosphate buffer saline (PBS; 0·1 M, pH 7·4), 0·5% bovine serum albumin and 0·3% Triton X-100. The incubation was done with mouse anti-thyroxine monoclonal antibody

(www.scbt.com) at 1:500 dilution (PBS 0·1 M, pH 7·4) for 18 h at 22° C. The sections were incubated with biotinylated secondary antibody and streptavidin-peroxidase complex (www.dako.com) for 45 min each and finally revealed with 3·3′-diaminobenzidine (DAB) in 0·1% PBS containing 0·02% H_2O_2.

PARTIAL SEQUENCING OF tshb 3′ END

Total RNA from one pituitary of an adult fish was extracted using TRIzol Reagent (Life Technologies; www.lifetechnologies.com) according to manufacturer recommendations. Total RNA was treated with DNase I (Life Technologies) and used as template for xomplementary (c)DNA synthesis using SuperScript II RNase H (Life Technologies) and an oligo(dT) Adaptor Primer: 5′-GGCCACGCGCGACTAGTAC $(T)_{15}$ g-3′. A forward primer (5′-TTGACTCCGACCCTGTCTTT-3′) was designed from a highly conserved coding region of the *tshb* of teleost searched by basic local alignment search tool (BLAST) at web servers of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). This primer and the reverse adaptor primer were used for amplification of the *tshb* subunit cDNA 3′ end. The PCR product was separated by electrophoresis, extracted by QIAEX II gel extraction kit (Qiagen; www.qiagen.com) and sequenced (Macrogen; www.macrogen.com).

The nucleotide sequence of cDNA encoding a partial sequence of *O. bonariensis tshb* subunit was deposited in GenBank with accession number KM362366.

REAL-TIME PCR ASSAYS

The relative levels of *tshb* subunit were measured by real-time (rt-)PCR assay following the standard curve method procedure published by Applied Biosystems (www.appliedbiosystems.com). Specific forward (5′-GCACCAAACCAGTCAGAGGT-3′) and reverse (5′-AACGAGGTTTTGGGAAGACA-3′) primers of *tshb* were designed to amplify a 105 bp fragment from the *tshb* 3′ end region previously obtained. The relative expression levels were normalized using β-actin as internal control (forward: 5′-CTCTGGTCGTACCACTG GTATCG-3′; reverse: 5′- GCAGAGCGTAGCCTTCATAGATG-3′; amplification size: 83 bp). The β-actin gene has already been used to normalize samples for DNA quantity in preview studies done in *O. bonariensis* larvae reared at different temperatures (Shinoda *et al.,* 2010; Fernandino *et al.,* 2012; Tovar Bohórquez *et al.,* 2017). The heads of six larvae were randomly sampled every week for total RNA isolation using TRIzol Reagent (www.lifetechnologies.com) following the manufacturer's instructions. The PCR mix consisted of 1 µl of cDNA (*c.* 100 ng), 1 pmol of each primer and 5 µl of fast start universal SYBR green master (www.lifescience.roche.com) in a final volume of 10 µl. Amplification reactions were performed in an Applied Biosystems Step One rt.-PCR systems (www.appliedbiosystems.com). Amplification of the target gene was done simultaneously with the β-actin gene in separate tubes and the results were analysed with the StepOne software version 2.2.2 (www.appliedbiosystems.com). The efficiency of the assay ranged between 85 and 100%. Dissociation curves analyses were run after each rt-PCR assay to ensure that there was only one product. A negative control without template was run for each primer pair.

THYROID HORMONE MEASUREMENTS

T_4 and T_3 whole-body concentrations were measured by ELISA following the manufacturer's protocols (RADIM; www.radim.com, for T_4 and Adaltis, www.adaltis.net, for T_3). Three pools of larvae (*c.* 100 mg each) were sampled weekly and placed in plastic 1·5 ml tubes. Larvae were homogenized on ice in 1 ml of ice-cold PBS and stored at 4° C overnight, according to Deane & Woo (2003). The homogenate was stirred and centrifuged at 2000*g* for 15 min at 4° C. The supernatant was retransferred to a clean tube and 0·5 ml of ice-cold PBS was added into the original tube, stirred again and centrifuged. The supernatant was immediately used for hormone measurements. A standard curve was run for each ELISA plate. The lower and higher limits of detection were 10 and 280 ng ml^{-1} for T_4 and 0·5 and 8 ng ml^{-1} for T_3, respectively. Parallelism between measured of T_4 and T_3 from serial dilutions of the homogenate and their respective standard curve was confirmed prior to enzime immunoassay development.

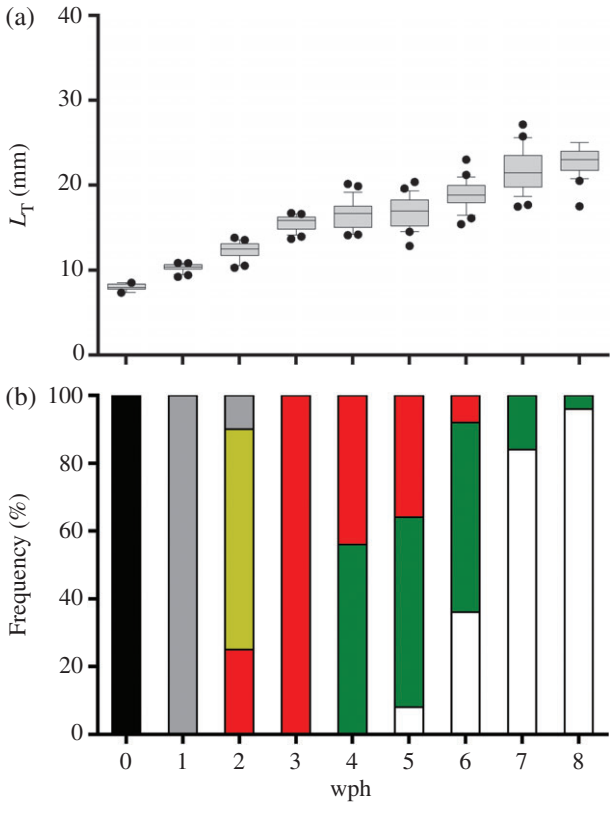

FIG. 1. (a) Total length (L_T) growth curve of *Odontesthes bonariensis* larvae reared during 8 weeks post hatching (wph) at 17° C. Box plots represent median (——), quartiles (box), 10th and 90th percentiles (whiskers) and outliers (●). (b) Frequency distribution of the developmental stages A–F (see Table I) of *O. bonariensis* as a function of larval age ($n = 25$ per sampling date). ■, stage A; ▭, stage B; ▭, stage C; ▭, stage D; ▭, stage E; □, stage F.

STATISTICAL ANALYSIS

Ordinary least-squares algorithm and Fisher's test were used to evaluate the linear regression between L_T and M_B. The effects of developmental stage on the thyroid axis component were tested using one-way ANOVA. Data with significant differences were further compared among the means using *post hoc* Tukey's HSD test ($P < 0{\cdot}05$). The data were checked for normal distribution with the Kolmogorov–Smirnoff test. Statistical analyses were performed using RStudio (www.r-project.org) and GraphPad Prism 6.01 (www.graphpad.com). Data are presented as the mean ± S.E.

RESULTS

FISH GROWTH AND DEVELOPMENTAL STAGES

The growth curve of *O. bonariensis* larvae and the frequency distribution of developmental stages through the larval–juvenile transition period are shown in Fig. 1. The onset of juvenile stage was highly asynchronous with 8% of the individuals reaching

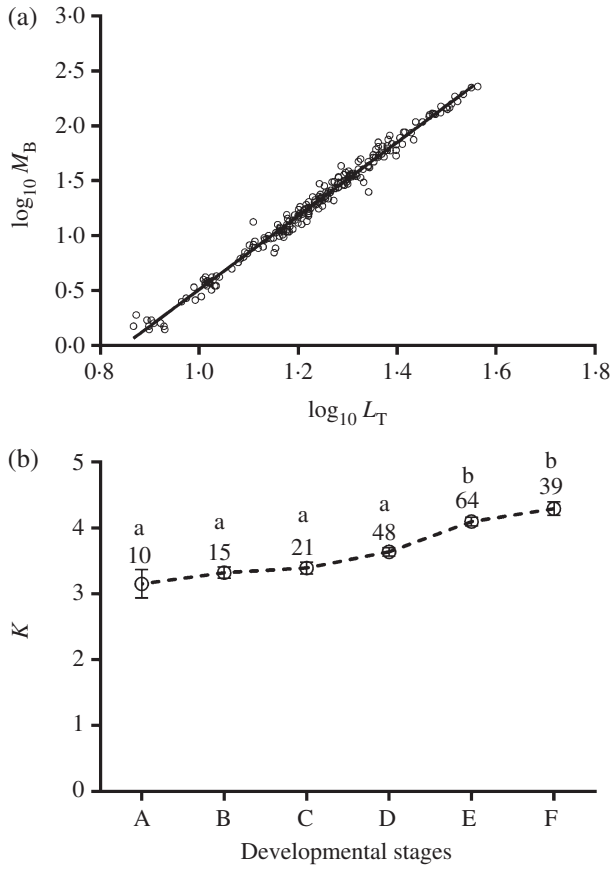

FIG. 2. (a) Double-logarithmic plot of total length (L_T) *v*. body mass (M_B) observed during early developmental stages of *Odontesthes bonariensis*: $\log_{10} y = 3{\cdot}348(\pm 0{\cdot}026)\log_{10} x - 2{\cdot}836(\pm 0{\cdot}033)$, $r^2 = 0{\cdot}987$, $F_{1,220} = 16\,366$, $P < 0{\cdot}001$. (b) Mean ± S.E. Fulton's condition factor (K) calculated during developmental stages A–F (see Table I) of *O. bonariensis*. The numbers in above each data point indicate sample size; different lower-case letter indicate significant differences resulting from Tukey's *post hoc* test ($P < 0{\cdot}05$).

juvenile stage early at 5 weeks post hatching (wph) and 4% of individuals keeping larvae characteristics even at 8 wph [Fig. 1(b)].

Linear double-logarithmic regression assessment between L_T and M_B showed a typical linear regression [Fig. 2(a)]. The parameters b and $\log_{10} a$ were $3{\cdot}348 \pm 0{\cdot}026$ and $-2{\cdot}836 \pm 0{\cdot}033$, respectively. The K was low at early stages and increased significantly at development stage E, stabilizing at stage F, when the fish reached the final phenotype [Fig. 2(b)].

ANATOMICAL LOCATION AND MORPHOLOGY OF THE THYROID GLAND

Thyroid follicles were located exclusively in the subpharyngeal region associated with the ventral aorta below the hyoid bone region (Fig. 3). Small follicles were first observed at hatching showing a homogeneously eosin-stained colloid with vacuoles

in its periphery [Fig. 3(a)]. These follicles were the first sign of endogenous production of thyroid hormones, since both follicle lumen and thyrocytes were highly positive T4 immunostained [Fig. 3(b)]. Follicular form at 8 wph was oval with a cuboidal epithelium [(Fig. 3(c)–(e)]. Follicle aspect ratio was significantly higher at the end of larval–juvenile transition period, showing that follicles switched from a rounded form at hatching to an oval form at juvenile stage (Fig. 4).

Significant effects of developmental stage on number of follicles per larva, mean colloidal area and total colloidal area were observed (Fig. 5). The number of follicles per larva decreased significantly at stage B and then increased continuously with maximum values at stage F [Fig. 5(a)]. An overall increase of the mean colloidal area along larvae development was observed. The first significant difference was obtained at stage D, reaching maximum values at stages E and F [Fig. 5(b)]. Similar results were observed for total colloidal area, with a significant increase at stage E, recording maximum value at stage F [Fig. 5(c)].

SEQUENCE CHARACTERIZATION AND EXPRESSION LEVELS OF tshb

A 563 bp fragment of *tshb* was obtained corresponding to a 207 bp coding block and a 356 bp 3′ untranslated region was obtained [Fig. 6(a)]. The predicted translation product of the coding region was a 68 amino acid sequence with six conserved cysteines [Fig. 6(b)].

A significant effect of developmental stage on *tshb* transcript levels was observed. The *post hoc* test indicated that transcript levels of *tshb* reached highest values at stage C and D and then declined sharply and remained constant at low values [Fig. 7(a)].

THYROID HORMONE CONCENTRATIONS

T_4 and T_3 hormones were detected early at hatching [Fig. 7(b), (c)]. Significant effects of developmental stage on T_4, T_3 and T_4:T_3 hormone ratios were observed. The *post hoc* test for one-way ANOVA showed a significant decrease of T_4 levels from hatching to stage C. Thereafter, a significant increase of T_4 level was observed at stage E, prior to achieving the juvenile stage and decreased significantly at stage F [Fig. 7(b)].

Whole-body concentration of T_3 increased slightly, but not significantly, from stage A to B and then decreased significantly at stage *C. prior* to achieve the juvenile phenotype, T_3 levels increased significantly at stage E and then decreased again statistically at juvenile stage [Fig. 7(c)].

Whole-body total T_4 molar concentrations were between 2·4 and 5 times higher than those of total T_3 throughout the experimental period [Fig. 7(d)]. The molar ratio of T_4:T_3 decreased significantly from hatching to stage B and then increased significantly at stage D. At stage E, the T_4:T_3 ratio significantly decreased in comparison with the previous stage and remained almost constant at juvenile stage [Fig. 7(d)].

DISCUSSION

Odontesthes bonariensis larvae showed high variability in developmental rate, with at least four stages observed at the same time. This asynchrony in early development is

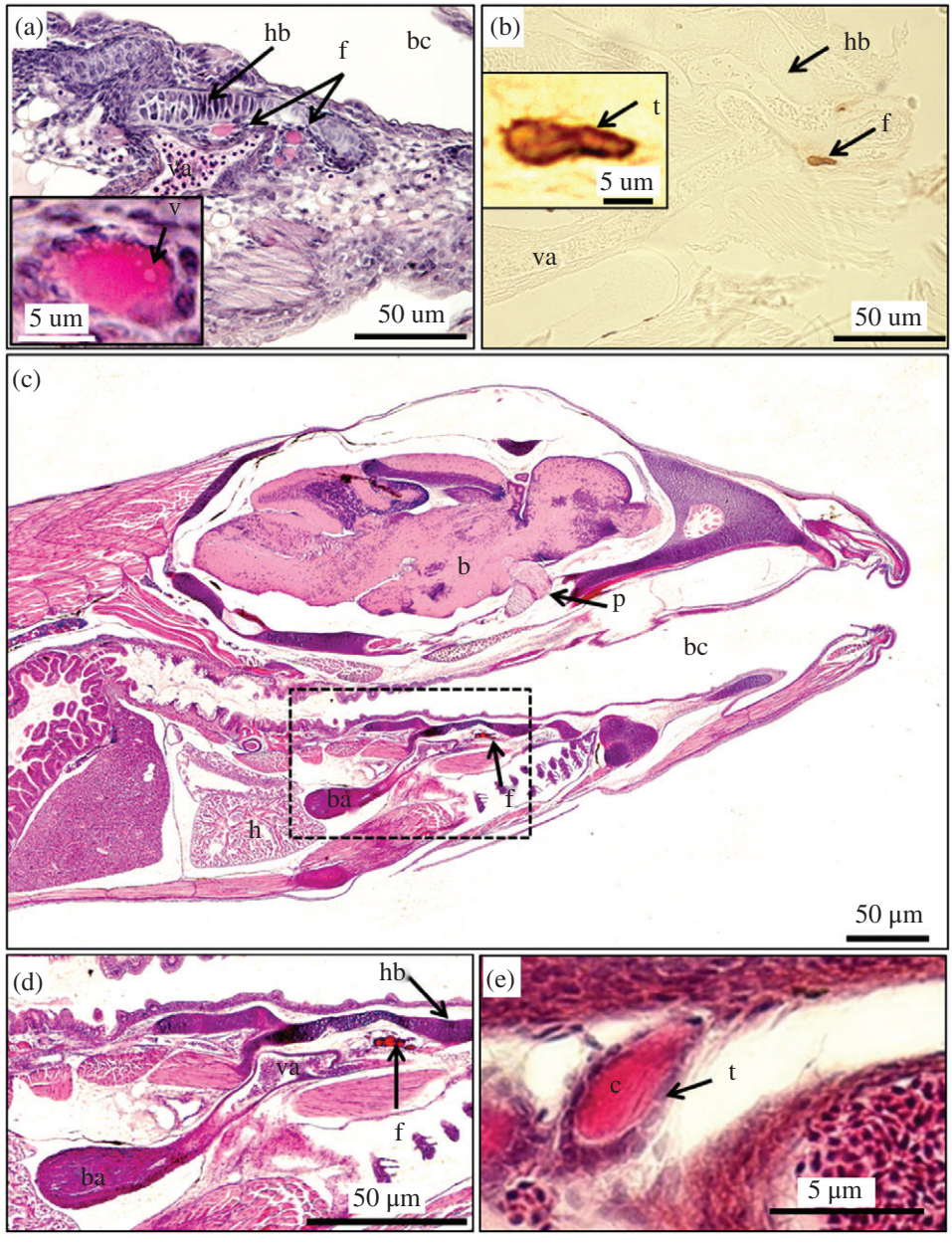

FIG. 3. Sagittal sections of *Odontesthes. bonariensis* larvae. (a) Thyroid follicles at 8 weeks post hatching showing vesicles in the perifery of the colloid. (b) The thyroid follicles in (a) highlighted by immunohistochemistry (anti-T_4 antibody) and presenting a secretory phase for T_4 immunostaining both within the follicle lumen and in the thyrocytes. (c) Sagittal section of head of larva (anterior to right) in which the dashed square in is extended in (d). (e) A thyroid follicle with oval form with a cuboidal epithelium. b, Brain; ba, bulbus arteriosus; bc, buccal cavity; c, colloid; f, follicles; h, heart; hb, hyoid bone; p, pituitary; t, thyrocytes; v, vesicle; va, ventral artery.

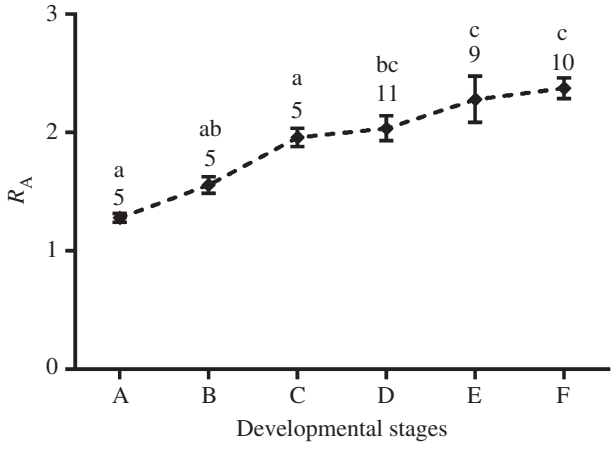

FIG. 4. Changes of thyroid follicles aspect ratio (R_A; mean ± S.E) observed during early developmental stages A–F (see Table I) of *Odontesthes bonariensis* ($F_{5,37} = 7{\cdot}6$; $P < 0{\cdot}05$). The numbers in above each data point indicate sample size; different lower-case letter indicate significant differences resulting from Tukey's *post hoc* test ($P < 0{\cdot}05$).

a major constraint in aquaculture of *O. bonariensis* since larger larvae require higher frequency and quality of food and rearing density. For instance, it has been observed that larger *O. bonariensis* larvae and juveniles use the bottom of the tanks, while the smaller larvae swim near the water surface (Zagarese, 1991). In addition, the larger larvae showed higher foraging efficiency and cannibalism (Zagarese, 1996). Therefore, despite the same rearing environment and even genetic background, the developmental rate may be affected by endogenous factors. These results suggest that the progress of development should be investigated in terms of morphological stages, or size, rather than age, as already discussed by Sæle & Pittman (2010).

The relationship between L_T and M_B has been widely studied in fishes, including *O. bonariensis* (Baigún *et al.,* 2009). Previous studies showed that this relationship changes not only seasonally but is also different among larvae, juveniles and adults fish (Froese, 2006). In the present study the significant increase of K from E stage may indicate a change of form related to the acquisition of juvenile phenotype.

In *O. bonariensis*, the presence of thyroid follicles in the subpharyngeal region associated with the ventral aorta, below the hyoid bone, is similar to other fish species (Falk-Petersen & Hansen, 2001; Wendl *et al.,* 2002; Raine *et al.,* 2005; Yamano *et al.,* 2007; Klaren *et al.,* 2008; Padrós *et al.,* 2011). In several species, however, non-pharyngeal ectopic thyroid follicles were also found (Leatherland, 1994). For instance, subpharyngeal follicles in common carp *Cyprinus carpio* L. 1758 comprised only 10% of the total thyroid tissue while the majority was restricted to kidney (Geven *et al.,* 2007). Regarding the morphology of the follicles, Raine *et al.* (2005) showed that the functional unit of the thyroid gland in juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) was tubular, not follicular. Nevertheless, no tubular structures were detected in sagittal sections and even in transversal sections of *O. bonariensis*. The form of this follicle changed from rounded at hatching to a more elongated form in the last stages, suggesting that this may be the definitive follicle form. The spherical and elongated form of follicles has been previously described in Mozambique tilapia

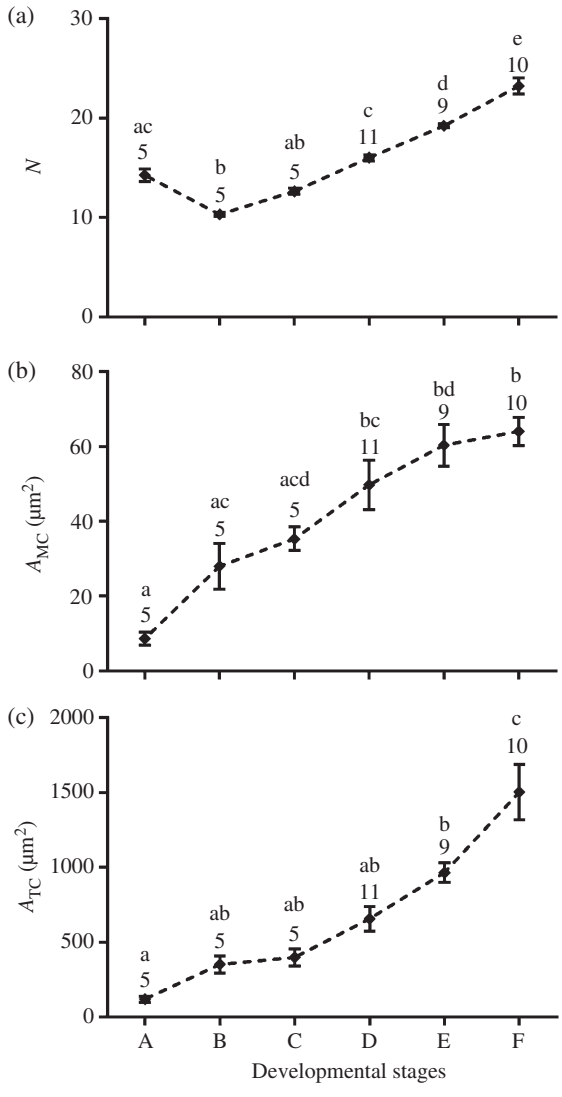

FIG. 5. Changes of morphological variables (mean ± S.E) of the thyroid follicles observed during early developmental stages of *Odontesthes bonariensis*. (a) Number (N) of follicles per larva ($F_{5,37} = 70{\cdot}1$; $P < 0{\cdot}05$), (b) mean colloidal area (A_{MC}) per larva ($F_{5,37} = 10{\cdot}3$; $P < 0{\cdot}05$) and (c) total colloidal area (A_{TC}) per larva ($F_{5,37} = 15{\cdot}3$; $P < 0{\cdot}05$) through the developmental stages A–F (see Table I). The numbers in above each data point indicate sample size; different lower-case letter indicate significant differences resulting from Tukey's *post hoc* test ($P < 0{\cdot}05$).

Oreochromis mossambicus (Peters 1852) and *C. carpio* by Geven *et al.* (2007) and in zebrafish *Danio rerio* (Hamilton 1822) by Schmidt & Braunbeck (2011). Nevertheless, no explanation about these changes has been proposed but it seems that this is a necessary process for the normal development of the thyroid axis.

Fish eggs contain significant amount of THs of maternal origin prior to the maturation of the larval thyroid gland (Einarsdóttir *et al.,* 2006; Walpita *et al.,* 2007; Hsu

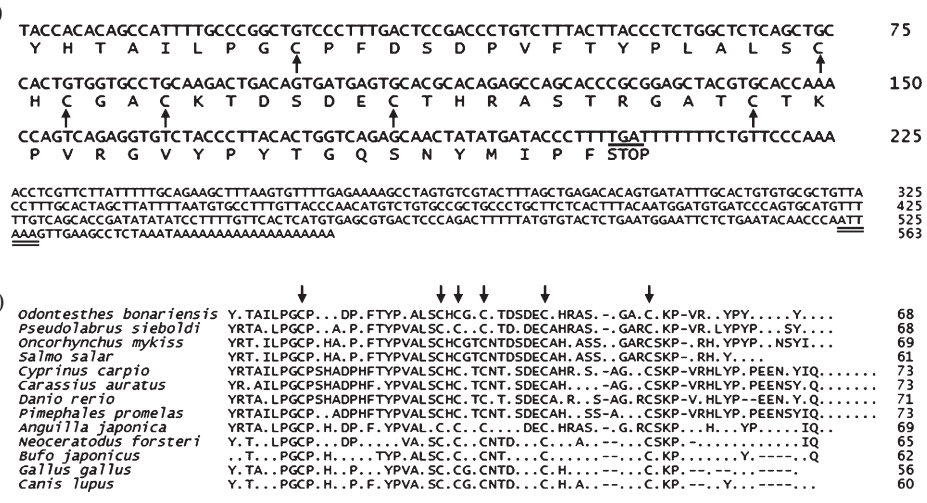

FIG. 6. (a) The nucleotide and deduced amino acid partial sequences of cDNA encoding the partial subunit of *Odontesthes bonariensis tshb*. The amino-acid residue numbers are shown on the right. The stop codon (TGA) is underlined and the polyadenylation signal (ATTAAA) is double-underlined. ↑, The cysteine residues. (b) Multiple protein sequence alignments for predicted Tsh protein. ↑, The conserved cysteine residues. NCBI GenBank accession number: *Odontesthes bonariensis* (pejerrey, KM362366); *Pseudolabrus sieboldi* (bamboo leaf wrasse, BAF81902); *Oncorhynchus mykiss* (rainbow trout, P37240); *Salmo salar* (Atlantic salmon, AAC77908); *Cyprinus carpio* (common carp, BAA20082); *Carassius auratus* (goldfish, BAA20081); *Danio rerio* (zebrafish, AAN08914); *Pimephales promelas* (fathead minnow, ABG72808); *Anguilla japonica* (Japanese eel, Q7ZZV4); *Neoceratodus forsteri* (Australian lungfish, CAE17336); *Bufo japonicus* (Japanese toad, BAB93563); *Gallus gallus* (domestic chicken, NP_990394); *Canis lupus familiaris* (domestic dog, NP_001003290).

et al., 2014). This has been well established in fish larvae where no functional thyroid tissue was developed during embryogenesis, while thyroid hormones were detected in significant quantities (Szisch *et al.,* 2005; Einarsdóttir *et al.,* 2006). Nevertheless, the eosinophilic thyroid follicles with peripheral vacuoles observed in *O. bonariensis* at the time of hatching suggest that the larvae could be able to synthesize THs at this moment as it was already reported in other species (Tanaka *et al.,* 1995; Raine & Leatherland, 1999). Tanaka *et al.* (1995) categorized fishes based in the developmental timing of thyroid gland into an early-appearance group, in which thyroid follicles are differentiated at hatching, or a late-appearance group, in which differentiation of the thyroid occurs at yolk absorption. Thus, *O. bonariensis* can be included in the early-appearance group in which thyroid follicles are differentiated at hatching.

The number of follicles per larva decreased significantly at stage B and then increased continuously. A decrease in thyroid follicle number was not expected; nevertheless, thyroid reorganization could be induced by fusion between neighbouring follicles as it was demonstrated in other vertebrates (Ries & Allegretti, 1965; Penel *et al.,* 1982; Uchiyama *et al.,* 1986). On the other hand, mean colloidal and total colloidal area per larva increased steadily from hatching until the juvenile stage, as it was reported in Senegalese sole *Solea senegalensis* Kaup 1858 (Klaren *et al.,* 2008).

The characterization of a cDNA fragment encoding a putative *tshb* subunit showed a deduced amino acid sequence containing six cysteine residues highly conserved in all

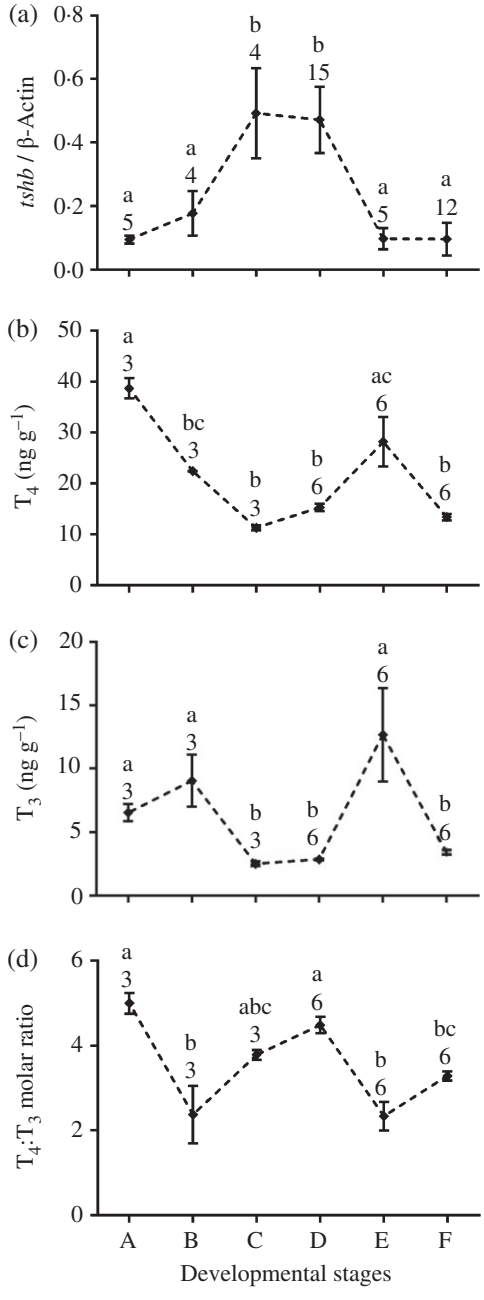

FIG. 7. (a) Changes (mean ± S.E.) of relative transcript levels of *tshb* ($F_{5,35} = 3{\cdot}8$; $P < 0{\cdot}05$), (b) whole-body concentration of T_4 ($F_{5,21} = 11{\cdot}4$; $P < 0{\cdot}05$), (c) T_3 ($F_{5,21} = 4{\cdot}2$; $P < 0{\cdot}05$) and (d) T_4:T_3 ratio ($F_{5,21} = 12{\cdot}8$; $P < 0{\cdot}05$) observed during early developmental stages A–F (see Table I) of *Odontesthes bonariensis*. The numbers in above each data point indicate sample size; different lower-case letter indicate significant differences resulting from Tukey's *post hoc* test ($P < 0{\cdot}05$).

glycoprotein hormones of vertebrates (MacKenzie *et al.,* 2009). Interestingly, the high *tshb* transcript levels during stages C and D were coupled with low levels of T_4 concentration. The significant increase of T_4 whole-body concentration at stage E may be related to the peak of *tshb* transcript levels observed at stage C, considering that there is a delay between gene expression and protein production. These results are in accordance with a negative feedback regulation over *tshb* expression by thyroid hormones already proposed in other teleosts (MacKenzie *et al.,* 2009).

In this study, fish with juvenile phenotype were first observed at 5 wph. Therefore, the increase in THs concentration at stage E, prior to achieving the juvenile phenotype, may be associated with the acceleration of metabolic processes necessary to complete metamorphosis. This is in agreement with several physiological and morphological changes reported in *O. bonariensis* during this stage. For instance, the transition of feeding stage from live food to artificial diet in this species can be done after reaching a body length of 20 mm (Strüssmann & Yasuda, 2005), which is generally attained between 5 and 6 wph. Moreover, the morphology and function of alimentary tract, as well as the ability to produce the digestive enzyme trypsin, is completely developed at this size (Toledo-Cuevas *et al.,* 2011). The increase in THs at stage E could also be related to gonadal differentiation during the transition from larva to juvenile and the acquisition of the final sex phenotype, since the first signs of ovarian differentiation in *O. bonariensis* reared under similar conditions have been observed between 4 and 7 wph (Ito *et al.,* 2005). In other species, similar morphological changes considered as the end of metamorphosis have been linked with peaks in T_4 concentration (Tagawa *et al.,* 1990; Brown, 1997; Hotta *et al.,* 2001; Szisch *et al.,* 2005; Yamano *et al.,* 2007).

Although, the PBS-based extraction method used in this study to extract all the thyroid hormone from the homogenized tissue might be less efficient than protocols with an alcohol step, it was possible to measure both T_3 and T_4 in all the developmental stages analysed. In *O. bonariensis* T_4 concentrations were higher than T_3 during larval development, as observed in other fishes (Kawakami *et al.,* 2003; Crane *et al.,* 2004; Klaren *et al.,* 2008; Schnitzler *et al.,* 2016), maybe due to low deiodinase activity. It is interesting to remark that this relationship is reversed in adults of *O. bonariensis* (Chalde & Miranda, 2012) and other fishes (Björnsson *et al.,* 1998; Deane *et al.,* 2001; Deane & Woo, 2003; Einarsdóttir*et al.*, 2006), where T_3 plasma levels were higher than T_4.

In summary, the overall results obtained in this study suggest that the thyroid axis is differentiated at hatching since *tshb* transcript levels, follicles with eosinophilic and T_4-immunoreactive colloids with peripheral vacuoles and detectable concentrations of both THs were observed at that moment. Moreover, the significant decrease of T_4:T_3 ratio from hatching to stage B may be indicative of deiodinase enzyme activity early during larval development. Furthermore, the development of the thyroid axis was related to several morphological and physiological changes during larval–juvenile transition. Thus, this study establishes a useful baseline for designing protocols to reveal sensitive windows in which thyroid action, manipulation or disruption can be of particular importance for several physiological processes during early development.

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