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The thyroid gland and thyroid hormones in sheepshead minnow (*Cyprinodon variegatus*) during early development and metamorphosis

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Abstract The sheepshead minnow is widely used in ecotoxicological studies that only recently have begun to focus on disruption of the thyroid axis by xenobiotics and endocrine disrupting compounds. However, reference levels of the thyroid prohormone thyroxine (T4) and biologically active hormone 3,5,3′-triiodothyronine (T3) and their developmental patterns are unknown. This study set out to describe the ontogeny and morphology of the thyroid gland in sheepshead minnow, and to correlate these with whole-body concentrations of thyroid hormones during early development and metamorphosis. Eggs were collected by natural spawning in our laboratory. T4 and T3 were extracted from embryos, larvae and juveniles and an enzyme-linked immunoassay was used to measure whole-body hormone levels. Length and body mass, hatching success, gross morphology, thyroid hormone levels and histology were measured. The onset of metamorphosis at 12-day post-hatching coincided with surges in whole-body T4 and T3 concentrations. Thyroid follicles were first observed in pre-metamorphic larvae at hatching and were detected exclusively in the subpharyngeal region, surrounding the ventral aorta. Follicle size and thyrocyte epithelial cell heights varied during development, indicating fluctuations in thyroid hormone synthesis activity. The increase in the whole-body T3/T4 ratio was indicative of an increase in outer ring deiodination activity. This study establishes a baseline for thyroid hormones in sheepshead minnows, which will be useful for the understanding of thyroid hormone functions and in future studies of thyroid toxicants in this species.

Keywords Thyroxine · 3,5,3′-Triiodothyronine · Sheepshead minnow · Development

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Introduction

Despite their susceptibility to endocrine disrupting compounds, (Blanton and Specker 2007; Brown et al. 2004), young, developing fish are still largely ignored in the study of thyroid disruption by xenobiotics.

Indeed, the ecotoxicological effects of thyroid disruptors might be most significant during early developmental processes where thyroid hormones play a prominent role (Crane et al. 2004).

The roles played by thyroid hormones in growth and development of fish have been reviewed previously (Power et al. 2001; Yamano 2005). The transition from larva to juvenile, metamorphosis, is a crucial developmental phase in fish. Larva-to-juvenile transitions commonly include the formation of adult fins and ossification of fin rays, maturation of internal organs and sensory systems, formation of scales, modification of pigmentation and allometric changes in body proportions (McMenamin and Parichy 2013). Thyroid hormones are key regulators of teleost metamorphosis. Whole-body concentrations of thyroid hormones show typical changes during these periods of teleost fish development, indicating that the ontogeny of thyroid hormones is related to specific morphological characteristics that represent early development. Indeed, increases in thyroid hormone levels are associated with larval metamorphosis in teleost fish (Chang et al. 2012; Crane et al. 2004; de Jesus and Hirano 1992; Johns et al. 2009; Kawakami et al. 2003; Klaren et al. 2008; Shiao et al. 2008; Szisch et al. 2005; Yamano 2005).

Fish larval stages are thus believed to be more sensitive to toxicants than adults are (Brown et al. 2004; Einarsdottir et al. 2006; Johns et al. 2009), and it is therefore important to evaluate the effects of endocrine disrupting chemicals on young developing fish. Furthermore, the study of the ontogeny of the thyroid function in teleosts is warranted as it may reveal sensitive windows during which thyroid disruption could be of particular importance.

The sheepshead minnow (*Cyprinodon variegatus*) is a killifish belonging to the family Cyprinodontidae. They have become a favoured model for laboratory studies because of their small size, rapid development, and ease of laboratory husbandry (Cripe et al. 2009). *C. variegatus* is a model species well-suited for assessing the effects of chemicals in saltwater environments (Cripe et al. 2009; Raimondo et al. 2009, 2015; Winder et al. 2012). The species is sexually dimorphic, females spawn continuously under appropriate conditions, and have relatively large demersal eggs with a high hatch rate. The generation time of sheepshead minnows is around two months.

Unfortunately, as to date there are no measurements of the normal, undisturbed levels of either triiodothy-ronine (T3) or thyroxine (T4) in adults and the early developmental stages of this species. We here describe the ontogeny and morphology of the thyroid gland in *C. variegatus* 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 *C. variegatus* during critical early developmental events as a basis for future investigations of thyroid disruption. The ontogenic patterns of thyroid hormones during development of the sheepshead minnow from embryo to adult may reveal sensitive windows during which thyroid disruption could be of particular importance.

Materials and methods

Animals

Adult sheepshead minnows (*C. variegatus*) were purchased from Aquatic Research Organisms (ARO Inc. New Hampshire, USA). Males and females were maintained in 150-L glass aquaria at 26 °C, at a photoperiod of 14 h:10 h (L:D) and fed daily with frozen brine shrimp (*Artemia* nauplii) and flake food. Couples of 2 males and 3 females were paired in spawning boxes for 2 h (Cripe et al. 2009). Embryos were collected from the spawning tanks, selected under a dissection microscope and transferred to petri dishes (50 per dish) in an incubator (BCR-25, Jiangsu Best Electrics Co., Ltd.) at 26 °C. Embryos were sampled on pre-hatch days −6 and −3, pooled from several incubation petri dishes and divided into five batches of 40 each. Sampled embryos were placed in pre-weighed microfuge tubes, and wet weight was recorded, after which they were snap-frozen on dry ice and stored at −80 °C. On day 0, embryos hatched and larvae were transferred to a glass tank (1 L working volume, 50 larvae per tank). From 0 to 21 days post-hatch (dph) larvae were fed on cultured brine shrimp, and from 14 to 21 dph they were also fed on flaked fish food. Samples consisting of 10–40 larvae were taken on the time points indicated in the figures. Juvenile fish were netted from tanks, rapidly killed in MS222 (500 mg/L), placed in 1.5–mL microfuge tubes, and wet weights recorded prior to snap-freezing on dry ice and storage at −80 °C. All sampling was carried out between 08:00 and 10:00 GMT.

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. A rabbit thyroxine-specific antibody was obtained from ICN Biomedicals (Zoetermeer, The Netherlands). Sections were deparaffinated in xylene and rehydrated 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. Sections were then 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. Afterward, 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®. Images were captured using a Zeiss light microscope and a MOC-510 Mueller-Optronic 5 megapixel CMOS camera. The diameters of individual thyroid follicles and epithelial cell heights were measured using ImageJ 1.44 software.

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 (0.01 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, and 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 (DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium) according to the manufacturer's instructions. Calibrators were prepared in the same barbital buffer matrix as the samples. A 4-parameter calibration curve was calculated with the immunoassay software Gen5. The intra-assay and inter-assay coefficients of variation for the tT4 and tT3 ELISA, as reported by the manufacturer, are 6.4 and 9.9, and 4.1 and 9.0 %, respectively. The reported cross-reactivity of the ovine anti-T4 antibody to D-T4 is 94 % (the reactivity to L-T4 is set at 100 % as a reference), and to L-T3 and D-T3 is 3.3 and 1.8 %, respectively. The reported cross-reactivity of the ovine anti-T3 antibody to L-T4 is <0.1 % (setting the reactivity to L-T3 at 100 %). Cross-reactivities of both antibodies to diiodothyronine, diiodotyrosine and iodotyrosine are <0.1 %. The reported sensitivities of the tT4 and tT3 ELISA are 6 ng/mL T4 and 1.6 ng/mL T3, respectively.

Statistics

All data are presented as mean ± standard deviation. Statistics were performed using R (version 3.1.1) (R Development Core Team 2014). All data were tested for normality. Kruskal–Wallis one-way analysis of variance on ranks was used with nonparametric multiple comparisons for each pair using Wilcoxon methods. Statistical significance was accepted at $p < 0.05$.

Results

Each spawning female produced 15–30 embryos per spawn; fertilization could be assessed 24 h after spawning by counting the number of opaque (fertile) and white (nonfertile) embryos. Fertilization rates were low and variable (40–60 %), but the hatching rates of fertilized eggs measured after an incubation time of 6 days were high (86 ± 6 %).

Weight gain became significant from day 12 dph onwards (Fig. 1). Thyroid hormones were detected in pre-hatch eggs at concentrations of 67 ± 14 ng T4/g and 3 ± 1 ng T3/g wet weight (630 ± 134 ng T4/g, 26 ± 7 ng T3/g dry weight). At hatching (0 dph), 1.5-fold increases of T4 and T3 levels were observed. After hatching, whole-body total T4 levels in *C. variegatus* larvae stabilized at pre-hatch levels at day 12 dph decreased to reach minimum levels at 21 dph (0.3 times pre-hatch levels; Fig. 2). Whole-body total T3 concentrations were one order of magnitude lower than those of total T4 (Figs. 2, 3). Concentrations were constant throughout the pre-metamorphosis stage after which total T3 levels at 12 dph increased 3.5-fold compared to pre-hatch levels), and decreased to pre-metamorphosis levels at 21 dph (1.25 times pre-hatch levels; Fig. 3). The profiles of total whole-body T4 and T3 levels normalized for wet body weight and dry body weight are basically identical, indicating that the fractional body water content was stable and did not confound hormone concentration measurements. The molar ratio of T3 to T4 was lowest in pre-metamorphic larvae (0.06 ± 0.04; Fig. 4). Between 12 and 18 dph, the T3/T4 ratio rose to a peak value (0.23 ± 0.12).

Expressed as amount per larvae, thyroid hormone profiles were different than those in which thyroid hormone levels were expressed as amount per body weight. Larval body weight was stable during the pre-metamorphic period, and the pre-metamorphic levels of thyroid hormones were also constant. From day 12 dph, we observed a clear increase in weight gain as the larvae practically double their weight every 3 days. Clear peaks in hormone levels could be identified at 12–15 dph for T4 (1.6-fold increase) and T3 (10-fold increase). As the net T4 production per larva is lower than the net weight increase per larva, the T4 levels decrease on a weight-normalized basis.

The first follicles were observed at hatching (0 dph), which indicates the precocity of these larvae (Figs. 5, 6). Thyroid tissue in *C. variegatus* larvae was exclusively located in the subpharyngeal region, surrounding the ventral aorta. Peak levels of whole-body T3 concentrations, measured at 12 dph, coincide with statistically significant increases of thyroid follicles size at this time point (Fig. 7). Thyrocyte cell height was constant and fluctuated only slightly throughout metamorphosis (Fig. 7).

Discussion

Endocrine disruption has been reported in fish populations around the world (Brar et al. 2010; Dong et al. 2014; Guo et al. 2015; Guo and Zhou 2013; Jarque and Piña 2014; Schnitzler et al. 2012). The aim of this work was to determine the ontogenic patterns of

Fig. 1 Mean wet (*top panel*) and dry weight (*bottom panel*) of sheepshead minnows from spawning up to 21 days post-hatch. Time points within each weight that are not significantly different from one another have been marked with the same letter. The *dashed line* represents day 0 (hatching), and the *shaded zone* represents metamorphic climax
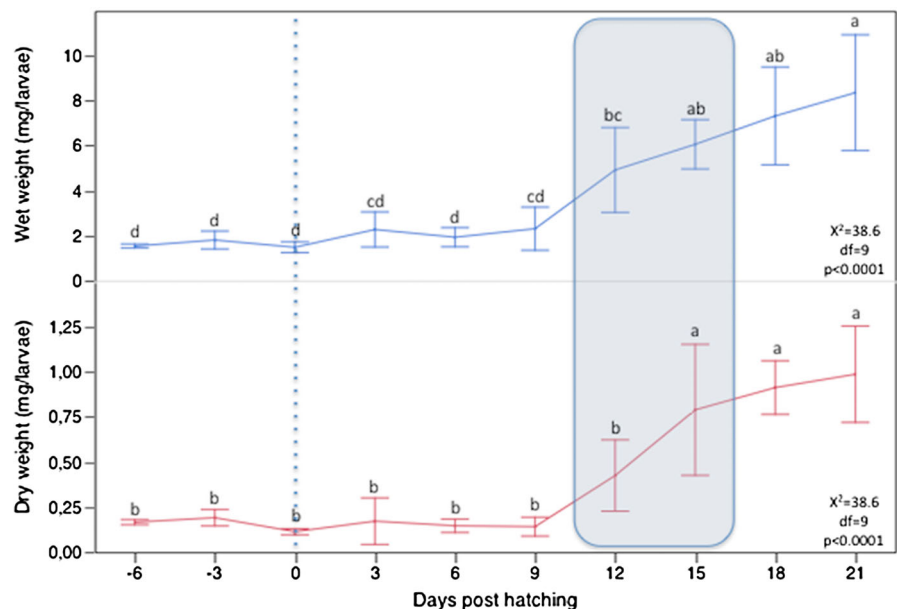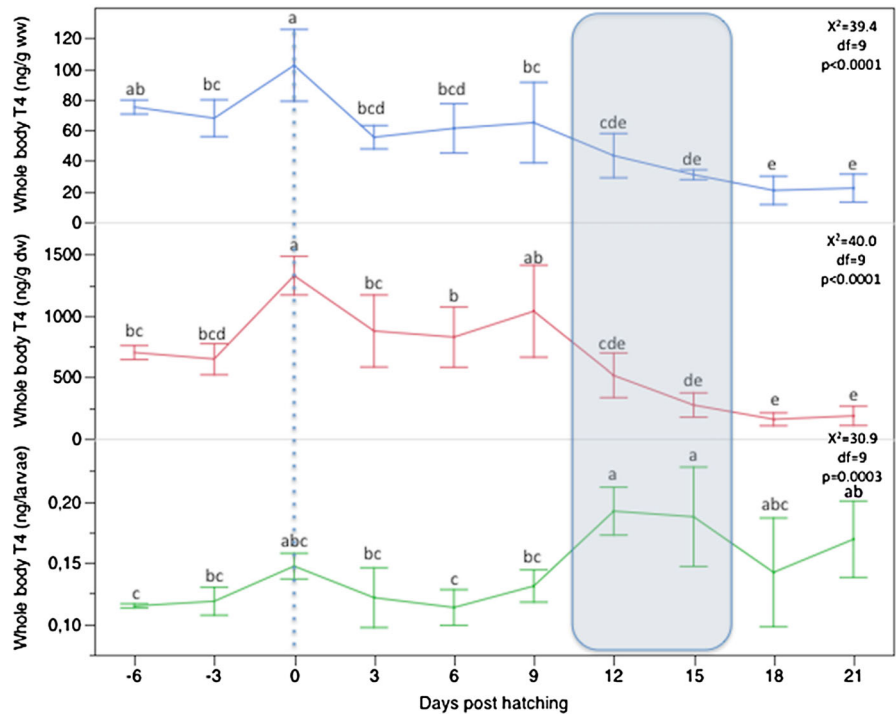
Fig. 2 Whole-body total thyroxine, expressed per gram wet body weight (*top panel*), per gram dry body weight (*middle panel*) and per larva (*bottom panel*), during the development of sheepshead minnows. *Each point* represents the average of five pooled samples comprising 0.1 g wet weight larval biomass. Data are expressed as mean ± SD. Time points within each hormone that are not significantly different from one another have been marked with the same letter. The *dashed line* represents hatch day, and the *shaded zone* represents metamorphic climax


Fig. 3 Whole-body triiodothyronine, expressed per gram wet body weight (*top panel*), per gram dry body weight (*middle panel*) and per larva (*bottom panel*), during the development of sheepshead minnows. *Each point* represents the average of five pooled samples comprising 0.1 g wet weight larval biomass. Data are expressed as mean ± SD. Time points within each hormone that are not significantly different from one another have been marked with the same letter. The *dashed line* represents hatch day, and the *shaded zone* represents metamorphic climax
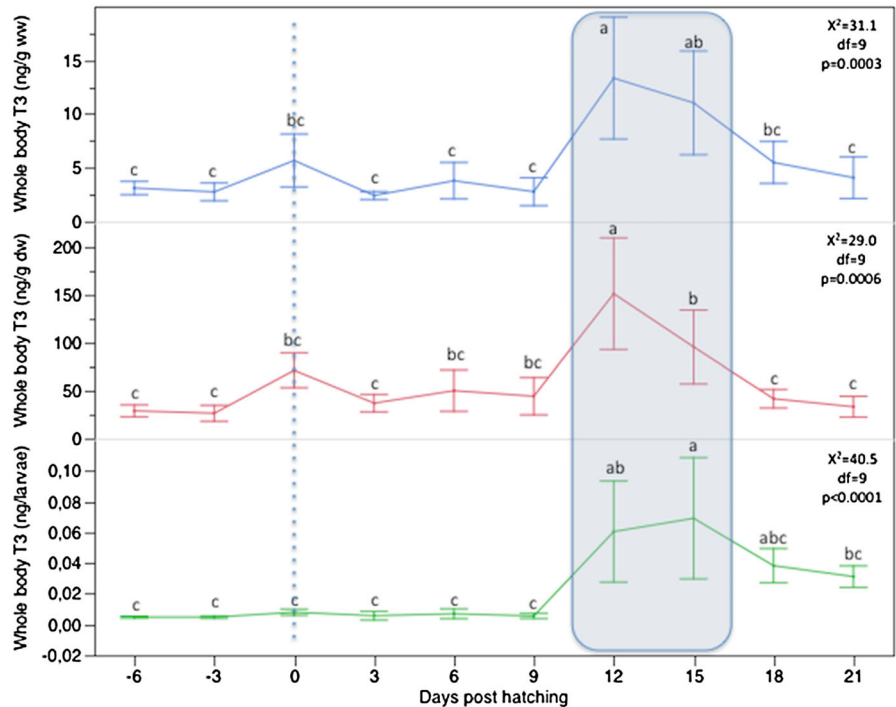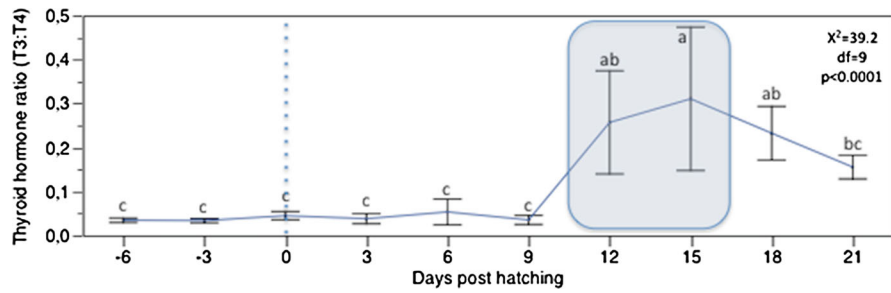
Fig. 4 Molar ratios of whole-body total T3 to total T4 concentrations (expressed in mole per gram dry body weight) during the development of sheepshead minnows. Ratios are calculated from the data in Fig. 2 and 3, and presented as mean $\pm$ SD. Time points that are not significantly different from one another have been marked with the same letter. The *dashed line* represents hatch day, and the *shaded zone* represents metamorphic climax

Fig. 5 Sagittal section (*top panel*) and transversal section (*bottom panel*) in sheepshead minnow at hatching, thyroid follicles are highlighted by immunohistochemistry (anti-T4 antibody 1/5000) and appear *black*. *A* eye and *B* mouth. The *scale bars* represent 100 and 50 μm (*top panel*) and 200 and 50 μm (*bottom panel*)
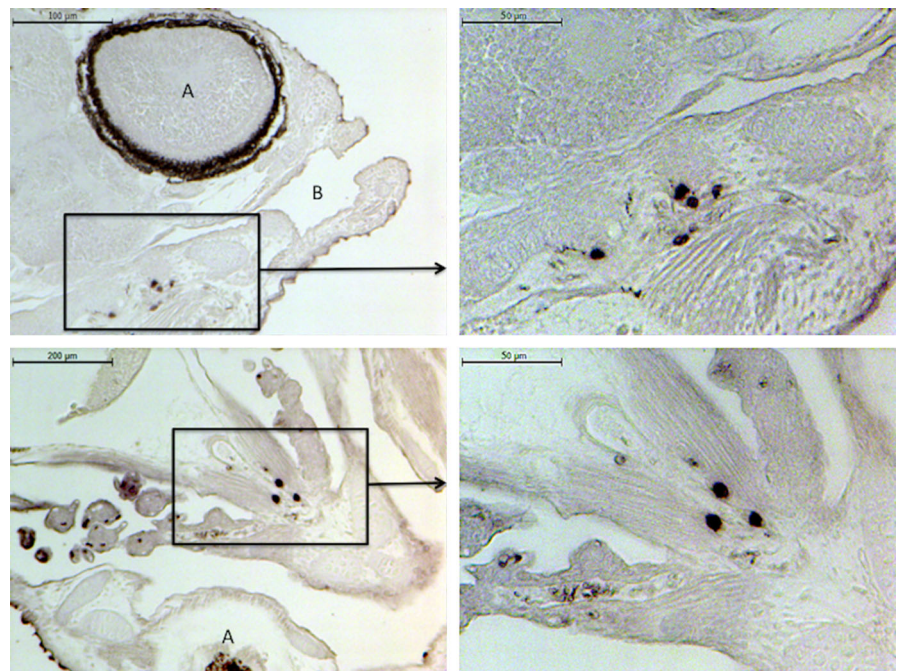
thyroid hormones during development of the sheepshead minnow from embryo to adult in order to reveal sensitive windows during which thyroid disruption could be of particular importance. The development of fish can be partitioned into periods of pre-hatch (from zygote undergoing cleavages to yolk-sac embryo), hatching, pre- and pro-metamorphosis (larvae), metamorphosis climax, and post-metamorphosis (juvenile and adult) (Raimondo et al. 2009). Whole-body hormone concentrations are generally normalized for grams of wet or dry weight (Crane et al. 2004; Klaren et al. 2008), or expressed per individual (Deane and Woo 2003; Reddy and Lam 1992), and the resulting profiles are likely to differ. We prefer to normalize the amount of hormones per individual, as this more accurately reflects larval hormone availability regardless of fluctuations in body weight.

Pre-hatch and hatching (between −6 and 0 dph)

Thyroid hormone content of eggs was high and reflects maternal transfer of thyroid hormones to the yolk (Ayson and Lam 1993; de Jesus and Hirano 1992; Greenblatt et al. 1989; Power et al. 2001). Thyroid hormones are primarily transferred in eggs by binding specifically to vitellogenin, a lipoprotein constituting

Fig. 6 Transversal sections in the pharyngeal region in sheepshead minnow at the right front end of the sub-pharyngeal region. *Left* Control thyroid follicles stained by immunohistochemistry (incubations without primary antibody anti-T4). *Right* Thyroid follicles stained by immunohistochemistry (antibody dilution 1/5000 anti-T4), colloids appear dark brown. The *scale bars* represents 200 μm
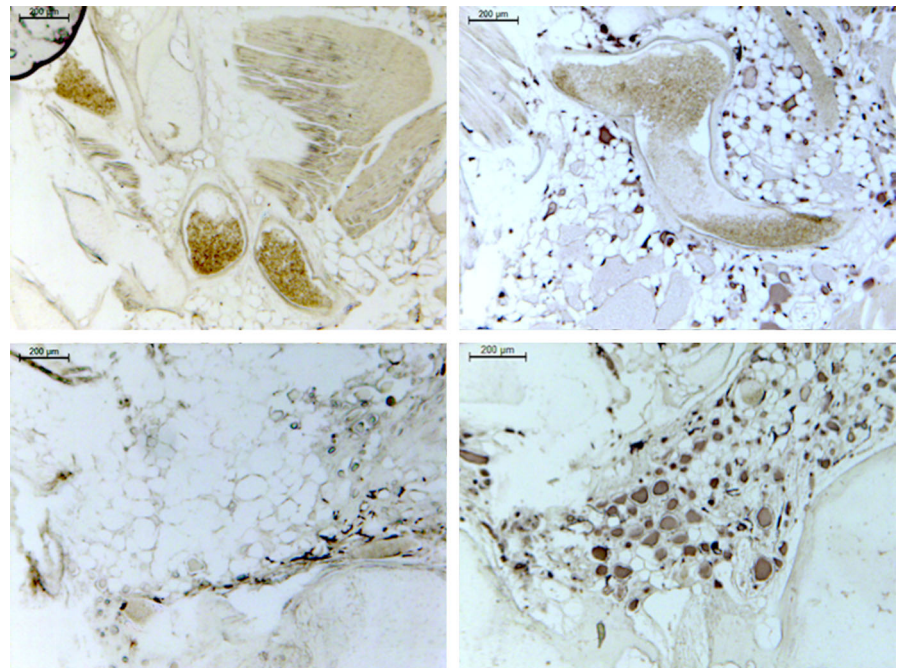

Fig. 7 Morphological parameters of the thyroid gland during the development of sheepshead minnows. Thyroid follicle size (*top panel*) and epithelial cell heights (*bottom panel*) are shown. Data are presented as the average of all follicles in three larvae per time point and expressed as mean ± SD. Time points that are not significantly different from one another have been marked with the same letter. The *dashed line* represents hatch day, and the *shaded zone* represents metamorphic climax
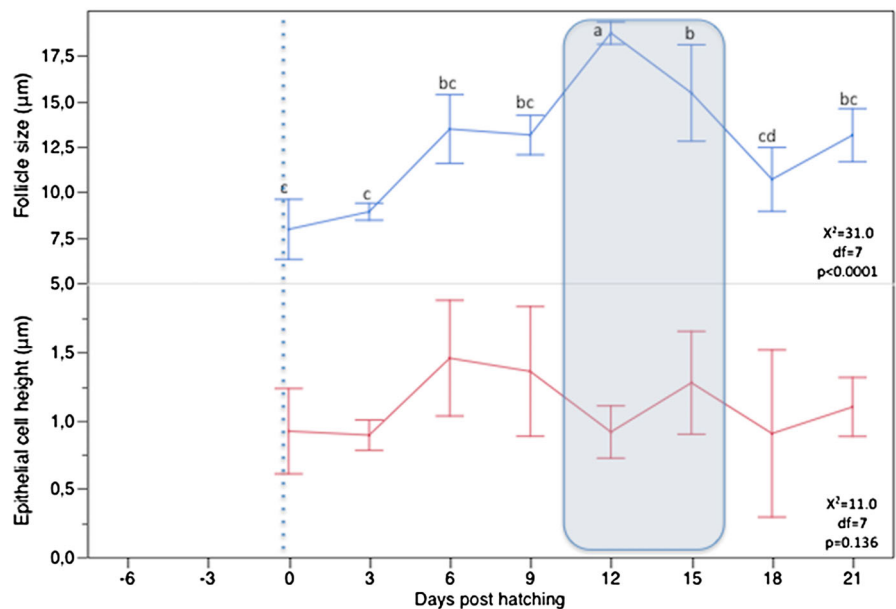
nutrient reserves for embryos during yolk deposition in early development (Greenblatt et al. 1989). Both T4 and T3 levels are stable prior to hatching, so we can only suggest that thyroid follicles had developed and that production of T4 may had commenced at this very early stage of development. The first thyroid follicles could be detected at hatching. Tanaka et al. (1995)

categorized fish species into two categories according to the timing of differentiation of thyroid follicles. The first category, in which thyroid follicles were differentiated already at hatching, was mainly composed of species with demersal eggs or ovoviviparous species (e.g., *Gadus macrocephalus* and *Clupea pallasii*), whereas the second category, in which differentiation

of the thyroid occurred just before yolk-sac absorption is completed, is composed of species with pelagic eggs (this is the case of many flatfish such as sole, *Solea senegalensis*) (Tanaka et al. 1995). The ecological and physiological significance of this classification is not known (Varsamos, et al. 2005), but the differences with which the thyroid system matures could confer different sensitivities to thyroid disruptors (McNabb 2006). We observed first small peaks in the levels of thyroid hormones T3 and T4 at hatching. These peaks may result from weight loss from chorion due to the hatching. In many teleost species, T4 concentration reaches its maximum when the larva begins exogenous feeding, as in salmonids (Greenblatt et al. 1989; Tagawa and Hirano 1987), zebrafish *Danio rerio* (Chang et al. 2012), the fathead minnow *Pimephales promelas* (Crane et al. 2004), goldlined seabream *Rhabdosargus sarba* (Deane and Woo 2003), tilapia *Oreocrhomis mossambicus* (Reddy and Lam 1992) and seabream *Sparus aurata* (Szisch et al. 2005).

Pre- and pro-metamorphosis (between 0 and 9 dph)

During the phase prior to metamorphosis (between 0 and 9 dph), the distinction between the yolk-sac larva stage and the early days of free-swimming larva is not clear in sheepshead minnow. Freshly hatched sheepshead minnow larvae are able to feed exogenously, while still having a yolk sac (Delgado et al. 2006; Mettee and Beckham 1978). The presence of thyroxine-positive thyroid follicles at hatching as well as the absence of a decline in thyroid hormones levels during yolk-sac resorption shows that the thyroid is already functioning at that stage (de Jesus and Hirano 1992; Power et al. 2001). Mean thyroid follicles diameter at 12 dph increased 2.25 fold compared to 0 dph. The size of the follicles and the height of the follicular thyrocyte epithelium is an indication of the secretory activity of the gland. A thyroid gland dominated by small follicles lined by cuboidal or columnar cells can be classified as actively synthesizing and secreting hormones, whereas less active glands show large follicles lined by flattened epithelial cells (Hallgren 2002). As we did not observe any significant changes in thyrocyte cell heights, we are not able to conclude on the secretory activity of the thyroid follicles, but the colloidal reserves of thyroid hormones were constantly increased during this period. There is thus a clear chronology in the activation of the thyroid gland

in association with the metamorphosis that begins already in pre-metamorphic larvae.

Metamorphosis (between 12 and 15 dph)

The onset of metamorphosis in *C. variegatus*, under our rearing conditions, was clearly marked by weight gain and the transition of larvae to juvenile that was characterized by development of pectoral fins, scales, and adult pigmentation, beginning at 12 dph. This event coincides with surges in whole-body thyroid hormone concentrations, in particular those of T3. The increase in T3 levels (10-fold per larva) during metamorphosis may be caused by an increased peripheral conversion of T4 to T3 (Yamano et al. 1991). The increases of the molar T3/T4 ratio observed between 12 and 18 dph is indicative for an upregulation of the peripheral deiodinase activity. Indeed, enzymatic deiodinase activity is involved in metamorphosis, as indicated by their differentially expression during zebrafish and rainbow trout development (Orozco and Valverde-R 2005; Walpita et al. 2009). Increases in whole-body thyroid hormone concentrations at metamorphosis were also observed in other fish species as well (Crane et al. 2004; de Jesus and Hirano 1992; Deane and Woo 2003; Einarsdottir et al. 2006; Szisch et al. 2005) and suggest that thyroid hormones play a critical role in the early development of sheepshead minnow. This is supported by the inhibition of the embryo to larval transition, stunted growth and retarded development shown by zebrafish during exposure to pharmacological inhibitors of thyroid hormone synthesis (Liu et al. 2011).

Post-metamorphosis (between 18 and 21 dph)

Whole-body T4 and T3 declined from day 15 onwards, most likely reflecting a decreasing requirement for thyroid hormones as development proceeds and fish reach juvenile stages. This phase was associated with the onset of dorsal and anal fins. A correlation between the development of adult fins and the peak of thyroid hormones was also observed in *Rhabdosargus sarba* (Deane and Woo 2003). Peaks in thyroid hormone levels were also associated with morphological changes of larvae from several different species during the transition to the juvenile stage (Chang et al. 2012; Crane et al. 2004; Perez-Dominguez and Holt 2006; Szisch et al. 2005).

In summary, our work provides baseline information on the changing levels of T4 and T3 during embryo and larval development. As this species is used as a model for endocrine disrupting chemicals by many laboratories, these baseline thyroid hormone data should be useful for future studies of the thyroid system in this species. The data indicate surges in thyroid hormones in the early stages of development, especially during the metamorphic climax (between 12 and 15 dph). During this period, fish larvae are highly vulnerable for effects of thyroid toxicants, because we observed as well an induced synthesis of thyroid hormones indicated by constantly increased colloidal reserves of thyroid hormones and an upregulation of the peripheral deiodinase activity. Both mechanisms are potential targets for thyroid disrupting chemicals and could, *in fine* modify thyroid hormone levels that are necessary for the normal progress of fish metamorphosis. We hypothesize that the early stages in development, when thyroid hormones are playing key roles in development, are the most sensitive to environmental toxicants that target the thyroid axis. Ecotoxicological and toxicological studies should target on this period, where the risks for thyroid disruption are higher, as the surge of thyroid hormones engage the different mechanisms of hormone synthesis, secretion and metabolization.

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