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Flatfish metamorphosis: A hypothalamic independent process?

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ABSTRACT

Anuran and flatfish metamorphosis are tightly regulated by thyroid hormones that are the necessary and sufficient factors that drive this developmental event. In the present study whole mount *in situ* hybridization (WISH) and quantitative PCR in sole are used to explore the central regulation of flatfish metamorphosis. Central regulation of the thyroid in vertebrates is mediated by the hypothalamus–pituitary–thyroid (HPT) axis. Teleosts diverge from other vertebrates as hypothalamic regulation in the HPT axis is proposed to be through hypothalamic inhibition although the regulatory factor remains enigmatic. The dynamics of the HPT axis during sole metamorphosis revealed integration between the activity of the thyrotrophes in the pituitary and the thyroid follicles. No evidence was found supporting a role for thyroid releasing hormone (*trh*) or corticotrophin releasing hormone (*crh*) in hypothalamic control of TH production during sole metamorphosis. Intriguingly the results of the present study suggest that neither hypothalamic *trh* nor *crh* expression changes during sole metamorphosis and raises questions about the role of these factors and the hypothalamus in regulation of thyrotrophs.

1. Introduction

Vertebrate metamorphosis is especially dramatic in anurans and flatfish, which change from immature tadpole/larval stages, to sexually immature juveniles with the morphological characteristics of an adult. In anurans, the aquatic and herbivorous tadpole develops into a terrestrial air-breathing carnivore. In flatfish, the pelagic "round" larvae becomes a "flat" benthic juvenile in synchrony with the migration of one eye to the opposite side of the head. Thyroid hormones (THs) are the necessary and essential factors regulating post-embryonic metamorphosis (revised in Denver, 2013; Power et al., 2008) and exert their effect through a whole-body and tissue/cell specific manner in both anurans and flatfishes (Becker et al., 1997; Campinho et al., 2007a, 2007b, 2012a; Denver, 1997; Denver et al., 2009; Huang et al., 1999; Inui and Miwa, 1985; Inui et al., 1989, 1995; Kaneko et al., 2005; Lema et al., 2009; Manchado et al., 2008; Manzon and Denver, 2004; Okada et al., 2007). In contrast to mammals where thyrotropin-releasing hormone (*trh*) acts as the hypothalamic regulator of the hypothalamus–pituitary–thyroid (HPT) axis (Fekete and Lechan, 2007; Fliers et al., 2006), in anurans, reptiles and birds development corticotropin releasing hormone (*crh*) has a more prominent role in regulating thyrotropin (*tshb*) secretion and T4 serum levels (De Groef et al., 2006; Denver, 2013).

In anurans, the central regulation of metamorphosis involves both the HPT and the hypothalamus–pituitary–adrenal gland axis (HPA) more commonly associated with stress (Kaneko et al., 2005; Manzon and Denver, 2004; Okada et al., 2007). In the case of the HPA axis environmental cues stimulate release of *crh*, which acts via its receptor, *crhr2*, in pituitary thyrotrophs and enhances *tshb* secretion, TH production and triggers metamorphosis (Denver, 1997; Manzon and Denver, 2004; Okada et al., 2007). In all teleosts studied so far, THs are the main regulators of pituitary *tshb* expression, in both adults and during metamorphosis, indicating that a central negative feedback mechanism exists at least at the level of the pituitary and thyroid gland (Campinho et al., 2012b; Chatterjee et al., 2001; Chowdhury et al., 2004; Larsen et al., 1998; Manchado et al., 2008; Manzon and Denver, 2004). However, regulation of the HPT axis by *trh* and/or *crh* remains to be convincingly demonstrated in teleosts and existing studies suggest that species-specific regulation of the HPT axis may occur (Chatterjee et al., 2001; Chowdhury et al., 2004; Han et al., 2004; Iziga et al., 2010; Kagabu et al., 1998; Larsen et al., 1998; Manchado et al., 2008; Matz and Hofeldt, 1999; Melamed et al., 1995).

In studies of HPT axis regulation in teleosts the results suggest primarily hypothalamic inhibition rather than stimulation (Ball et al., 1963; Chowdhury et al., 2004; MacKenzie et al., 1987; Peter, 1970; Sukumar et al., 1997). Nonetheless, a common feature of anuran and

Abbreviations: THs, thyroid hormones.

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flatfish metamorphosis is an increase or stable levels of *tsh* expression coincident with a surge in TH levels. These observations suggest that in flatfish during metamorphosis hypothalamic inhibition is relieved and negative feedback on the pituitary by plasma THs is either suppressed or its responsiveness is modified to a higher set-point (Einarsdóttir et al., 2005; Han et al., 2004; Kaneko et al., 2005; Korte et al., 2011; Manchado et al., 2008; Manzon and Denver, 2004; Yamaguchi et al., 1996). In the anurans during metamorphosis the set-point of the HPT axis is modulated by the action of *crh* on pituitary thyrotrophs, so that high levels of serum TH do not repress *tshb* expression (Manzon and Denver, 2004; Okada et al., 2007). Studies of HPT-axis regulation using the goitrogens, thiourea and methimazole (MMI) to block TH production and sole metamorphosis indicate that the negative feedback loop between the thyroid and the pituitary gland is functional well before metamorphosis (Klaren et al., 2008; Manchado et al., 2008; Ponce et al., 2010).

The present study was designed to evaluate the role of the hypothalamus, pituitary and thyroid in the regulation of flatfish metamorphosis. Expression of *crh, tshb* and *tg* were used as endpoint markers of endocrine activity of each HPT-axis compartment. The flatfish, Senegalese sole, that undergoes metamorphosis early in development (between 12–18 days after hatching) was used as the experimental model. Previous studies have demonstrated the dependence of sole metamorphosis on TH levels (Klaren et al., 2008; Manchado et al., 2008). Our data show that regulation of metamorphosis occurs at the level of the pituitary and the thyroid. Intriguingly the results of the present study suggest that neither hypothalamic *trh* nor *crh* expression changes during sole metamorphosis and raises questions about the role of these factors and the hypothalamus in regulation of thyrotrophs.

2. Materials and methods

All animal manipulations in the study were carried out in compliance with the Guidelines of the European Union Council (86/609/EU) and Spanish legislation for the use of laboratory animals.

2.1. Sole rearing and sampling during normal metamorphosis

Fertilized eggs were obtained from naturally spawning Senegalese sole broodstock (IFAPA Centro El Toruño). Eggs were collected in the morning (9:00 am) and transferred to a 1000 mL measuring cylinder to separate buoyant (viable) from non-buoyant (non-viable) eggs. Numbers of eggs in each fraction were estimated using volumetric methods (1100 eggs mL^{-1}). Eggs were incubated at a density of 3000 embryos L^{-1} in 15 L cylindro-conical tanks in an open seawater circuit with gentle aeration and full exchange of seawater every hour. Newly hatched larvae (1 day post-hatch (dph)) were transferred to a 400 L tank at an initial density of 45–50 larvae L^{-1} and maintained in the dark until the onset of external feeding at 3 dph after which a 16 h light:8 h dark photoperiod was used (light intensity 600–800 lux). Mean water temperature and salinity were 21.1 ± 0.3 °C and 34.2 ± 0.2 ppt, respectively. Larvae were fed rotifers (*Brachionus plicatilis*) enriched for 3 h with *Isochrysis galbana* (T-ISO strain) from 3 dph until 9 dph. Live microalgae (*Nannochloropsis gaditana* and *I. galbana*) were also added directly to water. *Artemia metanauplii* previously enriched for 24 h with *Isochrysis galbana* (T-ISO strain) were fed from 7 dph until the end of the experiment.

Pre-metamorphic (S0), initiation (S1), pre-climax (S2), climax (S3) and post-metamorphic Senegalese sole juveniles (S4) were collected using a previously established staging scheme (Fernández-Díaz et al., 2001). For whole-mount *in situ* hybridization (WISH) larvae were anesthetized in MS222 (0.125 mg/L, Sigma-Aldrich, Spain) and collected into ice-cold 4% PFA/1×PBS pH7 and kept at 4 °C overnight. Larvae were then washed in 1×PBS + 0.1% Tween-20 (Sigma-Aldrich) (PBT), bleached in 1×PBS/0.5% KOH and 3% H2O2 at room temperature (RT) and transferred to 100% methanol (MeOH) and stored at –20 °C until use.

2.2. Methimazole blocking of metamorphosis

Larvae were cultivated as described until 7 dph as described earlier. Thereafter, larvae were transferred into six tanks (15 L) at an initial density of 60–80 larvae L^{-1} and at 17.1 ± 0.9 °C and 37.1 ± 0.5 ppt. MMI dissolved in DMSO (Sigma-Aldrich, Ref. M8506) was added to three tanks to give a final concentration of 0.3 mM and the other three tanks were the untreated control and received only the vehicle (DMSO). After adding the drug, water was not exchanged for 24 h and then 20% of the water was renewed and MMI was added to maintain a constant concentration. Larvae were fed rotifers until 9 dph and *Artemia metanauplii* enriched for 24 h with *Isochrysis galbana* (T-ISO strain) from 7 dph until the end of the experiment. Larvae were sampled at 11 and 20 dph to determine dry weight and the rate of metamorphosis. Larvae were removed by netting from the tank bottom and the water column. Approximately, 20–25 individuals were randomly taken for metamorphic stage identification. The metamorphic index for each tank was calculated as follows: [(S1 larvae ×1) + (S2 larvae ×2) + (S3 larvae ×3) + (S4 larvae ×4)]/total number of larvae.

Larvae were collected into 4% PFA/1×PBS pH7 for whole mount *in situ* hybridization (WISH) 13 days after treatment using the procedure described earlier. For gene expression studies, larvae were netted, washed with DEPC water, excess water removed and several larvae pooled and frozen in liquid nitrogen and stored at –80 °C until analysis.

2.3. In situ *probe preparation*

Senegalese sole thyroglobulin (*tg*; AB297481) and thyroid stimulating hormone (*tshb*; AB297482) were cloned into pCR4Topo (Invitrogen) vector (Manchado et al., 2008) and used as the template for the cRNA DIG-labeled probes. The cDNA for sole corticotropin releasing hormone (*crh*; FR745427) was isolated by PCR from larval sole cDNA (forward primer GCACGAGGCAAGTAACTACAC; reverse primer: GGTCTCCTGTTGATAACAGCTG) and cloned into pGEM-T easy following the manufacturer's instructions. Clones selected for subsequent riboprobe synthesis were confirmed using Big-Dye capillary sequencing (ABI). The full-length cDNA for *tg, tshb* and *crh* cloned in pGEM-T easy was amplified by PCR with the vector primers M13 forward and reverse as previously described (Campinho and Power, 2013). *In vitro* production of DIG-labeled riboprobes was carried out using T7, SP6 or T3 RNA polymerase (Fermentas) following the manufacturer's instructions. Riboprobes were purified on G-50 (GE) microfuge columns and quantified in a Nanodrop spectrophotometer before being diluted in 50% formamide (VWR) and stored at –20 °C until use.

2.4. *Whole-mount* in situ *hybridization (WISH)*

WISH was carried out using an adaptation of the method of Thisse and Thisse (2008). Briefly samples in 100% MeOH were brought to room temperature and washed using an MeOH:PBS series (100% MeOH to 0% MeOH) and finally rinsed several times in PBT. Samples were then digested with proteinase K (10 μg/mL) in 1×PBS at RT from 20 to 40 minutes (mins) depending on the metamorphic stage of the larvae. After permeabilization samples were re-fixed in 4% PFA/1×PBS for 20 mins at RT, washed several times with PBT and then pre-hybridized for 2 hours at 68 °C in hybridization mix (HybMix). Pre-HybMix was discarded and replaced by pre-warmed HybMix containing 0.25 ng/mL of sole *tshb* or *tg* sense or anti-sense DIG-labeled cRNA probes and hybridized overnight at 68 °C. Samples were subject to stringency washes, Hyb(−): 2×SSC to 2×SSC + 0.1%