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Thyroid hormone mediates otolith growth and development during flatfish metamorphosis

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ABSTRACT

Flatfish begin life as bilaterally symmetrical larvae that swim up-right, then abruptly metamorphose into asymmetrically shaped juveniles with lateralized swimming postures. Flatfish metamorphosis is mediated entirely by thyroid hormone (TH). Changes in flatfish swim posture are thought to be regulated via vestibular remodeling, although the influence of TH on teleost inner ear development remains unclear. This study addresses the role of TH on the development of the three otolith end-organs (sacculus, utricle, and lagena) during southern flounder (*Paralichthys lethostigma*) metamorphosis. Compared with pre-metamorphosis, growth rates of the sacculus and utricle otoliths increase dramatically during metamorphosis in a manner that is uncoupled from general somatic growth. Treatment of *P. lethostigma* larvae with methimazol (a pharmacological inhibitor of endogenous TH production) inhibits growth of the sacculus and utricle, whereas treatment with TH dramatically accelerates their growth. In contrast with the sacculus and utricle otoliths that begin to form and mineralize during embryogenesis, a non-mineralized lagena otolith is first visible 10–12 days after hatching. The lagena grows during pre- and pro-metamorphosis, then abruptly mineralizes during metamorphic climax. Mineralization of the lagena, but not growth, can be induced with TH treatment, whereas treatment with methimazol completely inhibits lagena mineralization without inhibiting its growth. These findings suggest that during southern flounder metamorphosis TH exerts differential effects on growth and development among the three types of otolith.

1. Introduction

The flatfishes (Order Pleuronectiformes) begin life as bilaterally-symmetrical larvae that swim with an up-right posture. The larvae undergo an abrupt developmental transformation to the juvenile stage, termed 'metamorphosis', that is characterized by the migration of one eye to the opposite side of the head, extensive craniofacial remodeling, development of a highly lateralized swim posture, and transition from a pelagic to a benthic life-style (Schreiber, 2006). Like amphibian metamorphosis, virtually all morphological and behavioral changes of flatfish metamorphosis are thought to be mediated by one molecule, thyroid hormone, via receptors that function as nuclear transcription factors (Inui and Miwa, 1985; Miwa et al., 1988; Schreiber and Specker, 1998).

Although the morphology and physiology of the adult flatfish vestibular system has been studied extensively (Graf and Baker, 1983, 1985a,b, 1990; Graf et al., 2001; Helling et al., 2005; Jansen and Enger, 1996; Meyer et al., 1981; Platt, 1973a,b, 1983), the specific effects of thyroid hormones on the development of the flatfish inner ear during metamorphosis remain virtually unknown. This study addresses the development of one component of the flatfish vestibular system, the three otolith end-organs, during metamorphosis and the roles thyroid hormones play in their ontogeny.

In vertebrates, angular acceleration (e.g. head motion) is perceived by the semi-circular canals, whereas linear acceleration (e.g. gravity) is detected by otolith end-organs that communicate with the brainstem and cerebellum (Beisel et al., 2005). All vertebrates (with the exception of non-monotreme mammals) possess three pairs of otolith end-organs: the sacculus, lagena, and utricle; the lagena is absent in mammals other than the Monotremata (Khorevin, 2008). In juvenile and adult fish, these organs are located adjacent to the semi-circular canals, and each organ contains a mineralized otolith component consisting of calcium carbonate embedded in a gelatinous matrix (Popper and Lu, 2000) (Fig. 1). These mineralized otolith structures reside in fluid-filled pouches, where they are tethered to the underlying sensory epithelium and interact with sensory hair cells (Fig. 1). An otolith acts as an inertial mass on the otolithic pouch, which enhances the sensitivity of the underlying sensory hair cells to the forces of linear acceleration. In fishes, the otolith organs are implicated with roles in both balance and hearing (Khorevin, 2008; Platt, 1973a,b).

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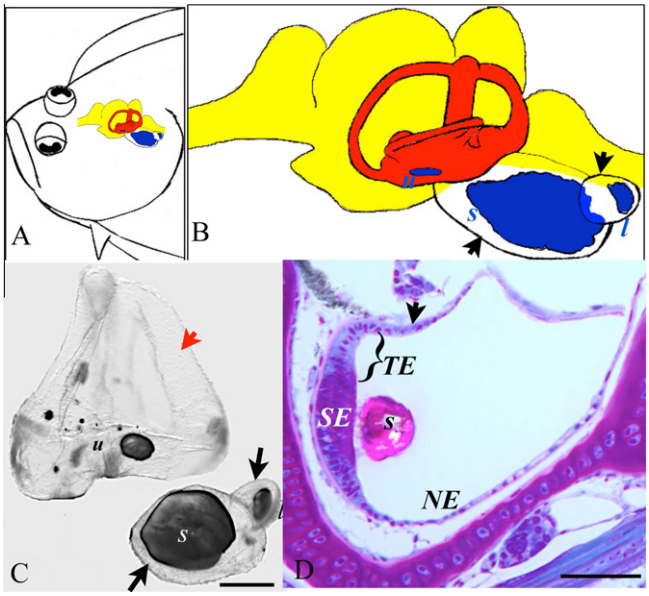

Fig. 1. Otolith and semi-circular canal anatomy in *P. lethostigma*. Left otolith and semi-circular canal morphology depicted from an adult (1 year post-hatch; (A and B)), and dissected from a juvenile (120 dph; (C)). Cross-section of the left sacculus from a pre-metamorphic larva (20 dph; (D)). Red fill in (A and B), and red arrowhead in C denote the left semi-circular canal; *u*, *s* and *l* denote the mineralized portion of the utricle, sacculus, and lagena otoliths, respectively. Black arrowheads denote sacculus and lagena organ membranes. *SE*, *TE*, and *NE* denote 'sensory epithelium', 'transitional epithelium', and 'non-sensory epithelium', respectively. Scale bars denote 0.25 mm in (C) and 100 μm in (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Thyroid hormones are known to be involved in mammalian vestibular and auditory development (Bradley et al., 1994; Bryant et al., 2002; Flamant and Samarut, 2003; Winter et al., 2007). Studies have also shown that thyroid hormones affect development of the sacculus otoliths in some fish: thyroid hormones have been directly implicated with roles in sacculus otolith growth during larval development of tilapia (Shiao et al., 2008) and tarpon (Shiao and Hwang, 2004, 2006), and disproportionate growth of sacculus otoliths compared with somatic growth has been reported during eel metamorphosis, a process known to be mediated by thyroid hormone (Correia et al., 2006; Lee and Byun, 1996; Shiao et al., 2001, 2002). However, the influence of thyroid hormones on the development of all three otolith classes (sacculus, lagena, and utricle) during metamorphosis for any teleost fish has not yet been reported.

Here we show that for southern flounder larvae (*Paralichthys lethostigma*) TH is both necessary and sufficient for growth of the sacculus and utricle otoliths during *P. lethostigma* early larval development and metamorphosis. In contrast to these otoliths that become mineralized during embryogenesis, the lagena otolith first emerges as a non-mineralized structure in the pre-metamorphic larva. Interestingly, unlike the sacculus and utricle otoliths, growth of the lagena is not under TH-control, though its abrupt mineralization during metamorphic climax is TH-induced. These findings suggest that during southern flounder metamorphosis TH exerts differential effects on growth and development among the three types of otolith.

2. Methods

2.1. Fish

Southern flounder (*P. lethostigma* L.) embryos (12–24 h post-fertilization) were obtained from the University of North Carolina Wilmington's aquaculture facility throughout the breeding season (October–April, 2005) and raised at the Carnegie Institution of Washington's Department of Embryology (Baltimore, MD) in 40–120 l aquaria (25 °C) using artificial saltwater (35‰ salinity). After hatching (48 h post-fertilization), larvae were fed live zooplankton (*Brachionus plicatilis*) and brine shrimp (*Artemia*) nauplii through the end of metamorphosis (approximately 31 days post-fertilization; dpf) according to methods described (Daniels, 2000; Daniels and Watanabe, 2003). Stages of larval development are based on the position of the migrating eye and pectoral fin morphology described in Schreiber (2006).

2.2. Treatments

Larval *P. lethostigma* at pre-metamorphosis (12 dpf) were treated with thyroxine (T4) (Sigma Chemical Co., St. Louis, MO, USA) added to the water (T4 final concentration, 30 nmol l^{-1}) for 7 days to induce metamorphosis. Methimazol (0.1 mM; Sigma), an inhibitor of endogenous thyroid hormone (TH) production (Brown, 1997), was dissolved directly into the water and administered for 4–7 weeks (starting at 9 dph pre-metamorphosis) to inhibit metamorphosis. Some fish treated for 7 weeks with methimazol (9–60 dph) were then treated with TH (T4 final concentration, 30 nmol l^{-1}) for 7 days to induce metamorphosis. The ability to effectively modulate endogenous TH levels during larval flatfish development via treatment of aquarium water with TH or pharmacological inhibitors of endogenous TH production has been previously described by us (Schreiber, 2006; Schreiber and Specker, 1998).

2.3. Histology, microscopy, and image analysis

For histological processing, larvae were fixed in 4% paraformaldehyde (dissolved in 1X PBS buffer) overnight at 4 °C. Samples were then infiltrated in 30% sucrose dissolved in 1X PBS overnight at 4 °C, embedded in OCT compound (Electron Microscopy Sciences), flash-frozen, and cryosectioned at a thickness of 10 μ onto VWR Superfrost slides. Masson's trichrome stain technique for collagen, cytoplasm, and nuclei was performed as described (Bancroft and Gamble, 2002). All photographs were taken with a Spot (Diagnostic Instruments, Sterling Heights, MI) digital camera using either a Leica MZFLIII stereo-microscope for low magnification bright-field and dark-field images, or a Nikon E800 compound microscope for high magnification phase-contrast and bright-field photography.

2.4. Measurements and statistical analyses

For this study, otolith 'size' is defined as the otolith saggital 2-dimensional surface area. To measure sacculus and utricle surface areas at multiple developmental stages, larvae were first euthanized via overdose of the anesthetic MS-222 (5 min exposure to 1% w/v MS-222 dissolved in rearing water), and otoliths (from both left and right sides) were removed using micro-dissection forceps. Otoliths were photographed on their 'sides', and 2-D surface areas were measured using ImageJ (National Institutes of Health, USA) imaging software. Lagena otoliths from larvae in late-metamorphic climax or older were measured as above. However, lagena otoliths from larvae younger than late-metamorphic climax were photographed *in vivo*, since the gelatinous consistency of larval lagenas made it virtually impossible to remove them without disintegration. Head length was measured by linear distance from the tip of the nose to the distal edge of the opercular opening that is closest in proximity to the spinal cord. To determine if otolith growth rates during metamorphosis change in a manner uncoupled from general somatic growth rate, otolith surface areas were plotted against 'Head size', defined as $(\text{head length})^2$. Measurements for