Vol. 278: 271–278, 2004 | MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser | Published September 7

Thyroid hormones are necessary for teleostean otolith growth

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ABSTRACT: Experiments were conducted to understand the effects of thyroid hormones and thiourea (TU) on otolith growth during metamorphosis of leptocephali. TU is an anti-thyroid hormone drug that inhibits the production of thyroxine (T_4) and triiodothyronine (T_3) in the thyroid tissue. Fully grown leptocephali of tarpon *Megalops cyprinoides* collected at the mouth of the Gong-Shy-Tyan Brook, N Taiwan, were immediately treated with 10 ppb T_3 and 300 ppm TU for the experimental period of 13 d. The newly grown otolith lengths measured from the maximum radius were 77.0 ± 12.0, 91.9 ± 7.2 and 47.9 ± 6.3 µm in the control, T_3 and TU groups, respectively. The newly grown otolith length of the T_3 group was significantly longer than those of the control and TU groups, and the control group had significantly larger length values than the TU group. The T_3 treatment stimulated otolith growth, increasing daily increments from 7 to 13 cm (control) to 9 to 16 µm (T_3) during the growth peak of metamorphosis. Maximum daily increments were only 6 to 10 µm in TU-treated tarpons. The otoliths of the fish treated with T_3 and TU had increments deposited daily. Otoliths of T_3-treated and control fish grew throughout the experimental period, but otoliths of TU-treated fish stopped growth beginning on Day 6 to Day 9. These results suggest that thyroid hormones are essential for otolith growth, probably by regulating the expressions and functions of the otolith protein matrix and ion transporters in the saccular epithelium.

KEY WORDS: Hypothyroidism · Thyroid hormone · Thiourea · Otolith · Metamorphosis · Leptocephalus · Tarpon

INTRODUCTION

Fish otoliths are accreting crystals mainly composed of metabolically inert calcium carbonate (Campana 1999). Age determination from reading daily otolith growth increments has wide application in life-history investigations in fish (Pannella 1971). Somatic growth back-calculated from otolith growth is a common and useful utility based on isomeric growth between otoliths and length or weight of fish (Volk et al. 1984, Neilson & Geen 1985, Penney & Evans 1985). However, further studies have discovered an uncoupling of somatic and otolith growth, usually under suboptimal conditions (Mosegaard et al. 1988, Wright et al. 1990). Temperature, starvation, and ontogenic development can cause somatic growth to stop or become negative while otolith growth continues (Campana & Neilson 1985, Tzeng & Yu 1992, Tsukamoto & Okiyama 1993). This uncoupling phenomenon has spurred numerous studies, and the prevailing hypothesis holds that the width of otolith daily growth increments better reflects metabolic rate than it does somatic growth (Wright 1991, Huuskonen & Karjalainen 1998).

In several species, including *Megalops cyprinoides* (Tsukamoto & Okiyama 1993), *Anguilla* eels (Shiao et al. 2001, 2002) and *Conger myriaster* (Lee & Byun 1996), the commencement of leptocephalus metamorphosis is characterized by an abrupt increase of daily growth increment width. However, Cieri & McCleave (2000) speculated that otolith growth of American eel *Anguilla rostrata* may stop and a portion of the periphery of the otolith may be resorbed during metamorphosis from leptocephali to glass eels. Metamorphosis of leptocephalus conger eel coincides with a thyroid

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hormone surge (Yamano et al. 1991). Thyroid hormone thyroxine (T_4) is secreted by thyroid follicle cells and converts to the biologically active form, triiodothyronine (T_3), by deiodination in the peripheral tissues (Eales 1985). T_3 action is mediated by the thyroid hormone receptors α and β, which belong to the nuclear receptor superfamily (Wu & Koenig 2000). Thyroid hormones are known to be involved in mammalian auditory development and adult homeostasis, including basal metabolic rate (Flamant & Samarut 2003). Exogenous thyroid hormones were demonstrated to promote metamorphosis of leptocephalus tarpon (J.-C. Shiao & P.-P. Hwang unpubl. data). Based on the facts above, we propose the following hypotheses: (1) the abrupt increase of otolith increment width during leptocephalus metamorphosis is stimulated by thyroid hormones, (2) variable thyroid hormone concentrations (hyper- or hypothyroidism) may affect otolith growth (fast or slow), probably in a dose–response manner. A determination of the relationship between thyroid hormones and otolith growth may lead to greater understanding of otolith growth, especially during leptocephalus metamorphosis.

Mugiya (1990) hypophysectomized gold fish *Carassius auratus*, and found severely and moderately retardant growth in their scales and otoliths, respectively. Removal of the pituitary gland causes abnormal stasis of somatic growth due to the dysfunction of many hormones, including thyroid hormones. The effects of hypophysectomy are difficult to attribute to specific hormones. Consequently, our knowledge of hormonal influence on otolith growth is still far from complete.

This study used exogenous thyroid hormone, T_3, and the anti-thyroid hormone drug thiourea (TU) to create hyper- or hypothyroidism in metamorphic tarpons, and evaluated the influence of these treatments on otolith growth. Metamorphic tarpons were kept in the same captive environment without feeding to reduce bias from other effects. Feeding is not required for metamorphic tarpons, which derive nutrients from the breakdown of lipid and proteoglycans accumulated in the leptocephalus stage (Pfeiler 1986). To our knowledge, this is the first report that directly measures the relationship between thyroid hormones and otolith growth.

MATERIALS AND METHODS

Fish. Fully grown leptocephalus tarpons *Megalops cyprinoides* were caught by a net set against the tidal current at the mouth of Gong-Shy-Tyan Brook, northern Taiwan, during the flood tide on June 17 and July 14, 2003. The leptocephali drifted into the estuary with the tidal current and were collected in the end of a funnel-shaped net while the water level rose from the beginning of the flood tide. The leptocephali were carefully transferred to a container without leaving seawater to avoid possible damage to the vulnerable body. The leptocephali were brought to the laboratory and distributed into several glass aquaria. Subsamples (n = 16 and 20) were randomly selected from captive tarpons, and their total lengths were measured to represent the initial total length for each group. The salinity and water temperature were approximately 30 ppt and 28°C at the sampling location.

Thyroid hormone and thiourea concentration. The dose–response effects of leptocephalus tarpons to T_3 and to TU have been evaluated by J.-C. Shiao & P.-P. Hwang (unpubl. data). Concentrations of 10 ppb T_3 and 300 ppm TU can promote or inhibit metamorphosis of leptocephalus tarpons. Accordingly, the leptocephali were reared in well-aerated 30 ppt artificial seawater containing 10 ppb T_3, 300 ppm TU, or without hormone or drug treatment as a control group, at a density of approximately 200 fish in 20 l of seawater. High concentrations of T_3 (100 ppm) were dissolved in ethanol, then diluted to 10 ppb in seawater. Ethanol alone had no effect on tarpon growth in a control group. Artificial seawater was made using a mixture of synthetic sea salts. Then, $1/10$ of the water was exchanged with seawater containing the same concentration of T_3 or TU every 2 d. Another group of leptocephali were immediately immersed in tetracycline solution (300 mg l^{-1}) after they were brought to the laboratory. After 24 h, the tetracycline solution was exchanged with artificial seawater. Tetracycline was incorporated in the otolith and created a fluorescent mark on the day when the fish were caught. During the experiment, water temperature was controlled at 25°C and the photoperiod was set at 12L:12D. The metamorphic tarpons were not fed and were sacrificed on Day 14 (after an experimental period of 13 d). Final total lengths were measured, then fish were frozen at –20°C until otoliths were extracted.

In order to evaluate the dose–response effect of otolith growth to TU, another 2 groups of metamorphic tarpons (35 fish for each group) were treated with low (30 ppm) and high (3000 ppm) TU concentrations. The treatment only lasted 7 d under the same rearing conditions mentioned above.

Otolith preparation. Otoliths were removed under a stereo microscope, dried in air and embedded with epofix resin. The otoliths were ground and polished until the core of the otolith was exposed on the otolith surface. A fluorescence microscope with incident light from a 50 W mercury lamp was utilized to detect the fluorescent ring on the ground surface of otoliths. The excitation wavelength of the incident light was limited by a band-pass filter (400 to 440 nm), and the emission

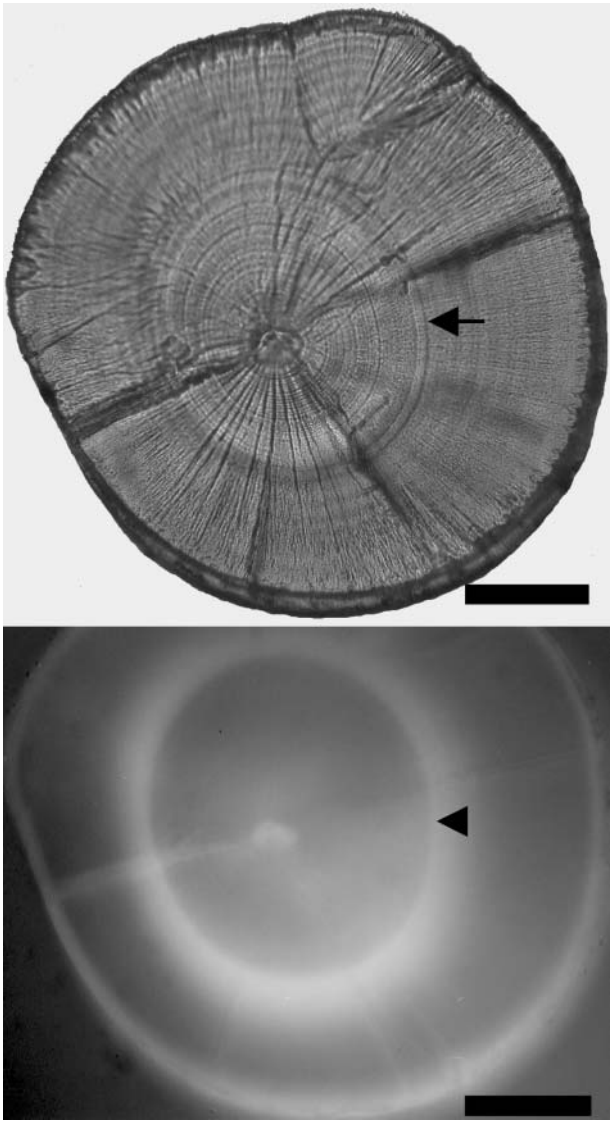

Fig. 1. *Megalops cyprinoides*. Tarpon otolith showing the tetracycline mark (arrowhead) on the captivity check (arrow). Scale bars = 50 µm

light by a long-pass barrier filter (470 nm). Then, dilute HCl (0.05 M) was used to etch the otolith for 20 s. Etching aided the reading of otolith increments by enhancing the contrast of the daily growth increments under a compound light microscope. Images of fluorescent rings and the whole etched otolith were recorded at 400× magnification under a light microscope equipped with a digital camera. Measurements of otolith length and increment width, as well as counts of daily growth increments, were processed on the images on a personal computer using the software by Image-Pro plus, Media Cybernetics (ver. 1994). The measurements were conducted from the maximum radius of each otolith.

Statistical analysis. Data are expressed as means ± SD (n = number of fish). Statistical differences among treatments were analyzed using 1-way analysis of variance (ANOVA). Tukey's pairwise comparison was used to identify groups that differed from others. Significance was set at $p < 0.05$.

RESULTS

Tetracycline mark and captivity check

Tetracycline was incorporated in the newly deposited otolith increment of *Megalops cyprinoides* and showed a yellow fluorescent ring under the fluorescent microscope (Fig. 1). All the otoliths examined were successfully marked by tetracycline (n = 40). After etching by HCl, the fluorescent ring formed a deeply etched check. This check is defined as a captivity check for convenience. The check was also found in other otoliths that were not marked with tetracycline. The coincidental appearance of the tetracycline mark and the check indicated that this check was deposited during the day of catch. Accordingly, otoliths are divided into the leptocephalus stage (before captivity check) and the metamorphic stage (from captivity check to otolith edge at the end of the experiment) (Fig. 2).

Otolith length and daily growth increment

The mean otolith length (from the core to the captivity check, Fig. 2) at the time when fish were brought into captivity was approximately 70 µm and showed no significant difference among the control, 10 ppb T_3 and 300 ppm TU groups ($p > 0.05$, Tukey's pairwise comparison) (Fig. 3). This result indicated that the fish were in a similar developmental stage when they were caught. The distances from captivity check to otolith edge were 77.0 ± 12.0 (n = 17), 91.9 ± 7.2 (n = 14) and 47.9 ± 6.3 (n = 16) µm in the control, T_3 and TU groups, respectively. The length of newly grown otoliths for the T_3 group were significantly longer than for the control and TU groups, and the control group was also significantly longer than the TU group ($p < 0.05$, Tukey's pairwise comparison) (Fig. 3, Table 1). This indicates that T_3 promotes and TU retards otolith growth.

Otolith daily increment width

Fig. 4 shows the typical temporal changes of increment width of 2 tarpons from each treatment. Increment width was relatively narrow during the wild

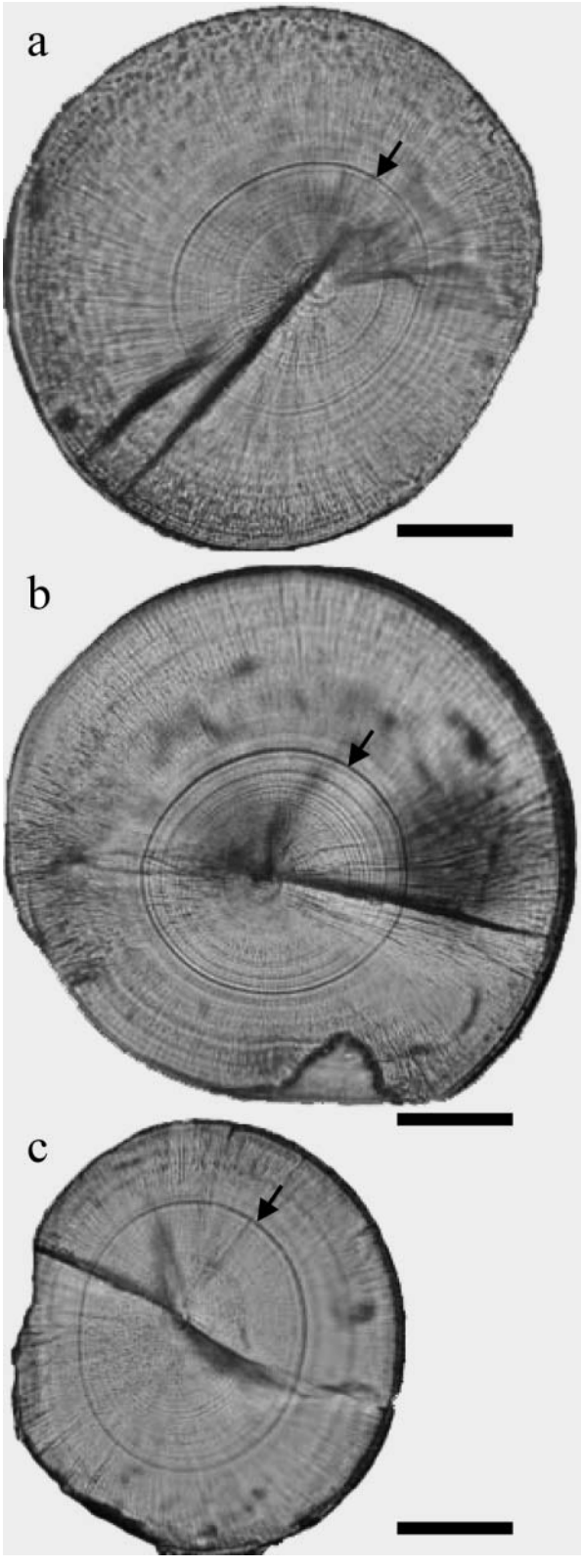

Fig. 2. *Megalops cyprinoides*. Microstructure of tarpon otoliths from different treatments (a) control, (b) 10 ppb T_3 and (c) 300 ppm TU. Arrows indicate the captivity check. Scale bars = 50 µm

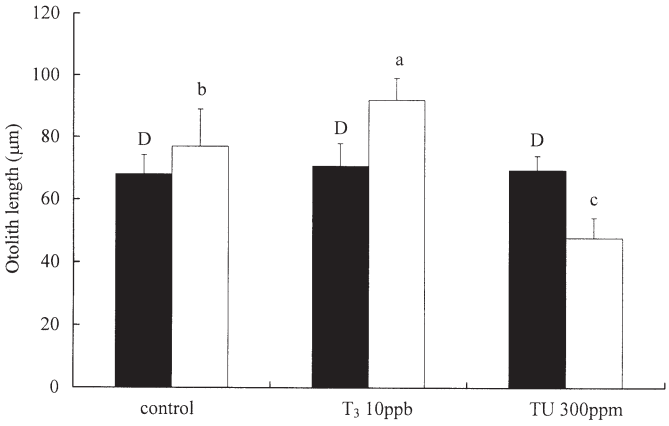

Fig. 3. *Megalops cyprinoides*. Tarpons show consistent otolith lengths at leptocephalus stage (from the primordium to captivity check, closed histogram) before different treatments. After treatment by thyroid hormone and thiourea for 13 d, tarpons show significant differences in mean otolith lengths of the metamorphic stage (from captivity check to otolith edge, open histogram). Error bar: 1 SD. Different letters indicate the significant difference among groups (Tukey's pairwise comparison, $p < 0.05$) in each stage

leptocephalus stage and fluctuated between 1 and 3 µm in all examined otoliths (Fig. 4). There was no difference of increment width during the wild leptocephalus stage among the control, 10 ppb T_3 and 300 ppm TU groups. After the captivity check, the increment width abruptly increased from approximately 2 to 7–13 µm (9.6 ± 2.1 µm, n = 17) in the control group and maintained this level for several days, then increment width decreased toward the end of metamorphosis (Fig. 4a). For the T_3 treatment, the increment width peaked at approximately 9 to 16 µm (12.0 ± 2.1, n = 15) for a few days, then decreased during late metamorphosis (Fig. 4b). Generally, the daily growth increment widths of tarpons in the T_3 treatment increased immediately and reached their peak faster than in the control group. The increment width peaked between 6 and 10 µm (8.5 ± 1.3, n = 17) in the TU group, then decreased rapidly at the otolith edge (Fig. 4c). Otolith growth stopped, beginning on Day 6 to Day 9 in the TU group. This indicates that TU retards otolith growth in the early metamorphic stage and completely inhibits otolith growth in the mid-metamorphic stages.

Daily age of tarpon

The mean ages of leptocephalus stage tarpon were 27.9 ± 3.6 d (n = 18), 29.2 ± 2 d (n = 13) and 28.1 ± 3.2 d (n = 15) in the control, 10 ppb T_3 and 300 ppm TU groups, respectively (Table 1). There was no significant difference in ages among groups, indicating the

Table 1. *Megalops cyprinoides*. At the beginning of treatments, total lengths (mean ± SD) of the same batch of tarpons are measured to represent the initial length for all groups. At the end of the experiments, final total lengths of the tarpons in each treatment are measured. Tarpon otolith increments were counted and otolith lengths were measured according to the developmental stage, the leptocephalus stage (from primordium to captivity check) and the metamorphic stage (from captivity check to otolith edge). Tarpons in control, 10 ppb T_3 and 300 ppm TU groups were reared for 13 d while tarpons in 30 and 3000 ppm TU were reared for 7 d (n = sample size)

	Control	T_3 10 (ppb)	TU 300 (ppm)	TU 30 (ppm)	TU 3000 (ppm)
Total length (cm)					
Initial length of fish	2.6 ± 0.12 (n = 16)	2.6 ± 0.12 (n = 16)	2.6 ± 0.12 (n = 16)	2.6 ± 0.29 (n = 20)	2.6 ± 0.29 (n = 20)
Final length of fish	1.9 ±0.13 (n = 16)	1.8 ± 0.09 (n = 16)	2.3 ± 0.20 (n = 16)	2.0 ± 0.14 (n = 27)	2.2 ± 0.12 (n = 32)
Leptocephalus stage					
Otolith increments (d)	27.9 ± 3.6 (n = 18)	29.2 ± 2.0 (n = 13)	28.1 ± 3.2 (n = 15)	27.3 ± 3.1 (n = 15)	27.5 ± 3.3 (n = 15)
Otolith length (µm)	68.0 ± 6.3 (n = 18)	70.7 ± 7.2 (n = 14)	69.3 ± 4.6 (n = 15)	66.0 ± 9.6 (n = 15)	64.6 ± 7.3 (n = 15)
Metamorphic stage					
Otolith increments (d)	13.2 ± 0.4 (n = 17)	13.0 ± 0.0 (n = 15)	7.1 ± 0.8 (n = 15)	7.0 ± 0.0 (n = 15)	3.5 ± 1.2 (n = 15)
Otolith length (µm)	77.0 ± 12.0 (n = 17)	91.9 ± 7.2 (n = 15)	47.9 ± 6.3 (n = 15)	55.6 ± 7.9 (n = 15)	26.1 ± 7.2 (n = 15)

tarpons had similar, but not identical, hatching dates. After the experimental period of 13 d, the newly deposited increments totaled 13.2 ± 0.4 (n = 17), 13 ± 0 (n = 15) and 7.1 ± 0.8 (n = 15) rings in the control, T_3 and TU groups, respectively. Otoliths of T_3-treated and control fish grew throughout the experimental period, but otoliths of TU-treated fish did not grow from Day 6 to Day 9 (Table 1).

To evaluate the dose–response effect of otolith growth to TU, 2 groups of metamorphic tarpon were treated with low (30 ppm) and high (3000 ppm) concentrations of TU. Ages of leptocephalus stage tarpon were 27.3 ± 3.1 d (n = 15) and 27.5 ± 3.3 d (n = 15) in the 30 and 3000 ppm TU groups, respectively. After the experimental period of 7 d, newly deposited otolith increments totaled 7.0 ± 0 (n = 15) and 3.5 ± 1.2 (n = 15) rings in 30 and 3000 ppm TU groups, respectively (Table 1). This indicates that otoliths could grow throughout the experiment at a low concentration of TU, but a high concentration of TU can completely inhibit otolith growth after a few days (herein, 3 d under 3000 ppm TU).

DISCUSSION

Metamorphic leptocephalus *Megalops cyprinoides* tarpon in this study showed an inverse relationship between somatic and otolith growth. Approximately 30% of total length is lost, coincident with a gain of 113% in otolith length during metamorphosis (Table 1, Fig. 3). Hence, otolith growth is independent of somatic growth during metamorphosis of tarpon. The increment width of otolith daily growth is influenced by temperature (Neilson & Geen 1982, Cheng et al. 1996) and feeding (Victor 1982, Wright et. al. 1990). These 2 factors influence increment width by altering the basal metabolic rate (Huuskonen & Karjalainen 1998). In the present study, the experimental animals were not given a supplemental diet and temperature was maintained at 25°C. Accordingly, temperature and feeding can be ruled out as factors responsible for the dramatic increase in otolith increment in metamorphic tarpons.

Exogenous T_3 (10 ppb) increased total otolith growth rate by approximately 20%, while the anti-thyroid hormone drug TU (300 to 3000 ppm) retarded otolith growth by approximately 30 to 40% during metamorphosis (Table 1, Fig. 3). This suggests a positive relationship between the thyroid hormone concentration and otolith growth. The ages of leptocephalus tarpons at capture ranged from 21 to 35 d. In captivity, the tarpons showed a synchronous increase of the daily growth increment widths from approximately 2 (premetamorphic stage) to 7–13 µm (metamorphic stage) under normal conditions. Yamano et al. (1991) measured a 6-fold and 13-fold increase of thyroxine (T_4) and T_3 concentrations, respectively, during the leptocephalus metamorphosis of conger eels. The changes of thyroid hormones were coincident to the 4-fold increase (0.3 to 1.2 µm) of otolith daily growth increment width observed by Lee & Byun (1996). Con-

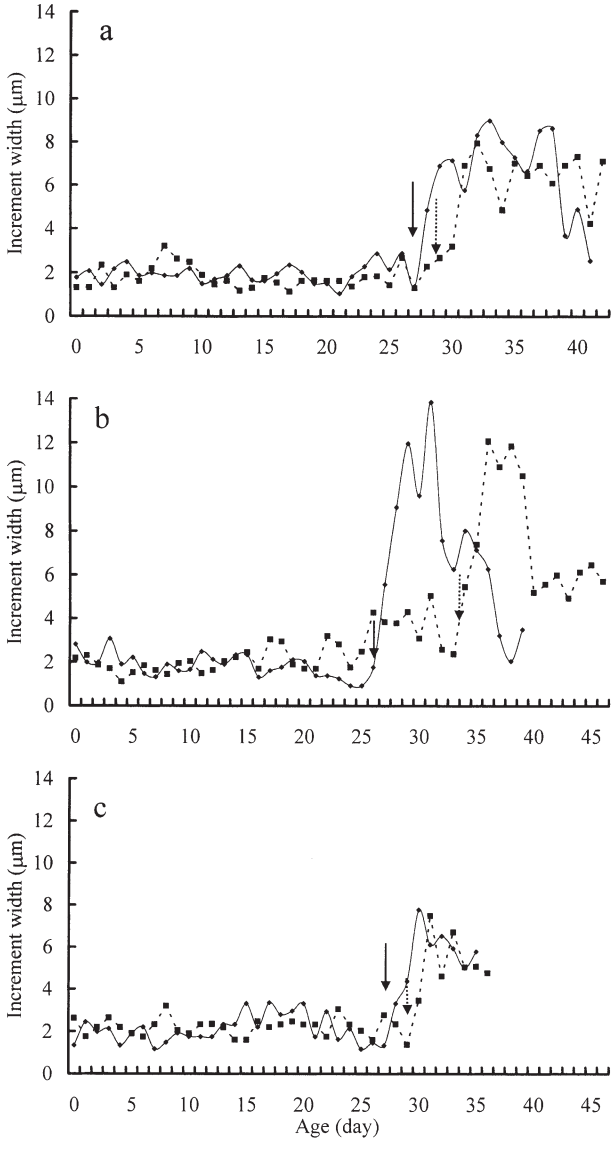

Fig. 4. *Megalops cyprinoides*. Temporal changes of increment width from primordium to edge of tarpon otolith. Profiles of 2 otoliths from each treatment are shown by continuous and dashed lines. Solid and dashed arrows indicate the position of captivity check in each otolith. Otolith growth stops on Days 7 and 8 in the 2 tarpons treated with 300 ppm TU as shown in panel (a) control, (b) 10 ppb T_3, (c) 300 ppm TU

sequently, the abrupt increase of daily growth increment width at the onset of metamorphosis of leptocephalus tarpon can be stimulated by a thyroid hormone surge, similar to that in conger eel. The variable increment width can be partially attributed to the changes in basal metabolic rate induced by hyperthyroidism and hypothyroidism. For example, hyperthyroidism will enhance the cellular activity, such as protein secretion and ion transport, then stimulate more calcium carbonate biomineralization. Metamorphic tarpon treated with high concentrations of TU (300 and 3000 ppm) showed a dose-dependent cessation of otolith growth (Table 1). Higher concentrations cause stasis of otolith growth earlier. This indicates that thyroid hormones are essential for otolith growth and further suggests a genomic mechanism in regulating otolith growth. TU in high concentrations seems able to completely inhibit the production of thyroid hormones; however, several increments were still added to the otoliths of TU-treated tarpons. This is because previously produced thyroid hormones can last for several days and sustain otolith growth before thyroid hormones are exhausted (Toft 1994).

To our knowledge, this is the first study to report that otolith growth can be completely stopped in the condition of hypothyroidism or promoted by exogenous thyroid hormones. Further study on the underlying mechanisms will be even more enlightening. Development of fish tissue stimulated by thyroid hormones is controlled at the receptor level by the differential expression of thyroid hormone receptors (Yamano & Miwa 1998). The thyroid hormone receptor, located in the nucleus, binds to T_3 and undergoes a change that allows it to bind to specific DNA sequences, known as thyroid hormone response elements (TREs), and activates the transcription and thus the translation of the target genes. Protein matrix, although it represents only 0.3 to 10% of total otolith weight (Degens et al. 1969, Asano & Mugiya 1993), determines the organization and properties of otolith structure. A novel gene, called starmaker, was recently reported to affect otolith biomineralization, and the suppression of the starmaker gene in zebrafish caused otolith crystal changes from aragonite to calcite and morphological abnormality from oval to star shaped (Söllner et al. 2003). Some studies (Gauldie & Nelson 1988, Davis et al. 1995, 1997, Takagi 2000, Murayama et al. 2002) have pointed out that the supporting cells in the sensory macula and transitional epithelial cells are the origin of protein secretion. The mechanism for thyroid hormone control of otolith growth is still unclear, but a process involving regulation of protein matrix production and ion transport from saccular epithelium into endolymph is very likely. We hypothesize that the genes that encode the protein matrix for otolith growth are regulated by thyroid hormone receptors. Abolishing the production of thyroid hormones by TU can inactivate the thyroid hormone receptors so that no protein matrix is produced and otolith growth ceases. Furthermore, hypothyroidism may cause dysfunction of the epithelial cells in the saccules. Two consequences are possible: one is the blockage of transport of calcium and bicarbonate ions into the endolymph (Tohse & Mugiya 2001) and the other is the saccules cannot maintain endolymph alkalinity (pH = 8) (Payan

et al. 1997), so that calcium carbonate biomineralization cannot proceed.

This study demonstrates that the thyroid hormone T_3 can promote the growth of otolith daily increments. Working with conger eels *Conger myriaster*, Yamano et al. (1991) observed the abrupt increase of thyroid hormones, T_4 and T_3, in early and late metamorphosis, respectively. A similar thyroid hormone surge can be expected during the leptocephalus metamorphosis of American eel *Anguilla rostrata*. Therefore, otolith increment width in metamorphic leptocephali abruptly increases as observed by Wang & Tzeng (2000), rather than ceasing under conditions of reabsorption as speculated by Cieri & McCleave (2000) for *A. rostrata*.

Acknowledgements. This study was conducted with financial support from the National Science Council of the Republic of China (Contract No. NSC 92-2811-B-001-068). We would like to extend our full gratitude to the following: C. H. Shen, H. H. Ho and H. L. Chen for assistance with the experiments and D. Cairns for suggestions on the manuscript.

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Editorial responsibility: Otto Kinne (Editor), Oldendorf/Luhe, Germany

Submitted: January 27, 2004; Accepted: April 27, 2004
Proofs received from author(s): August 30, 2004