The Journal of Experimental Biology 211, 1919-1926
Published by The Company of Biologists 2008
doi:10.1242/jeb.013748

Evaluation of thyroid-mediated otolith growth of larval and juvenile tilapia

Jen-Chieh Shiao[1,*], Su-Mei Wu[2,*], Yi-Ping Hwang[2], Done-Ping Wu[3] and Pung-Pung Hwang[4,†]

[1]Institute of Oceanography, College of Science, National Taiwan University, Taipei, Taiwan, Republic of China, [2]Department of Aquatic Biosciences, College of Life Science, National Chiayi University, Chiayi, Taiwan, Republic of China, [3]St Martin De Porres Hospital, Chiayi, Taiwan, Republic of China and [4]Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan, Republic of China

*These authors contributed equally to this work
†Author for correspondence (e-mail: pphwang@gate.sinica.edu.tw)

Accepted 1 April 2008

SUMMARY

Thyroid-mediated otolith growth in tilapia was evaluated by the ontogenic triiodothyronine (T_3) profile revealed by radioimmunoassay during the first month after hatching. Thyroid hormone receptor genes (*TRα* and *TRβ*) were cloned and only the expression of *TRα* mRNA, quantified by real-time PCR, was similar to the T_3 profile. Variations in otolith growth showed median correlation with the T_3 profile and *TRα* mRNA expression pattern. Hypothyroidism and hyperthyroidism were induced in tilapia juveniles and larvae by administration of different concentrations of thiourea (TU) and T_3, respectively, for 13 days. T_3 and TU had little effect on otolith growth during the larval stage. However, T_3 increased otolith growth and TU retarded, or stopped, otolith growth during the juvenile stage. Furthermore, TU treatment caused permanent changes in otolith shape in the ventral area. Otolith growth recovered slowly from hypothyroidism, requiring 2 days to form an increment during the first week. These results suggest that otolith growth, at least during the juvenile stage, is regulated by the thyroid hormones and the process may be mediated by *TRα*.

Key words, thyroid hormone, thyroid hormone receptor, thiourea, otolith, tilapia.

INTRODUCTION

The accreting and metabolically inert otoliths of teleosts that function as the vestibular and hearing receptor have widespread research application (Begg et al., 2005). Despite the importance of otolith-related techniques and their rapid expansion in applied research, knowledge of the mechanisms of otolith formation and growth is still scarce. Campana (Campana, 2005) states, after reviewing 862 recent otolith-related papers, that understanding the physiologically based otolith growth model is a major task for future study.

Otolith growth is influenced by exogenous factors, such as temperature (Volk et al., 1999) and feeding (Baumann et al., 2005) as well as by endogenous factors, e.g. thyroid hormones (Shiao and Hwang, 2004) and neuronal control (Anken et al., 2000). Among endogenous factors, hormones are important in regulating numerous physiological process and developmental events. Mugiya (Mugiya, 1990) demonstrated hormonal influence on otolith growth by hypophysectomizing goldfish (*Carassius auratus*). Among the hormones, thyroid hormones (THs) mainly function to control the growth, development, metabolism and homeostasis of vertebrates (Brent, 1996). Shiao and Hwang (Shiao and Hwang, 2004; Shiao and Hwang, 2006) further confirmed that thyroid hormones are necessary for otolith growth during metamorphosis of leptocephalus tarpon (*Megalops cyprinoides*). They suggested a positive correlation between thyroid status and otolith growth based on the fact that hyperthyroidism increases otolith growth while hypothyroidism retards or even stops otolith growth during the metamorphosis of tarpons (Shiao and Hwang, 2004; Shiao and Hwang, 2006).

The spectacular metamorphosis of the tarpon, flounder and conger eel is driven by a thyroxine surge (Miwa et al., 1988; Yamano et al., 1991), which is also presumably responsible for the abrupt increase of otolith daily growth rate during leptocephalus metamorphosis (Shiao and Hwang, 2004; Shiao and Hwang, 2006). Most teleosts do not show dramatic changes of morphology from larval to juvenile stage. However, the metamorphosis of many teleostean larvae is also a TH-dependent event, as shown in goldfish (*Carassius auratus*) (Reddy and Lam, 1992), zebrafish (*Danio rerio*) (Brown, 1997) and grouper (*Epinephelus coioides*) (de Jesus et al., 1998). Furthermore, gonadal maturation and reproduction may also be mediated by elevated thyroid status (Cyr and Eales, 1996). Accordingly, otolith growth may change at certain thyroid-mediated life history events. Otolith growth and its microstructure are usually regarded as the proxy of fish growth and as the recorder of their life histories. So far, the physiological basis, especially for endocrine-mediated growth changes of the otolith is still poorly understood.

Normal embryo development and fish growth depends on the programmed secretion of thyroid hormones and the expression of thyroid hormone receptors (TRs) (Yamano and Miwa, 1998; Liu and Chan, 2002). Disruption of thyroid hormonal secretion causes malformation of developing organisms, including fish (Elsalini and Rohr, 2003). Furthermore, teleosts display unique and characteristic otolith growth patterns, especially during the early stage of development. How the programmed thyroid secretions affect the ontogenic otolith growth is still unclear since no study has simultaneously examined the TH content, TR expression and otolith growth. Therefore, this study aims to evaluate (1) the relationship between otolith growth pattern and the programmed secretion/expression of intrinsic TH and TR, (2) the effects of abnormal thyroid secretion on otolith growth and morphology, (3) the recovery of otolith growth from hypothyroidism. The experiments used tilapia larvae and juveniles (*Oreochromis mossambicus*) under a manipulated laboratory environment.

MATERIALS AND METHODS

Fish

Tilapia [*Oreochromis mossambicus*, Cichlidae (Peters 1852)] larvae, juveniles and adults were reared in fresh water at 28±1°C under a 14 h:10 h light:dark photoperiod. Several pairs of adult tilapia were used to produce the larvae but the fish used in each experiment were from the same progenitors. Fertilized eggs were retrieved from the mouth of the female tilapia and incubated in the aquarium with strong aeration to make the larvae turn around for normal development. After the complete absorption of the yolk, larval tilapia was fed to satiation with commercial fish meal once a day in the morning and the remaining food was cleared after feeding in the afternoon. A stock of local running tap water in a circular tank was prepared for use. All tilapia larvae and juveniles were reared in tap water in 10 l aquaria with a density of approximately 25 fish per liter during the experimental periods. The water was moderately aerated and was replaced daily with fresh tap water containing the same concentration of chemicals. In the following experiments, fish were anesthetized with 0.1 mg ml^{-1} MS-222 solution (3-aminobenzoic acid ethyl ester; Sigma, St Louis, MO, USA) before they were killed for measurement and analysis.

Chemicals

High concentrations of 3,5,3′-triiodothyronine (T_3; 100 p.p.m.; Sigma) were dissolved in absolute ethanol then diluted to 10 p.p.b. or 25 p.p.b. in fresh water for use. Ethanol alone had no effect on fish growth (data not shown). A stock solution of 10 000 p.p.m. thiourea (TU) was prepared by dissolving 10 g TU powder in 1 l fresh water. TU is an anti-thyroid hormone drug that inhibits the production of 3,5,3′,5′-thyroxine (T_4) and T_3 in the thyroid tissue. The stock solution was diluted to 300, 600 and 900 p.p.m. for use.

Experiment 1

A batch of larvae from the same progenitors was reared in normal tap water until 30 days post-hatching (d.p.h.). Every 2 days after hatching, five fish were randomly picked for analysis and frozen in –80°C until T_3 quantification by radioimmunoassay (RIA; see below). Every 3 days after hatching, 8–10 fish were randomly selected for total RNA extraction and their *TRα* and *TRβ* mRNA were quantified by real-time PCR (see below). The remaining fish after 30 d.p.h. were used to examine otolith growth.

Thyroid hormone and thiourea treatment of juvenile tilapia

Experiment 2

In order to evaluate the effects of T_3 and TU administration on thyroid hormone content of tilapia juveniles, the hatched larvae were reared for 13 days to the juvenile stage, then fish were randomly transferred to one of three aquaria that contained either normal tap water or tap water with 10 p.p.b. T_3 or 300 p.p.m. TU for another 13 days. Then the fish were stored at –80°C until T_3 quantification.

Experiment 3

Hatched larvae were reared for 13 days to the juvenile stage. At 14 d.p.h., juveniles were immersed in 300 p.p.m. tetracycline solution (Sigma; pH adjusted to 7.4) in the dark for 12 h to create a fluorescent mark in the otolith. Fish were subjected to treatment the next day (15 d.p.h.). The juveniles were reared in 0, 300, 600 and 900 p.p.m. thiourea (TU), respectively for 13 days until 27 d.p.h. Before the otolith was removed for observation, total length (from the tip of the mouth to the end of caudal fin) was measured using a digital caliper (Mitutoyo, Kawasaki, Japan). Fish was blotted on the Kimwipes tissue to remove the water on the body surface and wet mass was measured using a digital balance.

Experiment 4

Another batch of larvae was also reared for 13 days and their otoliths were marked with 300 p.p.m. tetracycline at 14 d.p.h. as described above. At 15 d.p.h. the fish were subjected to 0 p.p.b. T_3, 0.1 p.p.b. T_3, 10 p.p.b. T_3, 0.1 p.p.b. T_3 + 300 p.p.m. TU or 10 p.p.b. T_3 + 300 p.p.m. TU for 13 days, until 27 d.p.h. All the fish from experiments 4 and 5 were sacrificed at 28 d.p.h. for morphological measurements i.e. total length (to 0.01 mm) and wet mass (to 0.01 mg), and for otolith examination.

Thyroid hormone and thiourea treatment of larval tilapia

Experiment 5

Fertilized eggs were incubated until hatching. Larvae at 3 d.p.h. were immersed in 300 p.p.m. tetracycline solution for 12 h before the treatment. Then fish were reared in normal tap water, 25 p.p.b. T_3, 300 p.p.m. TU or 25 p.p.b. T_3 + 300 p.p.m. TU from 4–16 d.p.h. Fish were sacrificed for measurement of total length, wet mass, otolith and T_3 contents on 17 d.p.h. However, otolith and T_3 contents were not measured for the group treated with 25 p.p.b. T_3 + 300 p.p.m. TU because of the loss of the samples during preparation.

Recovery of otolith growth from hypothyroidism

Experiment 6

Tilapia juveniles at 14 d.p.h. were immersed in 300 p.p.m. tetracycline solution for 12 h before being reared in 300 p.p.m. TU for 13 days. The fish were transferred to water without TU at 28 d.p.h. and the fish were immersed in 300 p.p.m. tetracycline solution for 12 h on 29, 31 and 33 d.p.h. (the second, fourth and sixth days after recovery from TU treatment). The fish were reared until 41 d.p.h. Total length and wet mass were measured and otoliths were extracted for examination.

T_3 quantification by radioimmunoassay (RIA)

Some of the juveniles that received the treatments described above were used for T_3 quantification using a commercial kit (DPC, Los Angeles, CA, USA). Five individuals were pooled, weighed (wet mass), and homogenized in a 99% methanol solution, vortexed for 1 min, then centrifuged at 86 ***g*** at 4°C. The radius of the centrifuge rotor was 74 mm. The soluble layer was collected and oven dried at 37°C overnight. Then, 200 μl EIA buffer (0.1 phosphate buffer, pH 7.4, 0.15 mol l^{-1} NaCl) was added to dissolve the extracted thyroid hormones. The extraction rate was 87.2%. Four plain 12×75 mm tubes for total counts (T) and nonspecific binding (NSB) were prepared in duplicate, as were 12 tubes for the standard (two for each), which contained T_3 concentrations of 0, 0.2, 0.5, 1, 2 or 6 ng ml^{-1}. These control samples were used to generate the calibration curve. Then, 100 μl of each control and extracted thyroid sample was mixed with anti-T_3, except for the T groups, and then mixed with 1 ml of I^{125}-T_3 in the sealed tube. All tubes were incubated at 37°C for 2 h, with gentle shaking. After removing all visible moisture, the immunoreactivity was read for 1 min using a gamma counter (MALLAC-1470, Turku, Finland). Results were obtained in terms of concentration from a logit-log representation of the calibration curve. Then the binding of each pair of tube contents was determined as a percentage of maximum binding (MB), with the NSB-corrected counts of the standards and samples taken as 100%:

$$\text{Per cent bound} = (\text{net counts}/\text{net MB counts}) \times 100\% .$$

Results for the unknowns were then determined from the calibration curve. The displacement curve for larval extracts was parallel to that of the T_3 standard. The linear regression coefficient for the logarithms of T_3 standard concentrations was –0.99. The regression coefficient

for the serial dilutions of T_3 extractions from larval whole bodies was –0.98. The coefficients of intra-assay and inter-assay variations were 6.3% (N=6) and 19.2% (N=3), respectively. T_3 content is given as the mean value of five pooled fish expressed per unit wet mass.

Cloning and quantification of thyroid hormone receptors

Total RNA was extracted from the whole juvenile tilapia following the standard protocol using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (5 μg) was reverse-transcribed for cDNA synthesis using a kit (Qiagen, Hilden, Germany). The resulting cDNA was amplified by PCR in a total volume of 50 μl with 2 μl cDNA template, 5 μl of 10× PCR buffer (100 mmol l^{-1} Tris–HCl, 500 mmol l^{-1} KCl, 15 mmol l^{-1} $MgCl_2$), 5 μl of 2.5 mmol l^{-1} dNTP, 2 μl of forward and reverse primers, 0.5 μl *Taq* DNA polymerase (5 i.u. μl^{-1}) and 35.5 μl distilled water. We used the same specific primer sequences as Marchand et al. (Marchand et al., 2001) for the species *Oreochromis niloticus*. For the *TRα* amplification, the forward primer sequence was 5′-GCTGCATCATCGACAAGATC-3′ and the reverse primer sequence was 5′GATCTGAGCTC-ATGAGAAGC3′. For *TRβ* amplification, the forward primer sequence was 5′AATGTGTTATTGACAAAGTG3′ and the reverse primer sequence was 5′-GATCGGATGAAAGCAGGATA-3′. The amplified cDNA fragments were inserted into the pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into competent cells (ECOS9-5) for amplification. The purified plasmids were subjected to DNA sequencing using an automatic DNA sequencer (ABI 3700, Applied Biosystems, Wellesley, MA, USA). Quantitative real-time PCR (qPCR) was carried out using a SYBR Green dye (Qiagen, Hilden, Germany)-based assay with an ABI Prism 7000 Sequence Detection System (Perkin-Elmer, Applied Biosystems, Wellesley, MA, USA) according to the manufacturer's instructions. Primers targeting the *TRα* and *TRβ* and the endogenous control gene, β-actin, were designed using Primer Express 2.0 software (Applied Biosystems). In each assay, 25 ng cDNA was amplified in a 20 μl reaction containing 2×SYBR Green Master mix, 300 nmol l^{-1} of forward and reverse primers, and nuclease-free water. The primers designed for the consensus of *TRα* and *TRβ* isoforms were as follows: *TRα*-forward: 5′-GCTCAGGGCTCA-CAGTGGAA-3′, *TRα*-reverse: 5′-AACGACACGGGTGATGGC-3′; *TRβ*-forward: 5′-GGCAACCACTGGAAGCAGAA-3′, *TRβ*-reverse: 5′-TGATAATTTTTGTAAACTGACTGAAGGCT-3′.

Otolith preparation

Sagittal otoliths were removed under a stereomicroscope (Olympus SZX 12, Tokyo, Japan), dried in air and embedded with Epofix resin (Struers, Copenhagen, Denmark). The embedded otoliths were then sectioned using a low-speed circular saw (Buehler Isomet, Evanston, IL, USA) to remove excess resin. The otoliths were then ground and polished on a grinder-polisher machine (Buehler Metaserv 2000, Evanston, IL, USA) at a speed of 300 r.p.m. with wet-polisher paper of 2 000-grit for the initial grinding and 2 400-grit for the final grinding until the core was exposed. The otolith was finally polished with a polishing cloth and 0.05 μm alumina (Buehler) to smooth the surface. During the grinding and polishing process, the otolith was periodically checked under a compound light microscope. A fluorescence microscope (Axioplan 2 Imaging MOT, Zeiss, Germany) with incident light from a 50 W mercury lamp and FITC filter sets was utilized to detect the fluorescent ring on the ground surface of otoliths. Then, dilute HCl (0.05 mol l^{-1}) was used to etch the otolith for 20 s. Etching increased the visibility of otolith increments by enhancing the contrast under a compound light microscope. Images of fluorescent rings and the whole etched otolith were recorded at 200× magnification under the light microscope equipped with a digital camera (AxioCam HRm Zeiss). From the images, measurements were made of otolith length and the width of individual increments, as well as counts of daily growth increments and these were processed on a personal computer using Image-Pro plus software (Media Cybernetics Inc. 1994, Silver Spring, MD, USA). The measurements were made along the maximum radius from the core to the posterior end of each otolith. To observe the otolith topology, the sagittal otoliths were removed from the fish, dried in the oven, and gold coated for observation by scanning electron microscopy (FEI Quanta 200 SEM, FEI, Hillsboro, OR, USA).

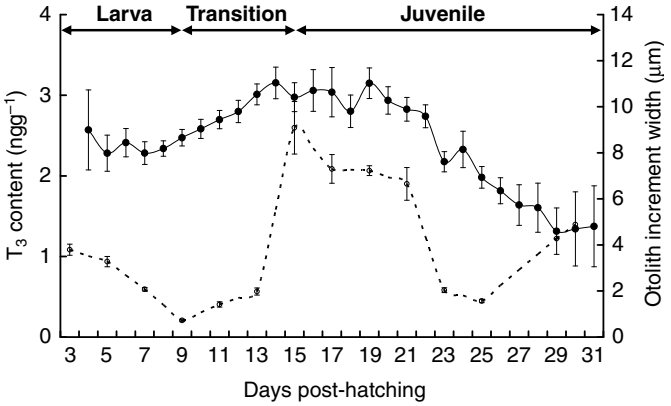

Fig. 1. Temporal change in T_3 content (N=5, open circles) and otolith growth (N=14, filled circles) of tilapia larvae and juveniles during the experimental period. T_3 content was measured every 2 days but samples at 27 day post-hatching were lost. The data are given as means ± s.e.m. for clarity.

Statistical analysis

Data were expressed as means ± s.d. (N=number of fish) but means ± s.e.m. were used in the Figs 1, 3 and 4 for clarity. Correlations between T_3 and daily growth increment (DGI) profiles as well as T_3 profiles and *TRα* mRNA expression patterns were analyzed by Pearson product moment correlation. Statistical differences among treatments were analyzed using one-way analysis of variance (ANOVA). Tukey's pairwise comparison was used to identify groups that differed from others if the data satisfactorily met the assumptions of normal distribution and equal variance. Otherwise, the Kruskal–Wallis test on ranks and Dunn's pairwise comparison were used to isolate the groups that differed from the others. Statistical significance was set at α<0.05.

RESULTS

T_3 contents, *TRα*, *TRβ* mRNA expression and otolith growth

T_3 contents of tilapia changed considerably from early larval to juvenile stage. The temporal profile during the experimental period between 3 and 30 d.p.h. was classified into five phases. Phase 1: a gradual decline during the larval stage from 3 to 9 d.p.h. Phase 2: rapid increase from 11–15 d.p.h., which corresponded to the transition of the tilapia from larval to juvenile stage. During the transition from the larval to juvenile stage, the yolk is completely absorbed and the fish starts active feeding and free swimming (Holden and Bruton, 1992; Anken et al., 1993). Phase 3: a high plateau around 15–21 d.p.h. Phase 4: a second decline from 21 to 25 d.p. Phase 5: a minor increase from 25 to 30 d.p.h. (Fig. 1).

The trend of otolith growth was generally similar to the T_3 profile. The width of the daily growth increment (DGI) slightly declined from 3 to 7 d.p.h., followed by a gradual increase to approximately 3 μm around 14 d.p.h. A high growth plateau was maintained until 22 d.p.h. then the daily otolith growth gradually decreased to approximately 1.3 μm by 29 to 31 d.p.h. (Fig. 1). A significant median correlation (correlation coefficient=0.681, P=0.021) occurred between the T_3 and DGI profiles during the period from 3 to 25 d.p.h.

The partial DNA sequence of tilapia *TRα* and *TRβ* genes were cloned and sequenced. The cloned sequences are 703 nucleotides and 730 nucleotides for *TRα* and *TRβ*, respectively. The sequences were submitted to GenBank with the accession no. EU048544 for *TRα* and EU048545 for *TRβ*. The sequences were confirmed from the NCBI database and phylogenetic tree analysis of the *TR* family from published species. Quantitative analysis of the *TRα* mRNA expression suggested a trend similar to the T_3 contents and otolith growth; decreasing from 3 to 9 d.p.h., followed by an increase until 15 d.p.h., then gradually decreasing until 27 d.p.h. However, *TRβ* mRNA expression was low, with no evident variation throughout the experimental period (Fig. 2). A significant median correlation was also observed for *TRα* and DGI profiles during the period from 6 to 27 d.p.h. (correlation coefficient=0.63, P=0.021).

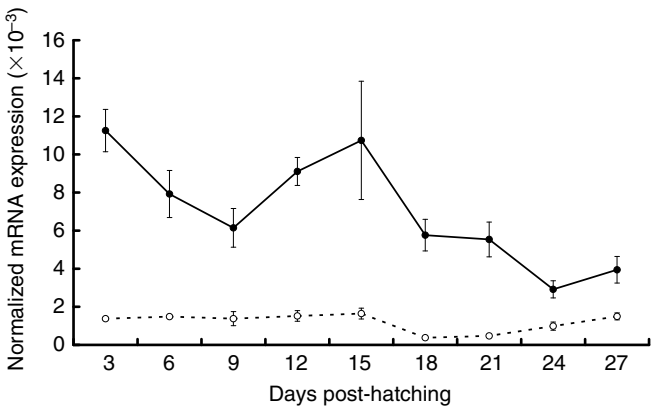

Fig. 2. Ontogenic expression of thyroid hormone receptor α (filled circles) and β (open circles) RNA during tilapia larval and juvenile stages (N=3). The mRNA expression was normalized in relation to β-actin. The data are given as means ± s.e.m.

Effects of TU and T_3 on tilapia juveniles

Compared with the control group (1.4±0.01 ng g^{-1}), 300 p.p.m. TU treatment caused a significantly low T_3 content (0.3±0.07 ng g^{-1}) in tilapia juveniles but immersion in 10 p.p.b. T_3 significantly increased the T_3 contents (9.7±2.13 ng g^{-1}) of tilapia juveniles by approximately sevenfold (P<0.05).

Tilapia juveniles, immersed in 300, 600 and 900 p.p.m. TU for 13 days, showed a retarded otolith growth in a dose-dependent manner (Table 1). The new growth in otolith radius was approximately 56%, 43% and 41% of the control group for the 300, 600 and 900 p.p.m. TU group, respectively. The newly formed otolith radii of TU groups were significantly smaller than for the control group (all P<0.05). Fish growth, i.e. TL and mass, was also significantly retarded by TU treatment. By contrast, 10 p.p.b. T_3 moderately increased the TL and mass and significantly increased the DGI (P<0.05), but 0.1 p.p.b. T_3 did not promote fish growth (Table 2). However, a high dose of T_3 (10 p.p.b.) compared with a low dose of T_3 (0.1 p.p.b.), slightly, but not significantly, increased otolith growth in the presence of 300 p.p.m. TU. This result was in general agreement with previous observations of TU-induced effects on tilapia larvae yolk absorption, growth and development (Reddy and Lam, 1992). The smaller otolith growth was attributed to fewer newly formed DGI (an average of two to four fewer rings; Table 3) and a slower otolith growth rate compared with the control group (see below).

Higher concentrations of TU, i.e. 600 p.p.m. and 900 p.p.m. evidently caused higher mortality of tilapia juveniles. Lower TU i.e. 300 p.p.m., 0.1 p.p.b. and 10 p.p.b.T_3 have no evident effects on fish survival compared with the control treatment (Tables 1 and 2).

A prominent growth difference of normal tilapia juveniles between experiments 3 (Table 1) and 4 (Table 2) was noticed. The larvae in each of these experiments were produced from different adults. The condition of the progenitors – sizes, nutrient levels and health – may affect development of their embryos.

Effects of TU and T_3 on tilapia larvae

For tilapia larvae, the simultaneous administration of 300 p.p.m. TU and 25 p.p.b. T_3 had no significant effect on fish or otolith growth. TU (300 p.p.m.) also had no negative effect on fish or otolith growth. T_3 of 25 p.p.b. significantly increased the otolith growth but not the DGI number. T_3 content of tilapia larvae were also significantly increased (approximately threefold) by 25 p.p.b. T_3 administration, but not significantly reduced by 300 p.p.m. TU (Table 4). In preliminary trials, 10 p.p.b. T_3 had no effect on fish and otolith growth (data not shown).

The mortality of larval tilapia was higher than that of juveniles. However, compared with the control group, 300 p.p.m. TU and 25 p.p.b. T_3 have no evident effect on larval survival (Table 4).

Otolith growth during TU treatment and recovery

TU treatment retarded otolith growth and slightly changed otolith morphology. After 13 days immersion in 300 p.p.m. TU, otolith growth was severely retarded on the ventral area (Fig. 3C,D,F). In most samples, the ventral part of the otolith stopped growing shortly after the beginning of the 300 p.p.m. TU treatment although otolith growth in other directions continued for another 6–9 days (Table 3). Otolith growth in the ventral direction did not resume even after fish were returned to normal water for 2 weeks. This indicated that TU treatment not only caused a temporary inhibition but also permanent damage to this organ, at least in the ventral area. The severe reduction of otolith growth in the ventral direction resulted in a flat margin (Fig. 3F). The normal otolith showed the convex margin in both ventral and dorsal directions. Furthermore, every tilapia larva and juvenile was successfully marked by tetracycline. All fluorescent rings were very distinct in the normal otolith (Fig. 3B) but in the experimental group, however, the second, third and fourth fluorescent rings, laid down during the recovery period, were less discernible, faint or only partially visible compared with the first prominent fluorescent ring laid down before exposure to 300 p.p.m. TU (Fig. 3D). This result indicated that $CaCO_3$ mineralization in the inner ear had not recovered to a normal level during the first week of recovery from hypothyroidism.

TU-treated fish showed a slower otolith growth rate than their counterparts in normal water (Fig. 4). There were one, six, eight and nine fish whose otolith only had 9, 10, 11 and 12 newly deposited rings, respectively, during the 13 day TU treatment (23–27 d.p.h.). A probable explanation for this was that respective otoliths stopped growth after 9 (N=1), 10 (N=6), 11 (N=8) and 12 (N=9) days of TU treatment. After the TU-treated fish were returned to normal water, otolith growth was observed on the second day in all individuals,

as determined by the tetracycline marking. It was unclear if the growth occurred on the first day during the recovery period since the fish was not marked by tetracycline on that day. Only three DGI were discernible among the three fluorescent rings deposited in the 5 day period. This indicated that otolith increase did not have a daily cycle during the approximately 1 week recovery from TU treatment. Furthermore, the otolith growth rate of the experimental group was only half that of the control group during the first 5 days of recovery. The otolith growth rate of TU-treated fish did not reach the same level as the control group after recovery for 1 week and remained approximately 2 μm day^{-1} slower than that of the control group. An otolith growth increment was not formed daily until approximately 1 week after recovery from the 300 p.p.m. TU treatment.

DISCUSSION

This study demonstrates that somatic and otolith growth show differential susceptibility to thyroid hormone levels at different developmental stages of tilapia larvae and juveniles. In this study, we only measured the functional thyroid hormone, T_3 since T_4 secreted in fish, as in other vertebrates, has to be converted into T_3 in order to bind to a nuclear receptor and exert its full biologic activity. We also only administrated T_3 to tilapia since T_4 supplementation *via* food had no effect on plasma T_3 and T_4 concentrations in tilapia (Geyten et al., 2005).

T_3 and TU treatments have no evident effects on somatic and otolith growth of tilapia larvae. As tilapia grows, T_3 treatment induces somatic growth and otolith growth during the juvenile stage, which can be retarded by TU treatment. TU (300 p.p.m.) and T_3 treatment does not cause an acute stress to fish, which is supported by the absence of any evident marks or checks in their otolith. We did not observe behavioral differences in T_3-treated fish compared with the control fish. Hypothyroidism induced by TU inhibition of the synthesis of thyroid hormones is progressive until the existing thyroid hormones are degraded. After TU treatment for several days, tilapia juveniles, but not the larvae, clearly show symptoms of hypothyroidism: less mobility, with stiff swimming behavior near the bottom and slow response to disturbances. The appetite of tilapia juveniles was also evidently affected after immersion in TU for several days. Reduced feeding slows somatic and

Table 1. Effects of thiourea treatment on tilapia juveniles

	0 p.p.m.	300 p.p.m.	600 p.p.m.	900 p.p.m.
Total length (mm)	13.7±1.1[a]	11.3±0.5[b]	10.7±0.4[c]	10.4±0.4[c]
Wet mass (mg)	35.1±11.0[a]	16.4±2.6[b]	12.9±1.4[bc]	10.9±1.6[c]
Mortality (%)	0	13.6	50.8	44.1
Otolith growth				
Core to TC (μm)	127.4±10.3[a]	127.8±8.5[a]	128.8±5.9[a]	127.1±7.2[a]
TC to edge (μm)	80.5±27.2[a]	45.4±6.1[b]	34.8±6.9[c]	33.2±4.8[c]
Newly deposited DGI	13.0±1.3[a]	11.0±0.9[b]	9.0±0.9[c]	9.2±1.1[c]

Fish were immersed in different concentrations of thiourea (TU, p.p.m.) from 15 to 27 days post-hatching (d.p.h.) and were then killed for measurement on 28 d.p.h. Data are shown as means ± s.d. (*N*=15–18). TC, tetracycline mark; DGI, daily growth increment. Values with different superscript letters are significantly different (*P*<0.05).

Table 2. Effects of thiourea, triiodothyronine or both on tilapia juveniles

	0 p.p.m.	0.1 p.p.b. T_3	10 p.p.b. T_3	0.1 p.p.b. T_3 + 300 p.p.m. TU	10 p.p.b. T_3 + 300 p.p.m. TU
Total length (mm)	18.4±1.0[a]	18.1±1.3[a]	19.2±1.2[a]	14.0±0.6[b]	13.9±0.5[b]
Wet mass (mg)	80.1±16.4[a]	76.9±20.7[a]	88.9±21.4[a]	25.2±4.9[b]	24.5±5.1[b]
Mortality (%)	0	0	3.7	0	7.4
Otolith growth					
Core to TC (μm)	191.3±5.6[a]	192.9±4.4[a]	192.2±4.8[a]	189.6±5.7[a]	189.9±5.2[a]
TC to edge (μm)	111.6±20.5[a]	113.1±20.8[a]	129.9±16.5[a]	45.7±11.8[b]	62.9±10.7[b]
Newly deposited DGI	13.2±0.4[b]	14.0±0.7[ab]	14.4±0.9[a]	9.0±1.6[c]	10.8±1.6[c]

Fish were immersed in different treatments from 15 to 27 days post-hatching (d.p.h.) and samples were killed for measurement on 28 d.p.h. Data are shown as means ± s.d. (*N*=22–26). TU, thiourea; T_3, triiodothyronine; TC, tetracycline mark; DGI, daily growth increment. Values with different superscript letters are significantly different (*P*<0.05).

Table 3. Newly deposited daily growth increment of the tilapia juveniles receiving different treatments of thiourea and triiodothyronine

DGI	0 p.p.m.	0.1 p.p.b. T_3	10 p.p.b. T_3	0.1 p.p.b. T_3 + 300 p.p.m. TU	10 p.p.b. T_3 + 300 p.p.m. TU
6	–	–	–	2 (8%)	–
7	–	–	–	2 (8%)	–
8	–	–	–	4 (16%)	–
9	–	–	–	8 (32%)	5 (22.73%)
10	–	–	–	6 (24%)	6 (27.27%)
11	–	–	–	2 (8%)	4 (18.18%)
12	–	–	–	–	4 (18.18%)
13	21 (80.77%)	5 (20.83%)	5 (19.23%)	1 (4%)	1 (4.55%)
14	5 (19.23%)	14 (58.33%)	7 (26.92%)	–	2 (9.09%)
15	–	5 (20.83%)	12 (46.15%)	–	–
16	–	–	2 (7.69%)	–	–

Fish were immersed in different treatments from 15 to 27 days post-hatching (d.p.h.). and samples were killed for measurement on 28 d.p.h. (*N*=22–26). DGI, daily growth increment; T_3, triiodothyronine; TU, thiourea.

Table 4. Effects of thiourea, triiodothyronine, or both on tilapia larvae

Larvae	0 p.p.m.	25 p.p.b. T_3	300 p.p.m. TU	25 p.p.b. T_3 + 300 p.p.m. TU
Total length (mm)	11.0±1.1[a]	11.0±0.9[a]	10.8±0.7[a]	10.9±0.6[a]
Wet mass (mg)	14.5±4.1[a]	14.6±4.9[a]	12.4±2.9[a]	12.6±1.9[a]
Mortality (%)	40	6.7	35.5	56.8
Otolith growth				
TC to edge (μm)	156.0±18.8[b]	171.7±18.8[a]	147.9±10.6[b]	156.1±13.5[b]
Newly deposited DGI	14.4±1.3[a]	15.0±1.0[a]	13.8±0.5[a]	–
T_3 content (ng g^{-1})	1.4±0.4[b]	4.8±0.9[a]	0.8±0.3[b]	–

Fish were immersed in different treatments from 4 to16 days post-hatching (d.p.h.) and were killed for measurement on 17 d.p.h. Data are shown as means ± s.d. (*N*=13–20). TU, thiourea; T_3, triiodothyronine; TC, tetracycline mark; DGI, daily growth increment. Values with different superscript letters are significantly different (*P*<0.05).

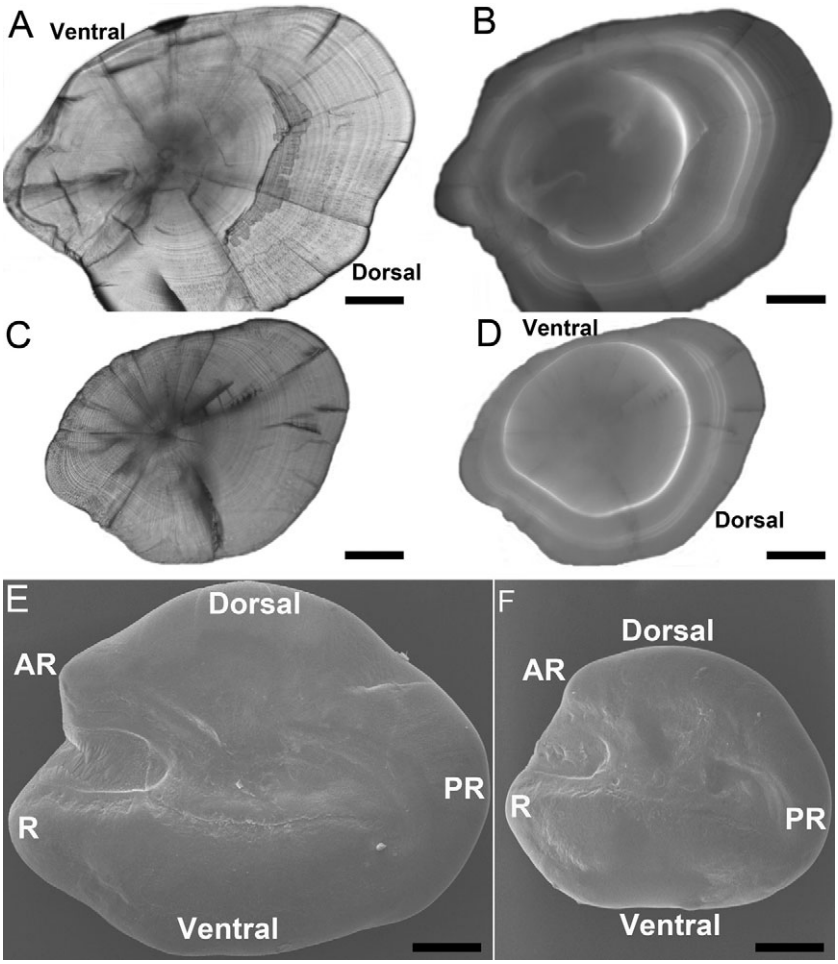

Fig. 3. Microstructure and morphology of otoliths from tilapia juveniles immersed in 300 p.p.m. thiourea (TU) from 15 to 27 days post-hatching (d.p.h.), followed by a recovery period in fresh water from 28 to 41 d.p.h. (C,D,F). Otoliths of normal fish are shown in A,B,E for comparison. The innermost fluorescent ring indicates the beginning of TU treatment. The outer three fluorescent rings were laid down on the second, fourth and sixth day after ending TU treatment. A–D are left sagittae; E and F are right sagittae. AR, antirostrum; PR, postrostrum; R, rostrum. Scale bars, 100 μm.

otolith growth, but this factor alone cannot cause the cessation of otolith growth, particularly on the ventral side of the otolith after a few days of TU treatment. Furthermore, the retarded somatic and otolith growth is only partially counteracted by T_3 administration, suggesting TU is toxic to tilapia juveniles. Extrathyroidal effects of TU, although not fully understood, have been found in killifish (Chambers, 1953), rainbow trout (Eales, 1981) and flounder (Schreiber and Specker, 1999). Furthermore, TU-like goitrogenic phenythiourea evidently delays the hatching of zebrafish (Elsalini and Rohr, 2003). TU was found to reduce protein synthesis in the liver and muscle of freshwater catfish (*Heteropneustes fossilis*) (Singh, 1979). If TU also inhibits protein secretions of otolith chambers, this would retard and change the otolith shape during the process of biomineralization as observed here in tilapia juveniles. Nevertheless, tilapia larvae (this study), metamorphosing flounder (Miwa and Inui, 1987) and metamorphosing tarpon leptocephali (Shiao and Hwang, 2006) are insensitive to TU, suggesting that the extrathyroidal effects of TU in teleosts may vary in different species as well as developmental stages.

Experiments on salmon have suggested that otolith growth is more closely correlated with metabolic rate than with somatic growth (Wright, 1991; Yamamoto et al., 1998). The coupling of metabolic rate and otolith growth can be detected as early as the embryonic stage of zebrafish (Bang and Grønkjær, 2005). At similar temperatures, developing zebrafish embryos can show significant intraspecific variation in metabolic rate (Bang et al., 2004). The relationship between metabolic rate and thyroid level are not fully known in fish. Some studies suggested that thyroid hormones do not increase metabolic rate in fish (Weirich et al., 1987; van Ginneken et al., 2007). Accordingly, thyroid hormones may increase otolith growth by stimulating somatic growth and differentiation rather than by directly enhancing the metabolic rate

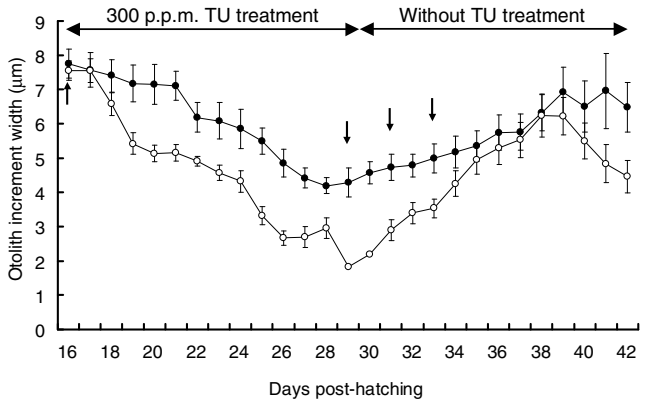

Fig. 4. Otolith growth rate during the 300 p.p.m. thiourea (TU) treatment from 15 to 27 d.p.h., followed by recovery in fresh water from 28–41 d.p.h. Filled circles indicate the control group without treatment; open circles indicate the experimental group (N=12 for each group). Data are shown as means ± s.e.m. for clarity. Arrows indicate the dates of treatment with tetracycline that produced the fluorescent rings as shown in Fig. 3.

of the fish. Kobuke et al. (Kobuke et al., 1987) first reported the presence of thyroid hormones in fish eggs, and their entry into tilapia oocytes is probably *via* diffusion from the maternal plasma (Tagawa and Brown, 2001). Maternal thyroid hormones are involved in the regulation of development and growth of teleosts (Tagawa and Hirano, 1987; Brown, 1997). However, T_3 has no biological function without binding to thyroid hormone receptors. Somatic T_3 content and *TRα* mRNA expression decrease during the larval stage in tilapia. The decrease in *TRα* mRNA expression suggests a low demand for T_3 by tilapia larvae and this phenomenon may explain why the overdose of T_3 induced by 25 p.p.b. T_3 administration has no prominent effects on somatic growth of tilapia larvae (Table 4). Differentiation of the thyroid gland can be detected in tilapia larvae as early as 3 d.p.h. by histological staining (S.-M.W., unpublished data), which is similar to the observation in zebrafish (Elsalini and Rohr, 2003). Therefore, the thyroid gland may start to synthesize T3 at an early stage in tilapia. In experiment 5, larval tilapia were reared in the presence of 300 p.p.m. TU from 4–16 d.p.h. TU, like phenylthiourea (Elsalini and Rohr, 2003) can almost completely inhibit the new synthesis of thyroid hormones. The measured thyroid content in TU-treated fish was 0.8±0.3 ng g^{-1} (Table 4), which is not significantly different from the normal fish (1.4±0.4 ng g^{-1}, Table 4). This result suggests that the maternal thyroid hormones in TU-treated fish may be still active, at least until 16 d.p.h., and be sufficient to supply larval development. In addition, the newly synthesized T_3, if there is any, is limited in larval tilapia since TU does not significantly reduce the somatic T_3 content of tilapia larvae (Table 4). Although the transition of tilapia larvae to juvenile does not involve a striking morphological change, as observed in flounder and eel, a thyroid hormone surge and increasing expression of *TRα* mRNA are found during larval metamorphosis at 9–15 d.p.h. (Figs 1, 2). This indicates that thyroid hormones have an evolutionarily conserved function in manipulating the metamorphic process from larval to juvenile stage in teleosts. The coupling of thyroid hormone surges and high *TRα* mRNA expression may stimulate tilapia growth and development, which is reflected in faster otolith growth. The disruption of T_3 levels, either by hypothyroidism or hyperthyroidism, at this stage can easily change somatic growth, and the changes are recorded in otolith growth.

After removal of TU, synthesis of thyroid hormones is gradually resumed, so that fish as well as otolith growth slowly recovers. There were only three tetracycline-marked increments deposited in the otolith (Fig. 3C,D) during an approximate 6 day period. This result suggests that an otolith growth increment is not deposited daily but in a 2 day cycle during the first week of recovery. This suggests that fish age may be underestimated due to the uncoupling of otolith growth increments and the real daily age of the fish under conditions of hypothyroidism. Thyroid hormone levels take about a week to return to normal based on observations of the otolith growth period. It is worth noting that otolith growth on the ventral side is not resumed after TU removal. The otolith grows by $CaCO_3$ mineralization on the protein matrix, which is directly regulated by the epithelial cells of the otolith sac (Payan et al., 1997; Payan et al., 1999; Takagi, 2000; Tohse and Mugiya, 2001). Failure to grow indicates that the function of the otolith sac might be permanently damaged on the ventral side when fish are exposed to 300 p.p.m. TU for 13 days. Ionocytes on the otolith sac are the cells responsible for transepithelial transport of HCO^-_3 and Ca^{2+} into the endolymph (Mugiya and Yoshida, 1995; Shiao et al., 2005), although Payan et al. (Payan et al., 2002) suggested a passive diffusion through a paracellular pathway for Ca^{2+} transportation. It is likely that TU treatment causes death or dysfunction to some ionocytes, leading to severely retarded otolith growth in the ventral direction. Disruption of ion and protein supplies from the plasma or cells into the endolymph may also cause the allometric growth of the otolith during hypothyroidism. These hypothetical explanations require further studies to determine the effects of hypothyroidism on the otolith sac at the cellular and molecular levels.

To our knowledge, this is the first study revealing consistent patterns among T_3 content, *TRα* expression, and otolith growth during the early stages of fish development. Yamano and Miwa (Yamano and Miwa, 1998) found ubiquitous expression of TR genes in the fish body, suggesting that development of each tissue of the flounder is controlled by thyroid hormone at the receptor level. Under TU-induced hypothyroidism, the slower otolith growth rate could be attributed to the retarded somatic growth, whereas the permanent changes on the ventral side of the otolith might be due to the cellular toxicity of TU. Dramatic changes in otolith increment width often occur at larval metamorphosis and settlement (Victor, 1986; Sponaugle and Cowen, 1994; Shiao et al., 2002). These changes cannot simply be attributed to environmental factors. This study demonstrates that otolith growth is influenced by T_3, especially during the larval to juvenile stages. To a certain extent, the characteristic ontogeny of otolith growth for each species may be also determined by programmed T_3 secretion and *TRα* expression.

The present work was financially supported by the Thematic Project of the Academia Sinica, contract no. AS-95-TP-B05 and by National Taiwan University to J.-C.S. We thank B. M. Jessop for suggestions on the draft.

REFERENCES

Anken, R. H., Kappel, T., Slenzka, K. and Rahmann, H. (1993). The early morphogenetic development of the chichlid fish, *Oreochromis mossambicus* (Perciformes, Teleostei). *Zool. Anz.* **231**, 1-10.
Anken, R. H., Edelmann, E. and Rahmann, H. (2002). Fish inner ear otoliths stop calcium incorporation after vestibular nerve transection. *Neuroreport* **11**, 2981-2983.
Bang, A. and Grønkjær, P. (2005). Otolith size-at-hatch reveals embryonic oxygen consumption in the zebrafish, *Danio rerio*. *Mar. Biol.* **147**, 1419-1423.
Bang, A., Grønkjær, P. and Malte, H. (2004). Individual variation in the rate of oxygen consumption by zebrafish (*Danio rerio*). *J. Fish Biol.* **64**, 1285-1296.
Baumann, H., Peck, M. A. and Herrmann, J. P. (2005). Short-term decoupling of otolith and somatic growth induced by food level changes in postlarval Baltic sprat, *Sprattus sprattus*. *Mar. Freshw. Res.* **56**, 539-547.
Begg, G. A., Campana, S. E., Fowler, A. J. and Suthers, I. M. (2005). Otolith research and application: current directions in innovation and implementation. *Mar. Freshw. Res.* **56**, 477-483.
Brent, G. A. (1996). Thyroid hormones (T_4, T_3). In *Endocrinology: Basic and Clinical Principles* (ed. P. M. Conn and S. Melmed), pp. 291-306. Totowa, NJ: Humana Press.
Brown, D. D. (1997). The role of thyroid hormone in zebrafish and axolotl development. *Proc. Natl. Acad. Sci. USA* **94**, 13011-13016.
Campana, S. E. (2005). Otolith science entering the 21st century. *Mar. Freshw. Res.* **56**, 485-495.
Chambers, H. A. (1953). Toxic effects of thiourea on the liver of the adult male killifish, *Fundulus heteroclitus*. *Biol. Bull. Bingham. Oceanogr. Coll.* **14**, 69-93.
Cyr, D. G. and Eales, J. G. (1996). Interelationships between thyroidal and reproductive endocrine systems in fish. *Rev. Fish Biol. Fish.* **6**, 165-200.
de Jesus, E. G., Toledo, J. D. and Simpas, M. S. (1998). Thyroid hormones promote early metamorphosis in grouper (*Epinephelus coioides*) larvae. *Gen. Comp. Endocrinol.* **112**, 10-16.
Eales, J. G. (1981). Extrathyroidal effects of low concentrations of thiourea on rainbow trout, *Salmo gairdneri*. *Can. J. Fish Aquat. Sci.* **38**, 1283-1285.
Elsalini, O. A. and Rohr, K. B. (2003). Phenylthiourea disrupts thyroid function in developing zebrafish. *Dev. Genes Evol.* **212**, 593-598.
Geyten, S. V., Byamungu, N., Reyns, G. E., Kühn, E. R. and Darras, V. M. (2005). Iodothyronine deiodinases and the control of plasma and tissue thyroid hormone levels in hyperthyroid tilapia (*Oreochromis niloticus*). *J. Endocrinol.* **184**, 467-479.
Holden, K. K. and Bruton, M. N. (1992). A life-history approach to the early ontogeny of the Mozambique tilapia *Oreochromis mossambicus* (Pisces, Cichlidae). *S. Afr. Zool.* **27**, 173-191.
Kobuke, L., Specker, J. L. and Bern, H. A. (1987). Thyroxine content of eggs and larvae of coho salmon, *Oncorhynchus kisutch*. *J. Exp. Zool.* **242**, 2-12.
Liu, Y. W. and Chan, W. K. (2002). Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. *Differentiation* **70**, 36-45.

Marchand, O., Safi, R., Escriva, H., Rompaey, E. V., Prunet, P. and Laudet, V. (2001). Molecular cloning and characterization of thyroid hormone receptors in teleost fish. *J. Mol. Endocrinol.* **26**, 51-65.
Miwa, S. and Inui, Y. (1987). Effects of various doses of thyroxine and triiodothyronine on the metamorphosis of flounder. *Gen. Comp. Endocrinol.* **67**, 356-363.
Miwa, S., Tagawa, M., Inui, Y. and Hirano, T. (1988). Thyroxine surge in metamorphosing flounder larvae. *Gen. Comp. Endocrinol.* **70**, 158-163.
Mugiya, Y. (1990). Long-term effects of hypophysectomy on the growth and calcification of otoliths and scales in the goldfish, *Carassius auratus*. *Zool. Sci.* **7**, 273-279.
Mugiya, Y. and Yoshida, M. (1995). Effects of calcium antagonist and other metabolic modulators on in vitro calcium deposition on otoliths in the rainbow trout *Oncorhynchus mykiss*. *Fish. Sci.* **61**, 1026-1030.
Payan, P., Kossmann, H., Watrin, A., Mayer-Gostan, N. and Boeuf, G. (1997). Ionic composition of endolymph in teleosts: origin and importance of endolymph alkalinity. *J. Exp. Biol.* **200**, 1905-1912.
Payan, P., Edeyer, A., de Pontual, H., Borelli, G., Boeuf, G. and Mayer-Gostan, N. (1999). Chemical composition of saccular endolymph and otolith in fish inner ear: lack of spatial uniformity. *Am. J. Physiol.* **46**, R123-R131.
Payan, P., Borelli, G., Priouzeau, F., de Pontual, H., Boeuf, G. and Mayer-Gostan, N. (2002). Otolith growth in trout *Oncorynchus mykiss*; supply of Ca^{2+} and Sr^{2+} to the saccular endolymph. *J. Exp. Biol.* **205**, 2687-2695.
Peters, W. (1852). Diagnosen von neuen Flussfischen aus Mossambique. *Monatsb. Akad. Wiss. Berlin* **275-276**, 681-685.
Reddy, P. K. and Lam, T. J. (1992). Role of thyroid hormones in tilapia larvae (*Oreochromis mossambicus*). I. Effects of the hormones and an antithyroid drug on yolk absorption, growth and development. *Fish Physiol. Biochem.* **9**, 473-485.
Schreiber, A. M. and Specker, J. L. (1999). Metamorphosis in the summer flounder, *Paralichthys dentatus*: thyroidal status influences salinity tolerance. *J. Exp. Zool.* **284**, 414-424.
Shiao, J. C. and Hwang, P. P. (2004). Thyroid hormones are necessary for teleostean otolith growth. *Mar. Ecol. Prog. Ser.* **278**, 271-278.
Shiao, J. C. and Hwang, P. P. (2006). Thyroid hormones are necessary for metamorphosis of tarpon *Megalops cyprinoides* leptocephali. *J. Exp. Mar. Biol. Ecol.* **331**, 121-132.
Shiao, J. C., Tzeng, W. N., Collins, A. and Iizuka, Y. (2002). Role of marine larval duration and growth rate of glass eels in determining the distribution of *Anguilla reinhardtii* and *A. australis* on Australian eastern coasts. *Mar. Freshw. Res.* **53**, 687-695.
Shiao, J. C., Lin, L. Y., Horng, J. L., Hwang, P. P. and Kaneko, T. (2005). How can teleostean inner ear hair cells maintain the proper association with the accreting otolith? *J. Comp. Neurol.* **488**, 331-341.
Singh, A. K. (1979). Effect of thyroxine and thiourea on liver and muscle energy stores in the freshwater catfish, *Heteropneustes fossillis*. *Ann. Biol. Anim. Biochim. Biophys.* **19**, 1669-1676.
Sponaugle, S. and Cowen, R. K. (1994). Larval durations and recruitment patterns of two Caribbean gobies (Gobiidae): contrasting early life histories in demersal spawners. *Mar. Biol.* **120**, 133-143.
Tagawa, M. and Brown, C. L. (2001). Entry of thyroid hormones into tilapia oocytes. *Comp. Biochem. Physiol.* **129B**, 605-611.
Tagawa, M. and Hirano, T. (1987). Presence of thyroxine in eggs and changes in its content during early development of chum salmon, *Oncorhynchus keta*. *Gen. Comp. Endocrinol.* **68**, 129-135.
Takagi, Y. (2000). Ultrastructural immunolocalization of the otolith water soluble-matrix in the inner ear of rainbow trout just-hatched fry. *Fish. Sci.* **66**, 71-77.
Tohse, H. and Mugiya, Y. (2001). Effects of enzyme and anion transport inhibitors on *in vitro* incorporation of inorganic carbon and calcium into endolymph and otoliths in salmon *Oncorhynchus masou*. *Comp. Biochem. Physiol.* **128A,** 177-184.
van Ginneken, V. J. T., Ballieux, B., Antonissen, E., van der Linden, R., Gluvers, A, van den Thillart, G. E. E. J. M. (2007). Direct calorimetry of free-moving eels with manipulated thyroid status. *Naturwissenschaften* **94**, 128-133.
Victor, B. C. (1986). Duration of the planktonic larval stage of one hundred species of Pacific and Atlantic wrasses (family Labridae). *Mar. Biol.* **90**, 317-326.
Volk, E. C., Schroder, S. L. and Grimm, J. J. (1999). Otolith thermal marking. *Fish. Res.* **43**, 205-219.
Weirich, R. T., Schwartz, H. L. and Oppenheimer, J. H. (1987). An analysis of the interrelationship of nuclear and plasma Triiodothyronine in the sea lamprey, lake trout, and rat: evolutionary considerations. *Endocrinology* **120**, 664-677.
Wright, P. J. (1991). The influence of metabolic rate on otolith increment width in Atlantic salmon parr, *Salmo salar* L. *J. Fish Biol.* **38**, 929-933.
Yamamoto, Y., Ueda, H. and Higashi, S. (1998). Correlation among dominance status, metabolic rate and otolith size in masu salmon. *J. Fish Biol.* **52**, 281-290.
Yamano, K. and Miwa, S. (1998). Differential gene expression of thyroid hormone receptor α and β in fish development. *Gen. Comp. Endocrinol.* **109**, 75-85.
Yamano, K., Tagawa, M., de Jesus, E. G., Hirano, T., Miwa, S. and Inui, Y. (1991). Changes in whole body concentrations of thyroid hormones and cortisol in metamorphosing conger eel. *J. Comp. Physiol. B* **161**, 371-375.