Materials and Methods

Materials

Reagents and enzymes

An RNA easy kit, PCR and plasmid purification kits were from Qiagen. MMLV-Reverse transcriptase, RNasin ribonuclease inhibitor, DNaseI, and Taq DNA polymerase were from Promega. A ABI PRISM dye terminator cycle sequencing ready reaction kit from Perkin Elmer. T4 DNA ligase and 100 bp DNA marker were purchased from New England Biolabs. Oligonucelotide primers were synthesized by the Bioservice (BIOTEC, Thailand) and Qiagen. Protein molecular weight markers were products of Pharmacia LKB Biotechnology (USA). All other chemicals used were analytical grade.

Bacterial and plasmid vectors

E.coli strain JM109 and pGEM-Teasy vector were purchased from Promega.

Animals

Green catfish (*M. nemurus*) eggs and larvae used in the experiments were provided and kept under hatchery condition from the Songkhla Inland Fisheries Station.

Methods

1. Animal breeding and larval care

De-chlorinated tap water was used for fish maintaining and preparation of solutions for all treatments, otherwise as stated.

1.1 Animal breeding

The mature female green catfish weighing 0.5 to 1 kg from the brood stock was obtained the first injection into the epaxial muscle with a combination of Lutinizing hormone releasing hormone analogue (LHRHa; 10 μg) (Suprefact, Hoechst AG, Germany), and dopamine antagonist (Motilium, Janssen) (10 mg/kg body weight). The second injection was conducted, 6 h after the first injection, with 20-25 μ g of LHRHa and 10 mg/kg body weight of dopamine antagonist. After 6-10 h from the second hormone injection, eggs were stripped, mixed with milt solution from the

mature male, and being placed evenly onto nylon net. After hatching (about 36-48 h), larvae were transferred to a nursery pond.

1.2 Animal care

The larvae were reared in a concrete pond (3 m x 2 m x 0.3 m) with continuous aeration for 45 days. They were fed with water flea (*Moina* sp.) from 3 to 7 days after hatch, then acclimated to a powder feed by receiving a mixture of water flea and powder feed (Power feed 2000, Whitecrane (V88) Aqua Product). Complete substitution with powder feed was performed when the fish were 10 days old. The powder feed was provided twice a day. The water level in the rearing pond was 20 to 30 cm in depth at the first period of rearing, then the level was increased to 45-50 cm at the time the powder feed was provided. Continuous flow of fresh water was supplied to maintain the level of water in the pond. To maintain the water quality, wastes and debris were removed every other day and 75 % of water in the pond was replaced every week. Fish were collected from the pond at the ages of 3, 7, 15, 30 and 45 days for further treatments.

2. Thyroid hormone solution preparation

Thyroid hormones in the form of sodium salt were dissolved in dimethylsulfoxide (DMSO) or methanol to obtain the stock solution of 10 mg/ml, and kept as the frozen aliquots at –20 °C until used. Working thyroid hormone solutions with a nominal concentration were obtained by diluting the stock solution with water. For the long-term hormone treatment studies, working hormone solutions were prepared in 20 L-gallon containers and kept at 4 °C until used.

3. Animal collection for experimental treatments

General procedures will be stated in this section. More specific issues will be detailed in each result. Experiment for each hormone treatment was conducted with 3 replicates. All catfish, whole body or organ tissues, were kept at –20 °C until further analysis.

3.1 For the hatching rate study

The experiment was conducted in a plastic box (12.5 cm x 15 cm x 5 cm), being attached with a rectangular wire-frame and nylon net at 1 cm above the bottom

of the box, leaving space for the aeration pipe to fit in (see picture in the appendix). Each box was added with 500 ml of water or water containing thyroid hormone, allowing the nylon net to be 2 to 3 cm underneath the solution surface. Fertilized eggs were evenly placed on the nylon net in the boxes containing thyroid hormones solution at various concentrations. The fish embryos were allowed to hatch with continuous aeration. After 48 h, numbers of embryos were recorded and hatching rate was determined.

3.2 For the survival rate study

For a short-term treatment, the catfish at specific ages were collected from the nursing pond and placed in a plastic container containing 8 L of T_3 solution. DMSO was used in the control group. Continuous aeration was supplied through out the treatment. After treatment for 24 h, fish were transferred to 1 L of fresh water. The survival rate was calculated by subtraction of the dead from the total number at the beginning of the experiment. Groups of 50 (for those at 3 or 7 days of age), 20 (for those at 15 days of age), and 10 (for those at 30 or 45 days of age) fish were assigned in each replicate. Two experiments with 2 replicates were performed for each treatment.

For a long-term treatment, fish at specific ages were exposed to 1 L of T_3 solution for 15 days. They were fed with powder feed twice a day. Each container was continuously aerated, and cleaned daily. Water and T_3 solution were renewed by three-forth substitution with fresh water and solution, respectively, every 2 days. Dead fish were recorded and removed everyday. Two individual experiments with 3 (for fish 15 and 45 days of age) and 2 (for fish 30 days of age) replicates were conducted. Groups of 20 (for those 15 and 30 days of age) and 10 (for those 45 days of age) fish were assigned in each replicate.

3.3 For the enzyme assays

Fish were transferred from the nursing pond at specific ages into a plastic container containing 8 L of T_3 solution (5, 20 and 80 ppm). After 24 h, they were transferred to a new container containing 1 L of fresh water or hormone solution. The exposure to the hormone was continued for another 3 days with aeration but without feeding. Three separate experiments were performed for each treatment and age with specific number of fish in each groups, i.e. 2000, 1500, 300, 40, and 30 for 3, 7, 15,

30, and 45 days of age, respectively. Up to 10 % of fish in each container was collected daily and immediately stored frozen at -80 °C until analysis.

3.4 For mRNAs assay

Fish with 15, 30 and 45 days old were treated and maintained similarly to the experiment for the long-term treatment. Three individual experiments with 3 replicates were carried out. Groups of 40 (for the 15-days larvae), 20 (for the 30-days larvae), and 10 (for the 45-days larvae) were assigned in each replicate. The larvae were exposed to T_3 for 15 days, then were collected, immediate frozen and stored at -80 °C until analysis.

4. Enzymatic activity assays

4.1 Protein extraction

Whole catfish body was homogenized in 10 mM Tris-HCl buffer pH 7.4, containing 1 mM EDTA, with ratio of the animal tissue: buffer (g : ml) at 1: 2.5. Cell debris was removed by centrifugation at 14,000 rpm for 90 min. Supernatant was collected and kept at -20 °C until used. For preparing the crude protein for G6PDH and LDH assays, the supernatant was aliquoted (300 µl) in 1.5 ml tube and saturated ammonium sulphate solution was added to obtain the final concentration of 85% of salt in the protein solution. The protein precipitate was collected by centrifugation at 10,000 rpm for 15 min, and was kept at -20 °C as pellet. When used, the pellet was re-dissolved in 300 µl of 50 mM Tris-HCl, pH 7.4. All steps were carried out at 4 °C, otherwise as stated.

4.2 Glucose-6-phosphate dehydrogenase (G6PDH) activity assay

The activity of G6PDH was determined from a change in absorption of NADPH at the wavelength of 340 nm, according to the method of Lupiáñez *et al.* (1987) and described by Corpas *et al.*(1995). In brief, 1 ml of total assay mixture composed of 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 20 mM G6P and 0.6 mM NADP. The change in absorption was measured for 5 min. One unit (U) of enzyme activity is defined as the amount of enzyme required to reduce one µmole of NADP per min., and the specific activity of the enzyme is the activity units per mg of protein. The enzymatic activity determinations were carried out in 3 replicates.

4.3 Lactate dehydrogenase (LDH) activity assay

The activity of LDH was assayed by monitoring the NADH production at 340 nm. The activity was determined in an assay mixture containing 10 mM Tris-HCl, pH 8.8, 0.1 M lactate and 10 mM NAD^+, according to the method of Adolf and Koko (1977) and described by Wahlefeld (1983). The increase in absorbance of NADH was monitored for 5 min. One unit (U) of the enzyme is defined as the amount of enzyme required to reduce one μmole of NAD^+ per min. The enzymatic activity determinations were carried out in 3 replicates.

4.4 Transglutaminase (TG) activity assay

The activity of TG was measured according to the procedure described by Folk and Cole (1966). The assay mixture of 0.5 ml contained 200 mM Tris acetate, pH 6, 100 mM hydroxylamine, 5 mM $CaCl_2$,10 mM glutathione, and 30 mM N-carbobenzyloxy-glutaminyl-glycine. The enzymatic reaction was performed at 37 °C for 30 min, then 0.5 ml of 15 %TCA and 5% $FeCl_2$ solution mixture was added to terminate the reaction. The reaction mixture was then centrifuged at 7,000 rpm for 20 min at room temperature, and clear supernatant was collected. The supernatant was measured at 525 nm. A standard curve was constructed at 0.01, 0.02, 0.04, 0.08, and 0.16 μmoles of glutamic acid -γ -hydroxamic. One unit (U) of enzyme activity is defined as the amount of enzyme required to produce one μmole of hydroxamic acid within 1 min at 37 °C. The specific activity of the enzyme is defined as the enzyme activity units per mg of protein. The enzymatic activity determinations were carried out in 3 replicates.

5. RNA extraction

Total RNA from the whole body of the catfish was extracted using RNeasy Mini Kit (QIAGEN, USA.), using the protocol of the company for animal tissue with some modifications. In brief, the whole fish was disrupted and homogenized in buffer containing guanidine isothiocynate using a glass-teflon homogenizer. For more complete homogenization, the partial homogenate was immediately transferred to QIAshredder spin column (QIAGEN, USA), and centrifuged for 2 min at 12,000 to 14,000 rpm. The flow-through clear tissue lysate was collected, and re-centrifuged at 12,000 to 14,000 rpm for 3 min. Then, 70% of ethanol was added to

precipitate the DNA. The lysate mixture, including DNA precipitate, was applied to an RNeasy column, and centrifuged at ≥ 10,000 rpm for 15 s. DNA and all other contaminated proteins were removed from the column by washing with solution containing guanidine salt and ethanol. The RNA bound to the silica membrane of the column was eluted in the final step with RNase-free water.

6. Removal of contaminated DNA with deoxynuleotidase (DNase)

In order to remove residual DNA that might still remain in the RNA solution, the digestion with DNase I was performed prior to RT-PCR, using the basic protocol recommended by the company with some modifications. In brief, 2 μg of RNA was digested with 2 units of DNase I in a reaction mixture (20 μl) in the presence of 20 units of ribonuclease inhibitor (RNase inhibitor). The digestion was allowed to occur at 37 °C for 30 min prior to the addition of 20 mM EGTA solution and incubation at 65 °C for 10 min to inactivate the DNase. The reaction mixture was kept at -20 °C, or immediately used for cDNA synthesis by the reverse transcription process.

7. Synthesis and amplification of specific DNA fragment by two-step reverse transcription and PCR (RT-PCR)

7.1 Synthesis of first strand complementary DNA (cDNA) by reverse transcription

The first strand cDNA was synthesized from total RNA by AMV reverse transcriptase (Promega) using random hexamer (Promega) to initiate the synthesis, according to basic protocol provided by the company with some modifications.

The standard reaction mixture in a total volume of 20 μl was set up for 2 μg of DNase treated RNA. Other components in the reaction mixture included the reverse transcriptase buffer, 20 units of RNase inhibitor, 1 mM of dNTPs mixture, 0.75 μg of random primers, and 20 units of MMLV reverse transcriptase. All components were added prior to incubation at 42 °C for 30 min. The reaction mixture then was immediately proceeded for the specific synthesis of the second strand cDNA and amplification of the DNA fragment by polymerase chain reaction (PCR), or kept until used at –20 °C .

7.2 Specific DNA amplification by polymerase chain reaction (PCR)

PCR was used to synthesize the second strand of the cDNAs and to amplify for specific gene fragments using first strand cDNAs obtained from the reverse transcription of total RNA. Pairs of oligonucleotide primer specifically designed for G6PDH, TG, deiodinaseIII, thyroid hormone receptor, and beta-actin (see Table 1) were used in amplification of the gene fragments, respectively. Synthesis of the second strand cDNA and amplification of the specific gene fragments were carried out simultaneously in 20 μl of the reaction mixture with the first strand cDNAs from 1 μl (one-twentieth) of the reverse transcription mixture. Other components in the reaction mixture included PCR buffer, $MgCl_2$ (1.87 mM), dNTPs (250 μM), 5′ and 3′ specific primers (0.5 μM each), and Taq DNA polymerase (1 unit). The synthesis and amplification were started with an initial denaturation of cDNAs at 94 °C for 5 min. This was followed by cycles of 94 °C for 30 s, annealing temperature for 1 min and 72 °C for 30 s. The final extension was carried out for 1 cycle of 72 °C for 5 min.

The PCR reaction products were separated on a 1.5 % agarose gel, and gel was post-stained with ethidium bromide and visualized under ultra-violet illumination. Each RT-PCR analysis was repeated three times for each set of RNA samples.

8. 5′ and 3′-RACE

Longer fragment of gene sequence was obtained by 5′ and 3′RACE as described in the 5′/3′RACE kit (Invitrogen) with some modification. First, MMLV-reverse transcriptase (Promega) and random hexamer primer (for 5′RACE) or oligodT_{15} (for 3′RACE) were utilized for the reverse transcription. Second, total RNA were used as template for the first strand cDNA synthesis. Third, polyT tail was added to the 5′end of the first strand cDNA that used for 5′RACE by activity of the terminal transferase (Promega).

9. Isolation and purification of DNA from agarose

The PCR reaction products were separated on 1.5% of low melting agarose gel containing 0.5 μg/ml of ethidium bromide. The gel area containing the DNA fragment of interest was excised with a sterile single-edged razor blade, and the DNA was recovered from the agarose by the method described by Sambrook *et al.*, 1989. In brief, a slice of agarose containing the DNA of interest was placed in a microfuge

Table 1 **Specific primers used in amplification of DNA fragments for the gene expression measurement by semi-quantitative RT-PCR**

Gene	Primer sequence 5′→3′	Annealing Temperature (°C)	Orientation
TRα	AGGAGGAGATGGTGAAGACG	58	sense
TRα	GGGTGATGGCTGGCGTGATG	58	antisense
G6PDH	TGGAACCGAGAGAGTGTTGCCTGTG	58.6	sense
G6PDH	TTCCGCTCATTCAGTGCCTTCCCAC	58.6	antisense
TG	ATGTCTCTGCTGGTGTTCAG	58	sense
TG	TGGCGACTAACTACCTCTCG	58	antisense
DeiodinaseIII	TCTGTAAGGCAGCGCACCTG	58	sense
DeiodinaseIII	AGCAACGAGTCGGCGATGTC	58	antisense

tube and 5 volumes of solution containing 10 mM Tris and 1 mM EDTA, pH 8.0 were added, then the tube was incubated at 65 °C for 5 min to melt the gel. The gel solution was cooled to room temperature and extracted with an equal volume of phenol/chloroform/isoamyl alcohol. The aqueous phase was transferred to a new tube and DNA was precipitated with 0.1 volumes of 3 M sodium acetate pH 5 and 2 volumes of ethanol. The DNA pellet was washed once with 70% of ethanol, dried up and dissolved in TE.

10. Cloning of DNA fragment

Purified PCR product was cloned into pGEM-Teasy Vector (Promega) with the method provided by the company. The ligation reaction included 25 ng of purified PCR product, 50 ng of pGEM-T easy vector, the Rapid Ligation buffer, and 3 Weiss units of T4 DNA ligase. Ligation was allowed to proceed for 1 h at room temperature, and the reaction mixture was transformed into JM109 High Efficiency Competent Cells (Promega). White colonies that generally contain the DNA insert plasmid were selected.

11. Purification of plasmids

E. coli plasmids were prepared by the alkaline lysis procedure published by Birnboim and Doly (1979), using the Perfectprep plasmid mini kit (Eppendorf, Germany). The plasmid was finally eluted with 10 mM Tris-HCl and 1 mM EDTA, pH 8.5.

12. DNA sequencing

Nucleotide sequence of thyroid hormone receptor was determined using an ABI Prism BigDye Terminator version 3.0 Ready Reaction Cycle Sequencing kit (Applied Biosystems). For the 20 µl reaction mixture, 500 ng of double stranded DNA was mixed with 3.2 pmole of primer (T7 promotor primer) in a total volume of 12 µl, and 8 µl sequencing reagent was added. The amplification was carried out for 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. The DNA separation according to size was performed in Applied Biosystems 3100 Genetic Analyzer Systems.

13. Determination of protein concentration

The concentration of protein was determined by the Microbiuret method, described by Itzhaki and Gill (1964), using bovine serum albumin as a standard.

14. Statistics

Data were analyzed by one-way or two-way ANOVA depending on numbers of factor to be determined. When significant differences between groups were found ($\alpha < 0.05$), multiple comparison tests of Newman-Keul and Dunnett were used to locate differences between the means. Some data were arc-sine transformed or log transformed, depending on an appropriate, to achieve normality, and some data were analyzed by the test of regression (at level of 95% confidence) prior to multiple comparison test.