

Results and Discussion

1. Effect of thyroid hormones on the green catfish hatching rate

Although the effect of thyroid hormones on the hatching ability also has been studied in several fish. The responses to the hormone involvement in hatching rate of the animals were inconsistent. It can be ranged from positive to negative result, found in one to another specie (Ayson and Lam, 1993; Lam and Sharma, 1985; Takawa and Hirano, 1991). So, the effect of thyroid hormones on hatching rate was attempted in the green catfish with the aim to reveal whether or not the hormone could increase its hatching rate.

To obtain the insight of the effect of thyroid hormone action on the catfish eggs, experiments were conducted using T_3 and T_4 as the exogenous forms of the hormone. The dosage of thyroid hormone and the exposure period to the hormone of the catfish eggs were also observed.

1.1 Effects of the exposure period to T_3 on hatching rate

The experiment was carried out using concentration of T_3 at 6.25, 200 and 300 ppm with the exposure to T_3 for 1 h and until hatch (36-48 h).

The percentages of hatching in all treatments and the control were ranged from 62 to 67 %. Fertilized eggs that incubated in the presence of T_3 until hatched showed no difference in percentage of hatching from that exposed to T_3 for 1h (Figure 1). Neither the hatching rate of fish was significantly changed when exposed eggs to different concentrations of T_3 (Figure 1). No significant difference was confirmed by the two-way analysis of variance (ANOVA) at $p > 0.05$. The hatching percentages, with SD (as indicated by vertical bar), at the concentrations of T_3 at 6.25, 200, and 300 ppm is shown.

1.2 Effect of T_3 dosage on the hatching rate

To determine the effect of T_3 doses on the hatching rate, fertilized catfish eggs were exposed to the hormone at concentrations of 6.25, 50, 100, 200, and 300 ppm until hatch. The hatching rate was determined and compared with that of the control.

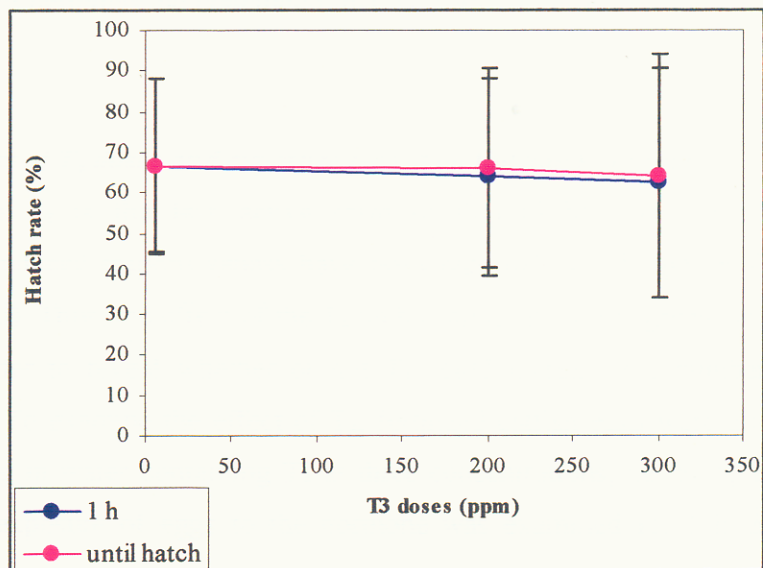


Figure 1 Effect of exposure time to T_3 on hatch rate. Fertilized eggs were layered on a nylon net and immersed in water contained various doses of T_3 (6.25, 200 and 300 ppm). Eggs were left to expose to T_3 for 1 h (●) or until hatched (36-48 h) (●). Number of the hatched out fish larvae was counted and the percentage of hatching was calculated. Vertical bar indicates the standard deviation (SD).

Figure 2 showed the hatching rate, in percentage. The hatching rate of the eggs that exposed to T_3 was ranged from 42 to 49 % while that of the control was around 56 %. However, by one-way analysis of variance, it revealed no significant difference ($p > 0.05$) between the treatments and the control. Together with the result from 1.1, it could definitely be concluded that T_3 exerts no effect on the hatching of the green catfish.

1.3 The effect of T_4 doses and exposure period on the hatching rate

Although T_4 is the less active form of thyroid hormone found in all vertebrates. T_4 -induced responsiveness have been reported in fish species (Tripathi and Verma, 2003). Thus, the effect of the T_4 on hatching rate of the green catfish was examined in order to reveal whether or not T_4 could increase the number of the hatched catfish. To examine the effect of T_4 , fertilized catfish eggs were allowed to perform hatching in water containing T_4 at three concentrations, i.e. 6.25, 200, and 300 ppm. The exposure of eggs to the hormone was examined for 1 h and until hatched.

The percentage of hatching obtained was ranged from 33 to 38% as shown in Figure 3. The hatching rates of eggs exposed to T_4 for 1 h were slightly higher than that with longer exposure to the hormone. However, the two-way analysis of variance at $p > 0.05$ revealed no statistical difference between exposure to T_4 for 1 h and until hatch. Moreover, the hatching rates among of treatments with exposure to different concentrations of the hormone (Figure 3) also was not statistically different ($p > 0.05$).

2. The effect of thyroid hormone on green catfish survival

Thyroid hormones not only affect the embryonic stage, but also play a major role during development of fish. The enhancement of the survival of fish larvae by thyroid hormone supplement was observed in several fish species. The experiments, thus, were performed to reveal the effects of T_3 on survival at several post-hatched ages of the green catfish, i.e. 3, 7, 15, 30 and 45 days. The developmental periods that thyroid hormone is a necessity for life of the fish and the effective doses of the hormone in increasing the survival rate of the fish were examined.

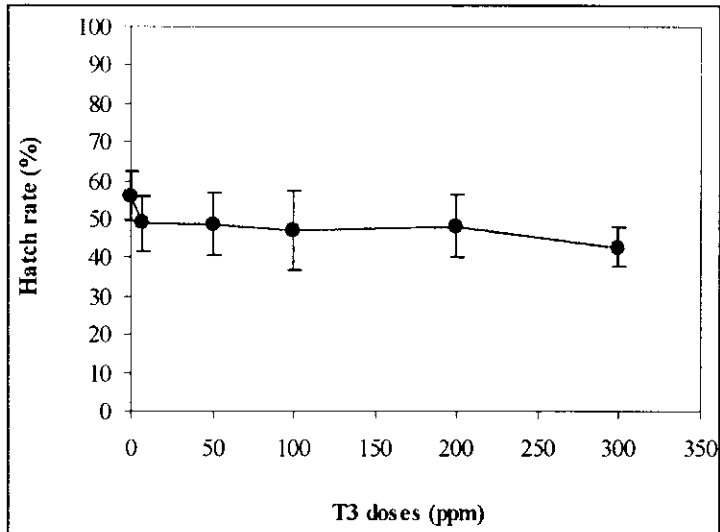


Figure 2 Effect of thyroid hormone doses on hatch rate (%) of the catfish. Eggs were exposed to various doses of T₃ (6.25, 50, 100 and 300 ppm) until hatch. Eggs in the control (at 0 ppm of T₃) were immersed and allowed to hatch in normal water contained 30 ppm of DMSO. Vertical bar indicates the standard deviation (SD).

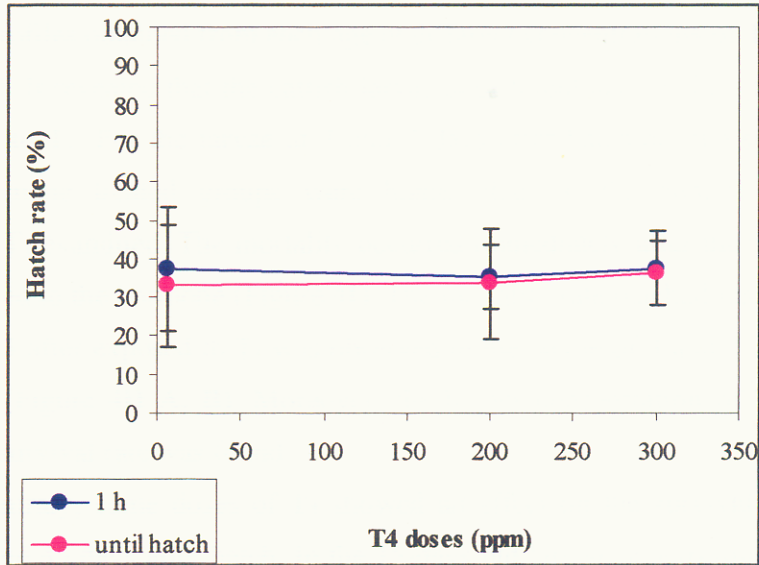


Figure 3

The hatching (%) rate of the catfish eggs exposed to T₄. Eggs were fertilized and immersed in water contained 6.25, 200 and 300 ppm of T₄. Number of the larvae hatched out from eggs exposed to T₄ for 1 h (●) or until hatch (36-48 h) (●) was determined and the percentage of hatching was calculated. Vertical bar indicates the standard deviation (SD).

2.1 Effect of short-term treatment of T_3 on the survival profile of the larvae

To determine the effect of short-term treatment of T_3 on the survival profile, the larvae at 3, 7, 15, 30, and 45 days after hatch were exposed for 24 h to T_3 at various concentrations. Thereafter, they were transferred to maintain in normal water without feeding, and the numbers of viable fish were recorded daily.

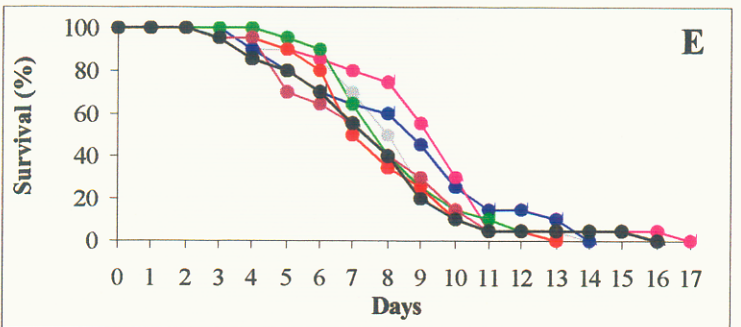
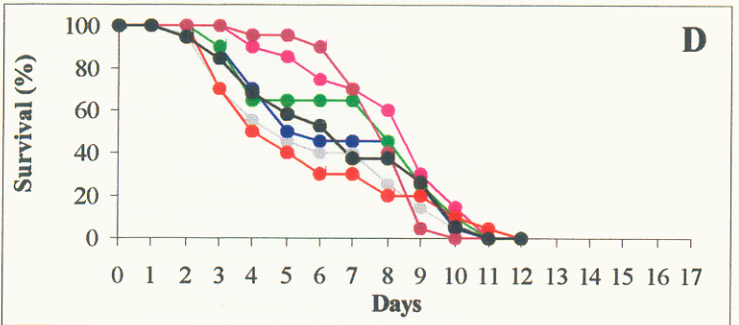
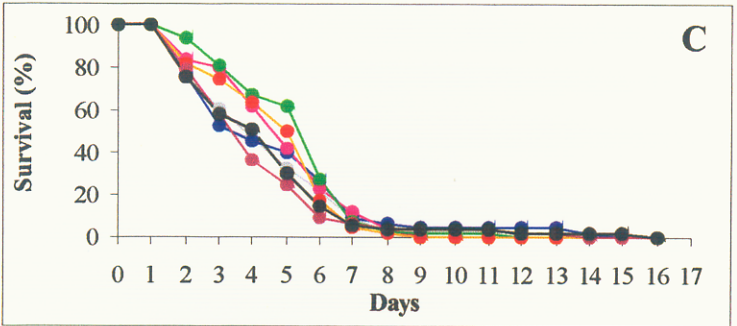
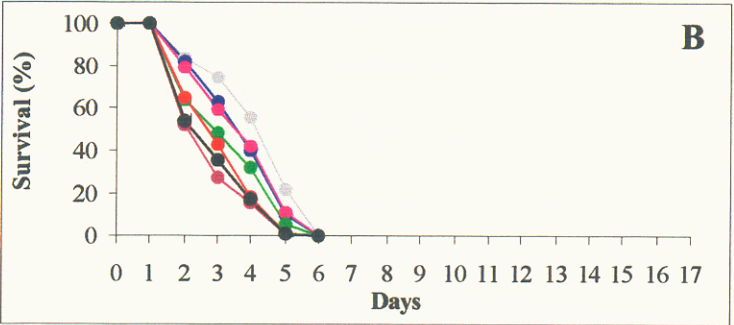
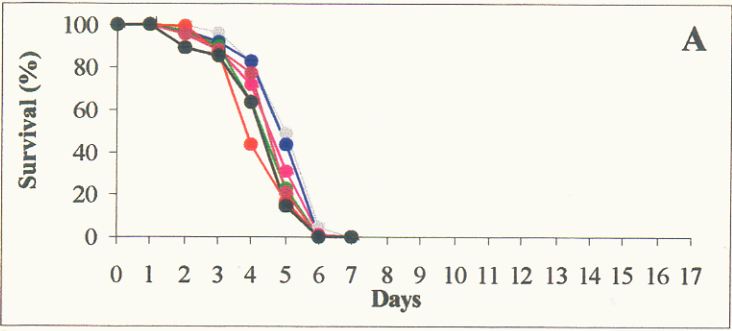
Survival profiles obtained from each age at different concentrations of T_3 were shown in Figure 4. It revealed that the catfish larvae at different ages had a variation in responsiveness to T_3 . For the larvae at 3 and 7 days after hatching, the survival rates of the hormone treated groups were higher than in the control at all concentrations of T_3 examined. The mortality occurred immediately after the day the larvae were exposed to the hormone (Figure 4I). The mortality rate reached maximum on day 5 and day 3 after exposed to T_3 was observed in the larvae of 3 and 7 days of age, respectively (Figure 4II A, B). Moreover, the higher concentration of T_3 , the more decrease of survival rate was found.

On the contrary, some doses of T_3 showed an effect on the survival of the larvae of 15, 30 and 45 days after hatch. In the 15-days old larvae, an exposure to T_3 with concentrations of 10 to 80 ppm more decrease the mortality rate of the treatments than the control (Figure 4I C, 4II C). While, higher mortality was observed in the larvae exposed to the hormones at 5, 160, and 320 ppm. In the 30-days old larvae, the hormone treated larvae, except that exposed to 80 ppm of T_3 , showed higher survival than the control (Figure 4II D).

Specific change of the survival profile was observed in fish of 45 days old. Higher and lower rates of mortality than the controls could be observed within the same concentration of T_3 (Figure 4I E, and 4II E). At 5 ppm of T_3 , the mortality rate gradually increased and reached to the maximum after the larvae were exposed to the hormone for 6 days. Then, the rate decreased and reached to the maximum at day 9 of the exposure (Figure 4II E). The opposite changing of the survival profile occurred at 20 ppm of the hormone (Figure 4II E). The survival increased during the first 4 days of exposure to the hormone. Thereafter, the mortality rate increased and brought the survival down to lower than that in the control. At 10 ppm of T_3 , the survival of the larvae was higher than in the control, whereas at the hormone higher than 80 ppm, the survival was lower than that in the control (Figure 4II E).

So, the results obtained in this section could direct to the conclusion that T_3 exerted an involvement in survival rate of the larvae of the green catfish. The

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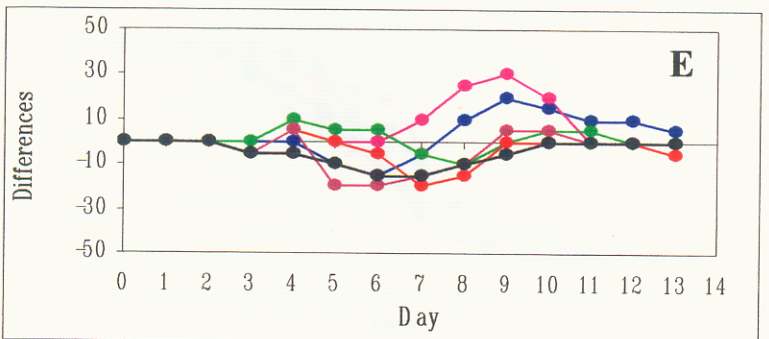
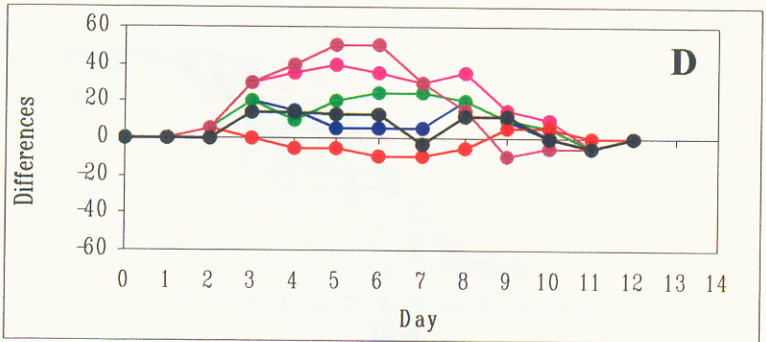
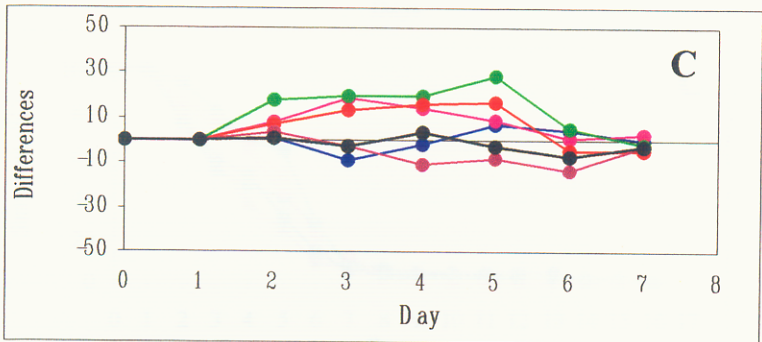
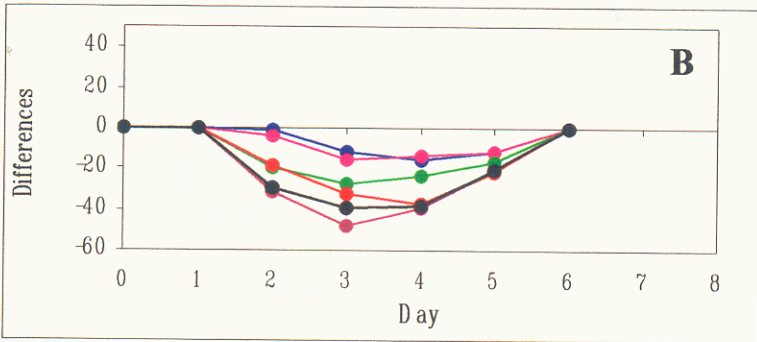
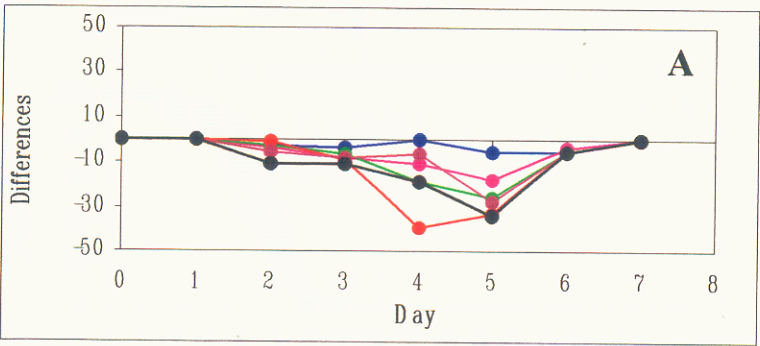


Figure 4 The effect of T_3 doses on survival of the catfish larvae. Fish of 3(A), 7 (B), 15(C), 30(D) and 45 (E) days old were exposed to T_3 at concentrations of 5 ppm (●), 10 ppm (●), 20 ppm (●), 80 ppm (●), 160 ppm (●) and 320 ppm (●). Normal water in the presence of DMSO was used for the control. Survival of the larvae was recorded daily.

- I : Survival percentage of each treatments were plotted and compare to that of the control (●).
- II : The difference plot of survival percentage between the treatments and the control.

enhancement effect on survival occurred at only definite concentrations of the hormone. e.g. 10-80 ppm and 10-20 ppm for the 15-days old and the 45-days old larvae, respectively. Moreover, within the examined concentration of T_3 , only the larvae at older than 15 days could conduct a positive responsiveness to the hormone, i.e. decreased in the mortality rate.

2.2 Effect of long-term treatment of T_3 on the survival rate of the catfish larvae

According to the results obtained in section 2.2, it clearly shown that short-term treatment with T_3 enhanced the survival of the larvae. The effective doses of the hormone were 10 to 80 ppm, and the larvae at 15 days old and those with an older age reflected the positive response to the hormone action. The question arose whether and how the survival of the larvae would be if they obtain the hormone for a longer period. To answer this question, the green catfish larvae at 15, 30, and 45 days were reared in water containing 10, 20, and 80 ppm of T_3 for 15 days with feeding as described in Methods.

As shown in the Figure 5A, the survival of the 15-days old larvae was lower than the control at all examined concentrations of the hormone. At 10 ppm and 20 ppm of the hormone, the mortality gradually increased from the first day of the exposure throughout the experiment. Whereas, rapid decline observed in the larvae exposed to 80 ppm of T_3 . No larva survived on the day before the last day of the experiment. The differences of survival between the 15-days old larvae with long-term T_3 treatment and the control was statistically significant, confirmed by regression analysis and followed by Dunnett test at $p \leq 0.05$. However, it should be noted that the larvae treated with 20 ppm of T_3 at short period, i.e. within the first week of the treatment, showed higher survival than the control (Figure 5A).

For the 30-days old larvae, significant enhancement of survival ($p \leq 0.05$) was observed at 10 and 80 ppm of T_3 (Figure 5B). The mortality rate occurred, but at lower rate than that found in the control. At 80 ppm of the hormone, the mortality rate of the larvae was steady from day 11 of treatment. While, at T_3 of 20 ppm, the rate was remarkably increased after the larvae were exposed to the hormone longer than 5 days (Figure 5B). The survival between the treatment and the control was statistically different by Dunnett test at $p \leq 0.05$.

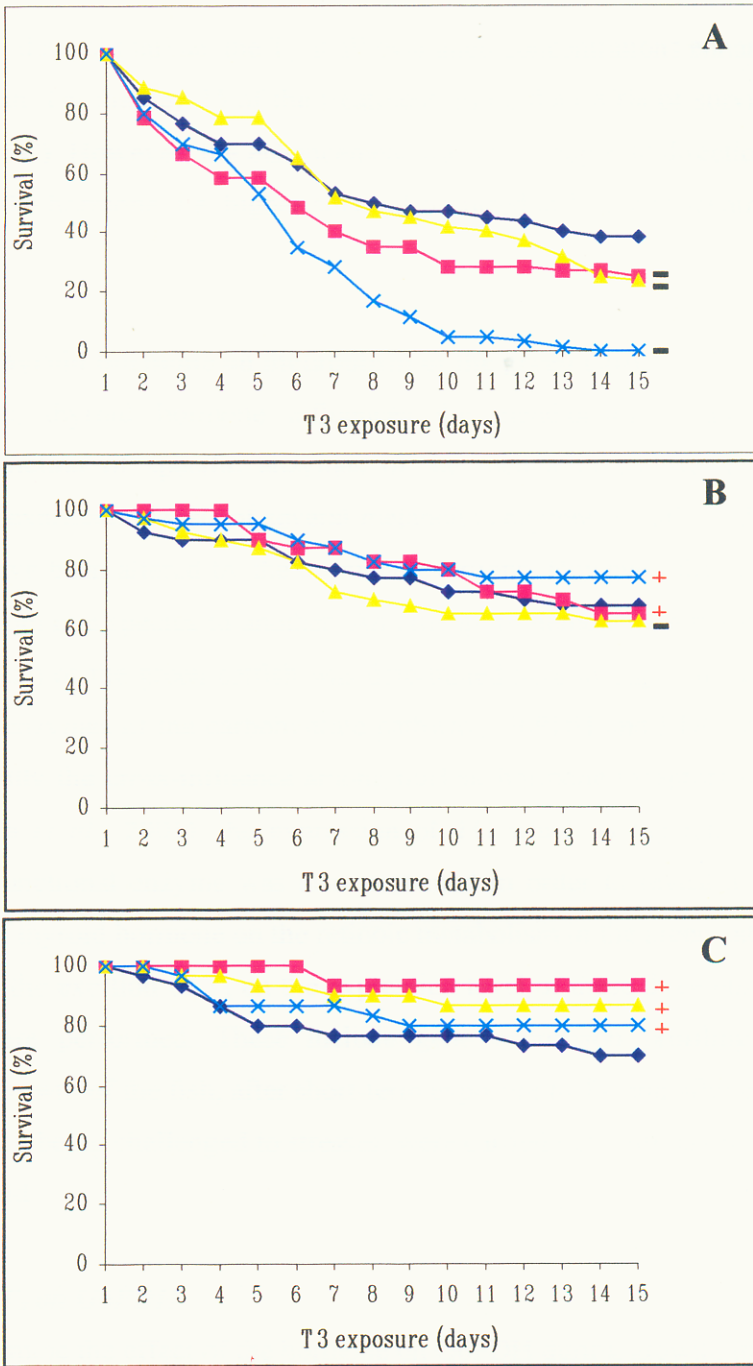


Figure 5 The effect of long-term treatment with various T_3 doses on survival of catfish. Fish with ages of 15(A), 30(B) and 45(C) days old were reared in the presence of 10 ppm (■), 20 ppm (▲) and 80 ppm (×) of T_3 , while fish in the control (♦) was reared in the normal water. The number of fish survived was recorded everyday for 15 days. Statistical survival differences ($p \leq 0.05$), by Dunnett test, of the treatments that higher or lower than the control were indicated by plus (in red) or minus (in black).

For the 45-days old larvae, the T_3 at all examined concentrations enhanced the survival of the fish. On the day 15 of the treatment, more than 80% of survival was obtained (Figure 5C). However, the enhancement was in an opposite direction to the concentration of the hormone, i.e. higher survival occurred at lower concentration of T_3 .

3. The effect of thyroid hormone on metabolism of the green catfish

In vertebrates, thyroid hormone influences the metabolism of animals by regulating the function of specific enzymes of the metabolic pathways. In fish, activities and the expression levels of several metabolic enzymes in carbohydrate and lipid pathways have been altered by the hormone exertions. However, the effect was so far different from one species to another. An attempt to demonstrate the influence of T_3 on metabolism of the green catfish was determined here through an expression level of two key enzymes of the carbohydrate pathway, G6PDH and LDH. As it has been shown recently the cross-influence among nuclear hormone receptors signaling pathways (Bonilla *et al.*, 2001), the expression of TG which is the retinoic acid-dependent multifunctional enzyme was also examined in order to reveal a possible cross-influence of thyroid hormone on the retinoic metabolism in the green catfish.

To determine the exertion of T_3 on the expression level of these three enzymes, 3 investigations were performed. These include gene expression profiling of the enzymes during growth of the fish, after short-term exposure to T_3 , and after exposure to the hormone and being challenged to stresses, were carried out with larvae 3, 7, 15, 30, and 45 days old.

3.1 Effect of T_3 on G6PDH

To determine the effect of the hormone on G6PDH during growth, the larvae at specific ages were collected, crude protein was prepared, and the G6PDH activity was assayed as described in Methods. The specific activity of the enzyme observed in each age of the larvae is shown in Figure 6A. The expression pattern of G6PDH was non-linear to the age of the larvae throughout the growth period of observation, however quite stable in a range of 20 to 30 $\mu\text{mole NADPH}/\text{min}/\text{mg protein}$. Level of the enzyme increased in a gradual manner during the growth from 3 to 15 days after hatch, then declined gradually thereafter. The minimal level of the enzyme was observed at 30 days of age. Then the level of G6PDH increased once. At 45 days of

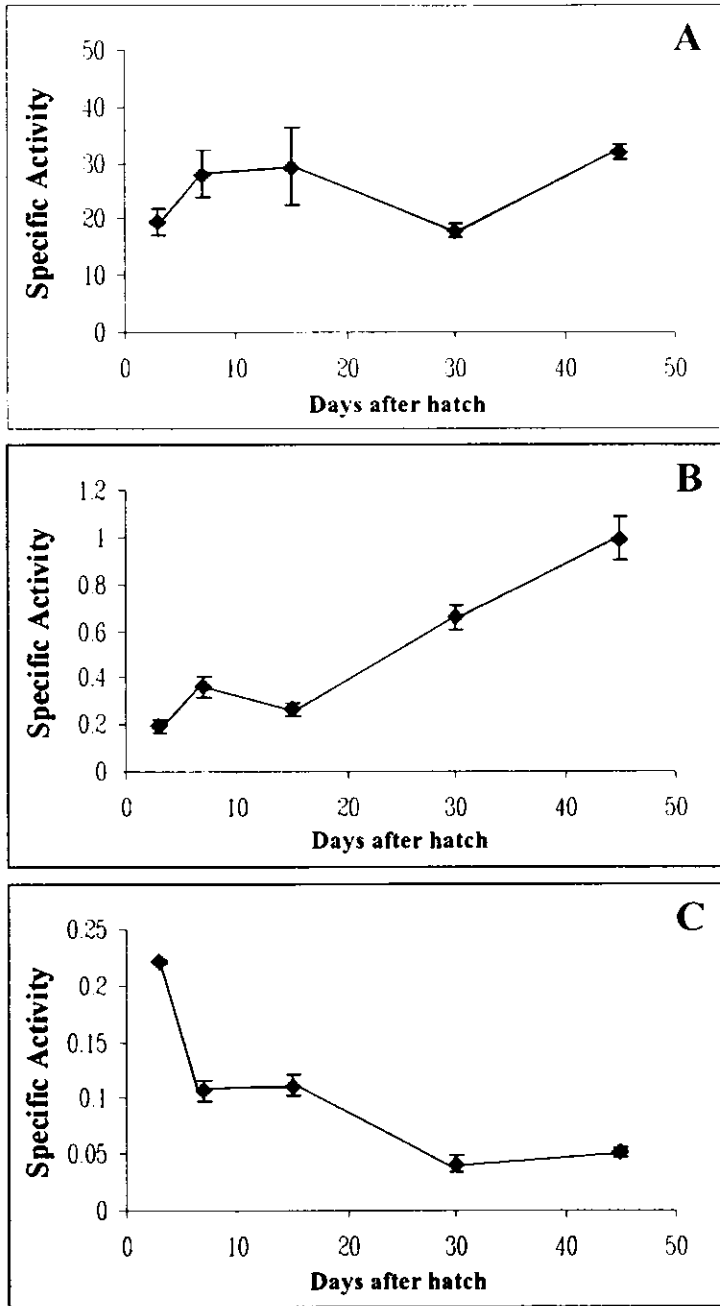


Figure 6 The specific activity profile of Glucose-6-Phosphate Dehydrogenase (A), Lactate Dehydrogenase (B), and Transglutaminase (C) during development of the green catfish. Crude protein of catfish whole body at ages of 3, 7, 15, 30 and 45 days old were extracted and activity of each enzymes was assayed. The specific activity is defined as $\mu\text{mole NADPH}/\text{min}/\text{mg}$ protein for glucose-6-phosphate dehydrogenase, $\mu\text{mole NADH}/\text{min}/\text{mg}$ protein for lactate dehydrogenase, and $\mu\text{mole hydroxamic acid}/\text{min}/\text{mg}$ protein for transglutaminase, respectively. Each experiment was analyzed in triplicate. Vertical bar indicates the standard deviation (SD).

age, the synthesis of the enzyme was slightly higher than that found in other ages (Figure 6A).

The effect of the short-term treatment with T_3 on the level of G6PDH was determined at 5, 20, and 80 ppm of the hormone, and the larvae were exposed for 24 h. Alteration of the G6PDH synthesis level was demonstrated in Figure 7. The enzyme level of the treatments was significantly higher than the control ($p \leq 0.05$) in the 3-days old larvae after they were exposed to 20 ppm of the hormone (Figure 7A). Whereas, the enzyme level was significantly higher than in the control found in all treatments of the 7-days old larvae (Figure 7B).

In the 15-days old larvae, the G6PDH level in the treatments was higher than in the control after they obtained 20 and 80 ppm of T_3 (Figure 7C). The difference of the enzyme level between the treatments and the control was statistically significant by Dunnett test at $p \leq 0.05$. Hence, it is conceivable that T_3 at 20 ppm can exert the most enhancements on the protein level of G6PDH in the catfish during 3-15 days of growth.

The opposite effect was observed in the 30-days old larvae. The level of G6PDH in the treatment was significantly lower ($p \leq 0.05$) than that in the control after they were exposed to 5 ppm of T_3 (Figure 7D). However, the synthesis level of the enzyme in the 45-days old treatments was significantly higher than in the control at the same concentration of T_3 used (Figure 7E)

To investigate an alteration of the G6PDH level in the T_3 treated catfish during being challenged to the depletion of an energy supply, the catfish larvae at 15, 30, and 45 days of age were reared in water containing 5-80 ppm of T_3 for 4 day without feeding. The alteration of level of the enzyme was shown in Figure 8. In the 15-days old larvae of the control group, the enzyme gradually increased from day 1 to day 2, dropped in day 3, and then was persistent through day 4 (Figure 8A; first row). This should indicate raising in the consumption of blood glucose in the fish during the first few days of the food depletion. Similar alteration was found in the larvae exposed to 5 ppm of T_3 (Figure 8B; first row). Different alteration of the G6PDH level was observed in the larvae exposed to 20 and 80 ppm of the hormone. Different alteration of the G6PDH level was observed in the larvae exposed to 20 and 80 ppm of the hormone. The enzyme level did not increase as in the control and the larvae exposed to 5 ppm of T_3 . Significantly unchanged ($p > 0.05$) was detected during day 1

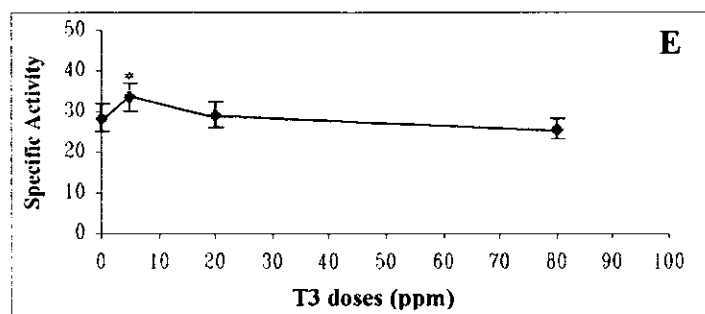
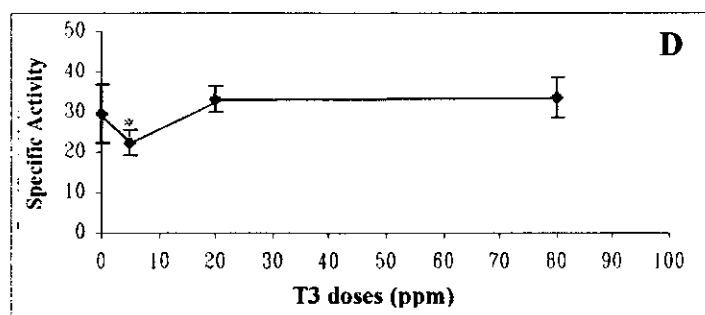
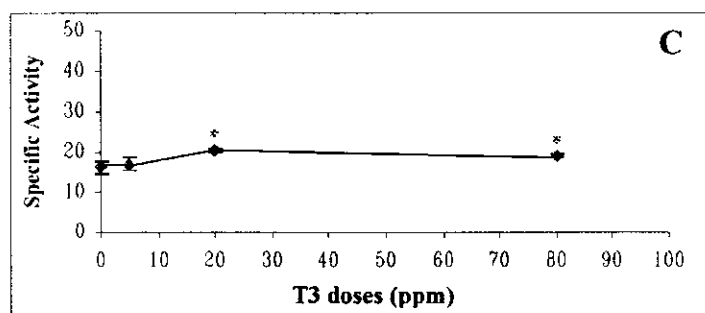
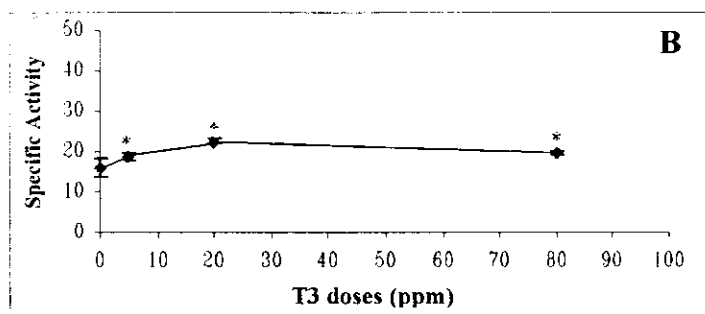
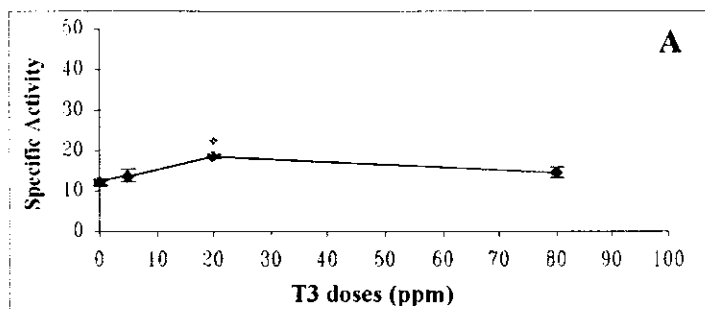


Figure 7 Effect of T₃ on G6PDH. The larvae of 3(A), 7(B), 15(C), 30(D) and 45 (E) days old were exposed to 5, 20, and 80 ppm of T₃ for 24 h. The specific activity of the enzyme was expressed in μ mole of NADPH/min/mg protein. Each enzyme assays was performed in triplicate. The vertical bar represents the standard deviation (SD). An asterisk indicates statistical difference ($p \leq 0.05$) determined by Dunnett test of the enzyme specific activity between the treatment and the control.

to day 3 of the treatment. Then, the level significantly decreased ($p \leq 0.05$) in day 4 of the challenging period (Figure 8C, D; first row). So, it obviously showed that G6PDH in the larvae obtained T_3 at high doses kept steadily, i.e. not much change, at low level during the first few days the fish faced to energy lacking.

In the 30-days old larvae, an extreme increase of the G6PDH was detected from day 1 to day 2 in the control (Figure 8A; second row). Similar elevation of the enzyme level also observed in the larvae exposed to T_3 at concentration of 5 ppm (Figure 8B; second row). The level of G6PDH in the larvae exposed to 20 and 80 ppm of the hormone was not altered significantly during the first 3 days. A significant ($p \leq 0.05$) decrease of the level was detected in day 4 in the 20-ppm T_3 treatment (Figure 8C; second row). While it was continuously unchanged through day 4 in the 80-ppm treatment (Figure 8C, D; second row). Moreover, the level of G6PDH in 80-ppm T_3 treatments existed higher than that found in the 20-ppm T_3 treatment (Figure 3.9C, D; second row).

In comparison to that observed in the 30-days old larvae, the control of the 45-days larvae showed less changes of the level of G6PDH (Figure 8A; third row). A significant decrease of the enzyme occurred on last day of the observation. Different alteration of the enzyme level was found in all the 45-days old larvae exposed to T_3 , comparing to that found in the 15- and 30-days old fish. Steadiness of the enzyme level during the last final days rather than in the first few days was experienced.

In compilation of the results generated in this section, it clearly demonstrated the same necessity of the G6PDH in all ages of the green catfish. Thyroid hormone (T_3), particularly at 20 ppm, increased the level of the enzyme after the catfish obtained the hormone for a short period. However, it was regulated to the lower during the fish were challenged with pressure, i.e. lacking of an energy, for a long period. The thyroid hormone at specific amount increased ability of the animal in down regulating the synthesis of the G6PDH, which is probably a mechanism to redirect the utility of glucose for biosynthesis, e.g. fat, into energy production. Thus, should bring the glucose breakdown to the more effective pathway for an energy production used in prolonging the viability of the fish under the energy exhausted condition.

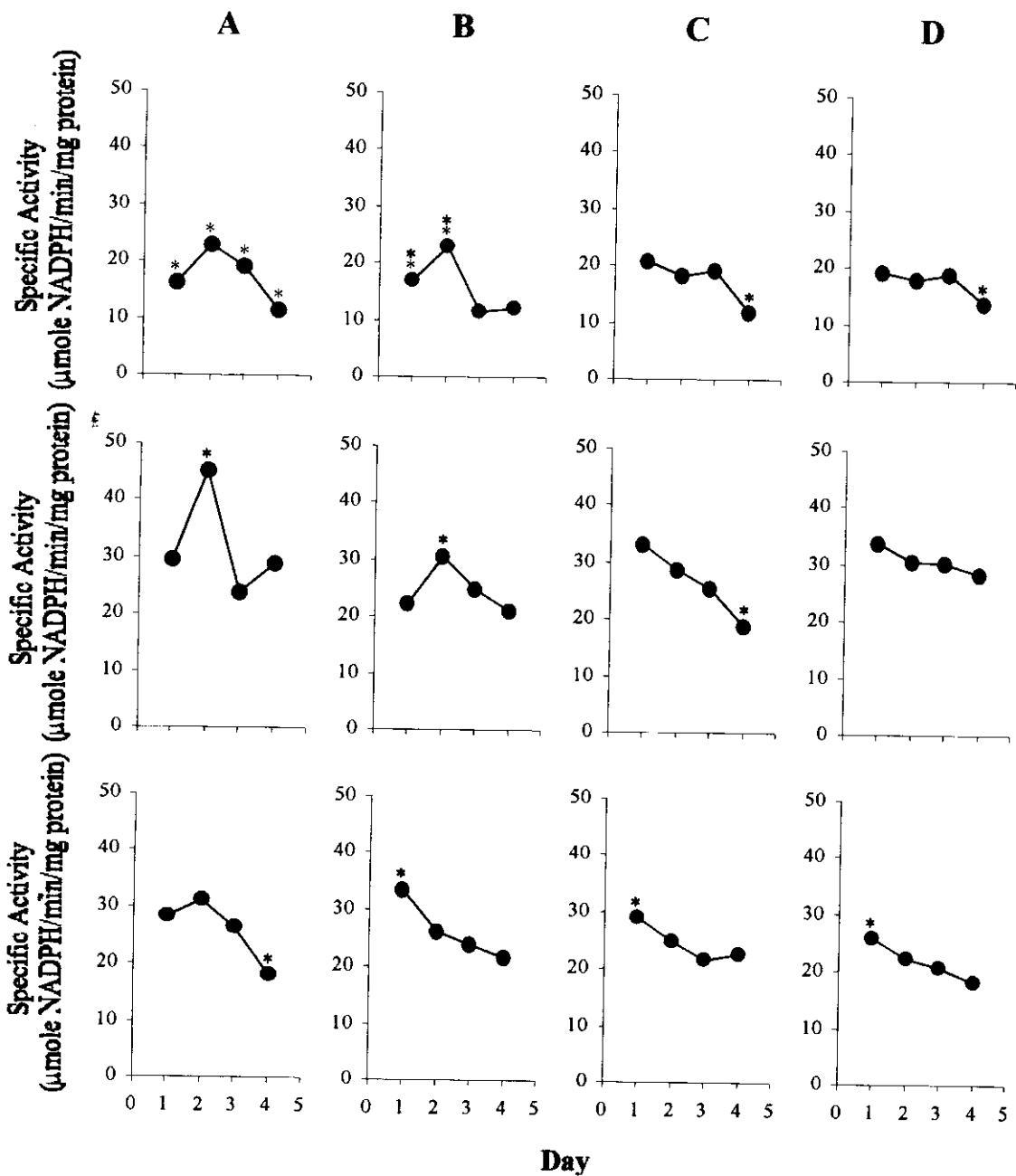


Figure 8 The profile of G6PDH activity alteration. The larvae of 15 (●), 30 (●) and 45 (●) days old were exposed to T₃ for 4 days. Fish were sampling out each day and activity of the enzyme was determined. **A**, **B**, **C**, and **D** indicate column of figures that enzyme activity obtained from the larvae exposed to T₃ at 0, 5, 20, and 80 ppm, respectively. Differences of the enzyme level on each day, within same fish age and T₃ concentration, were considered statistically significant when $p \leq 0.05$ by Newman-Keuls test. Specific activities labeled with green asterisks are significantly different from each other. While specific activity labeled with black asterisk is significantly different from that with unlabeled.

3.2 Effect of T₃ on LDH

LDH is the key enzyme of an anaerobic energy-production of glucose breakdown. This energy production pathway is very important for cells that are sensitive to rapid ATP needs, or the oxygen depletion is experienced. The level of LDH during the growth of the green catfish was shown in Figure 6B. The enzyme level was linear correlated to the age of the fish. Only an obvious decrease of the enzyme occurred at 15 days of age, then a drastic increase of the enzyme immediate occurred since there.

The short-term treatment with T₃ showed different effects on the level of LDH of the larvae with different ages. The enzyme levels in the 3- and 15-days old larvae were not significant changed ($p > 0.05$) from the control at every examined concentrations of the T₃ (Figure 9A, C). The significant increase of LDH was observed in the larvae of 7, 30, and 45 days of age. In the 7-days larvae, the enzyme was significantly higher than that of the control when the fish exposed to 5 and 80 ppm of T₃ (Figure 9B). While T₃ at 5 ppm extremely enhanced the level of LDH found in the 30-days fish, (Figure 9D), and at 5 and 20 ppm of T₃ significantly increased the enzyme levels of the 45-days old catfish (Figure 9E).

An alteration of the LDH profile during the larvae experienced stress condition, i.e. exhausted of energy supply, was shown in Figure 10. For the 15-days catfish, the enzyme in the control group gradually increased from day 2 to 4 (Figure 10A; first row). While, the LDH increased from day 2 to day 3 and, then, remained constant in the larvae treated with T₃ at 5 ppm (Figure 10B; first row). At T₃ of 20 and 80 ppm, level of the enzyme was maintained at approximately 0.3 $\mu\text{mole NADH/min/mg protein}$ throughout the period of being challenged to the pressure (Figure 10C, D; first row).

More steady level of the LDH was remarked in the control larvae with older of age. Only slight increase of the enzyme was observed in day 4 of the 30-days old larvae (Figure 10A; second row). Whereas, the enzyme was entire unchanged in the control of 45-days fish (Figure 10A; third row). The level of LDH was constant in the 30-days larvae exposed to T₃ at almost all concentrations (Figure 10B, C, D; second row). Slightly increased of the enzyme level in day 2, and then decreased to an original level and remained at low level in the following days found only when treated the larvae with 80 ppm of T₃ (Figure 10D; second row). For the 45-days fish, T₃

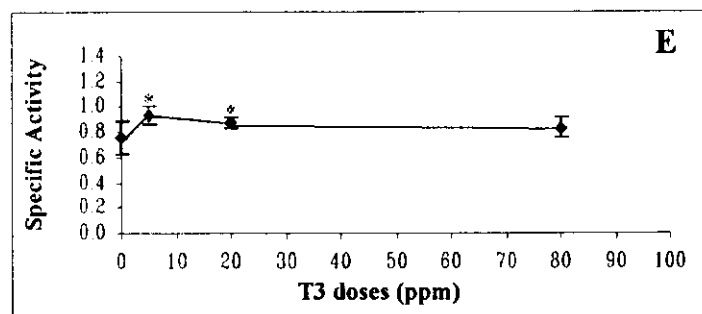
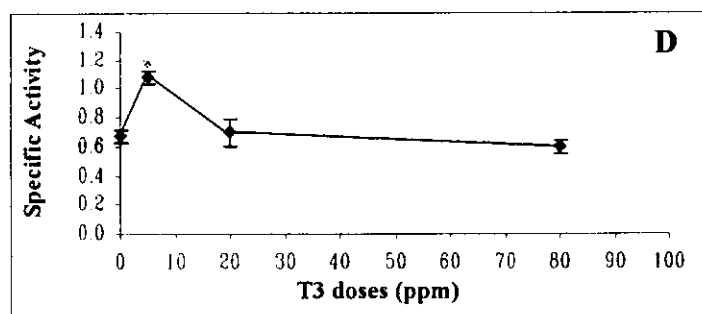
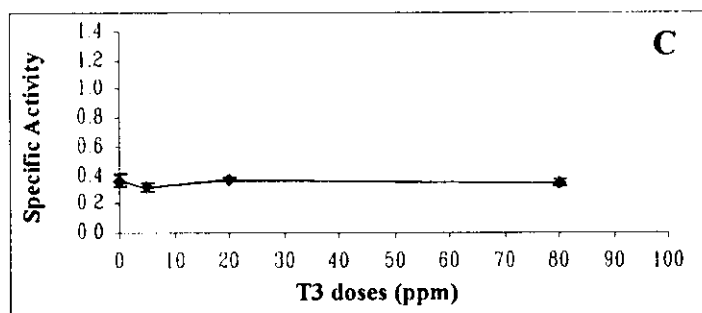
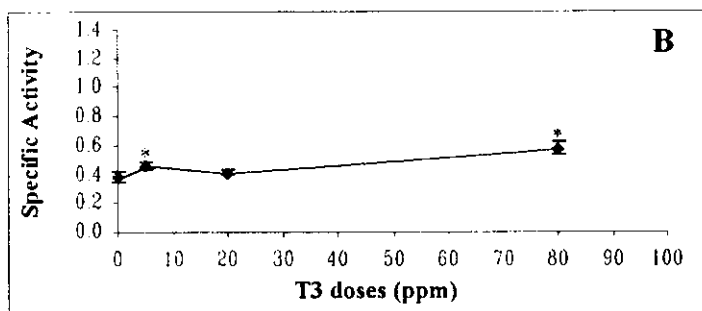
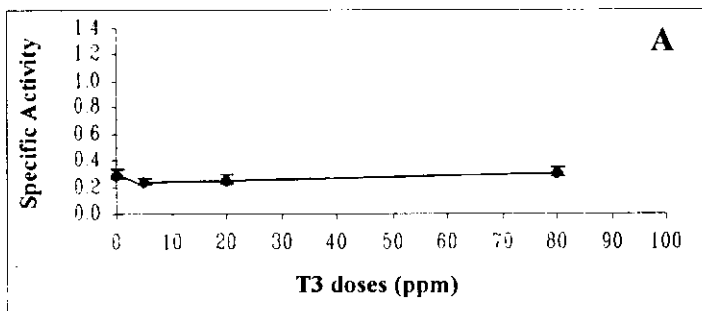


Figure 9 Effect of T₃ on LDH. The catfish larvae with age of 3(A), 7(B), 15(C), 30 (D) and 45 (E) days were reared in water contained 0 (control), 5, 20, and 80 ppm of T₃ for 24 h. The specific activity of LDH was expressed in μ mole of NADH/min/mg protein. Each enzyme assays was performed in triplicate. The vertical bar indicates the standard deviation (SD). An asterisk indicates statistical difference ($p \leq 0.05$), determined by Dunnett test, of the enzyme specific activity between the treatment and the control.

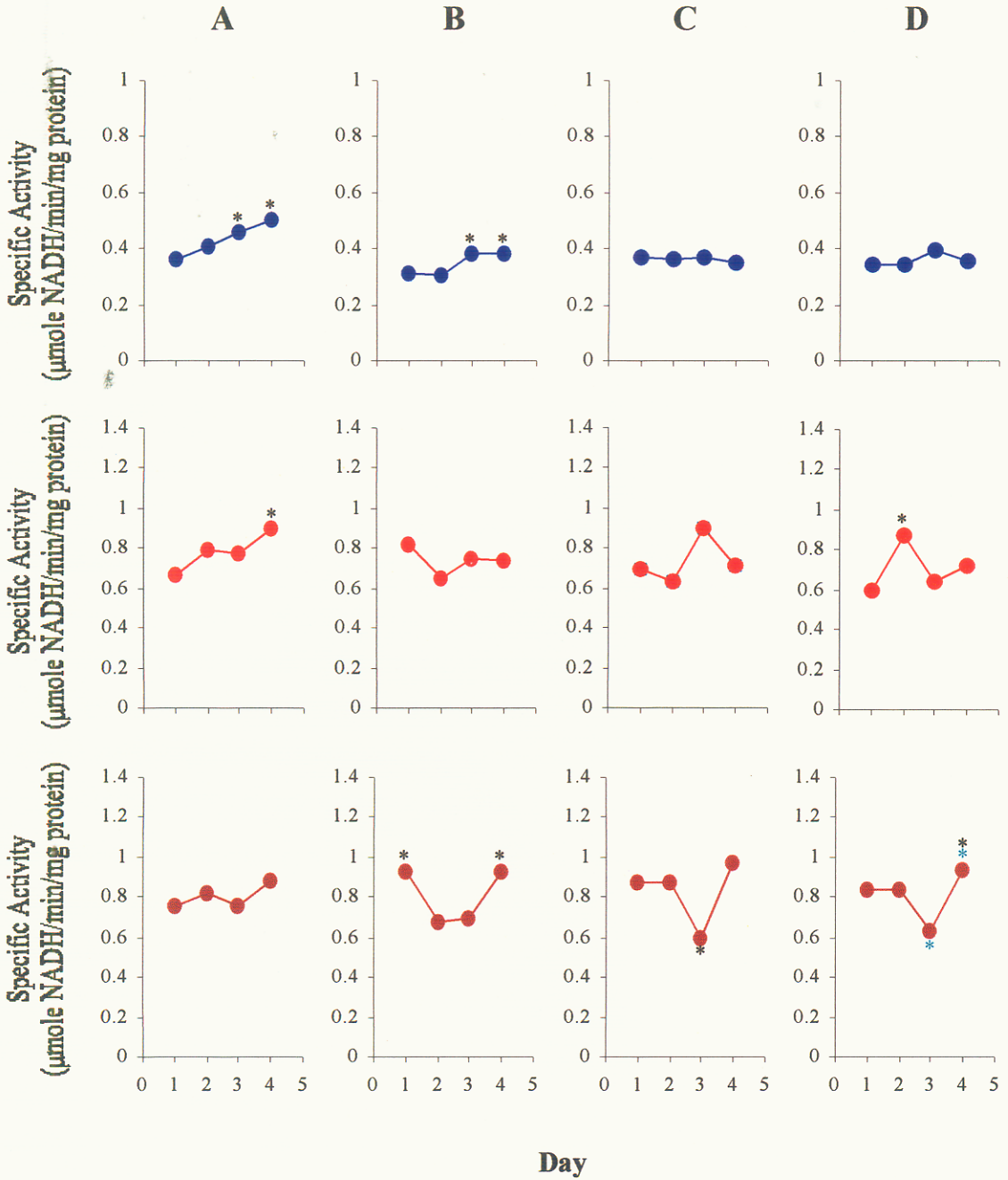


Figure 10

The profile of LDH activity alteration. The larvae of 15 (●), 30 (●) and 45 (●) days old were exposed to 0, 5, 20, and 80 ppm of T₃. Fish were sampling out each day, for 4 days. **A**, **B**, **C**, and **D** indicate column of figures of the enzyme activity obtained from the larvae exposed to T₃ at 0, 5, 20, and 80 ppm, respectively. Differences of the enzyme level on each day, within the same age and T₃ concentration, were considered statistically significant when $p \leq 0.05$ by Newman-Keuls test. Specific activities labeled with green asterisks are significantly different from each other. While specific activity labeled with black asterisk is significantly different from that without labeled.

significantly decreased the level of LDH on day 2 and/or day 3 depending on concentration of the hormone. At 5 ppm of the hormone, the enzyme significantly declined ($p \leq 0.05$) on day 2, unchanged through day 3, then ascend on day 4 to the same level as detected in day 1 (Figure 10B; third row). While at 20 and 80 ppm of T_3 , brief descending of the level occurred in day 3, (Figure 10C, D; third row). On day 4, declining to the previous level and to the slightly higher level of the enzyme were detected in the fish exposed to 20 ppm and 80 ppm of T_3 , respectively.

The data obtained the experiments in this section could indicate the needs of LDH during the growth of the green catfish. The fish with older of age require higher level of the enzyme for their metabolic activities. Thyroid hormone at specific concentrations altered the synthesis level of the enzyme at specific ages when the larvae obtained the hormone for a short period. For example, T_3 at 80 ppm is the most effective dose for the 30-days fish but the hormone at 5 ppm is the most effective dose for the 45-days fish. And, similarly to the regulation of G6PDH, the synthesis of LDH had a tendency to be regulated to a lower or unchanged level during the period of being challenged to pressures. Incorporation with the observation of the survival increase in T_3 treated larvae, it could be conceivable that the lowering or constantly maintaining the level of the LDH is a mechanism influenced by the thyroid hormones to direct the breakdown of the blood glucose to the more effective energy production pathway.

3.3 Effect of T_3 on TG

To determine the expression level of TG during growth of the green catfish, the larvae at 3, 7, 15, 30, and 45 days of age were collected and the activity of the TG was determined as described in Methods. The level of TG of the fish was shown in Figure 6C. The decrease of the enzyme level occurred during growth of the larvae. The level of TG was highest in the larvae at 3 days of age. Thereafter, the level drastically decreased, and was maintain steadily from 7 to 15 days of age. The TG level declined again, with more steep, from 15 to 30 days of age. The level, hitherto, was constant at slightly higher level of TG in the 45-days larvae. Thus, it clearly demonstrated that TG has less function during the early developmental period of the green catfish.

The thyroid hormone exertion on TG when the catfish larvae obtained T₃ for a short period was demonstrated in Figure 11. A significant decrease of the enzyme level was observed in the 3-days old larvae that exposed to T₃ at 5 and 20 ppm (Figure 11A). While that of the T₃ treated 7-days old catfish was significantly lower than the control at all examined concentrations of the hormone (Figure 11B). Similar regulation of the TG by the thyroid hormone was detected in the 15- and 30-days larvae (Figure 11C, D). The decrease of the enzyme occurred at T₃ of 20 and 80 ppm. A considerable increase of the TG level was only observed in the 45 days catfish that received 80 ppm of the thyroid hormone (Figure 11E). Thus, down regulation of the enzyme synthesis might be applicable to all observations in which the larvae were treated with the thyroid hormone for 24 h.

An alteration of the TG level during being challenged to stress was shown in Figure 12. For the larvae at 15 days of age, the enzyme level was unchanged in the control and that exposed to 5 and 80 ppm of the T₃ (Figure 12A, B, D; first row). However, at 20 ppm of T₃, the synthesis of TG was maintained steadily during the first few days at lower amount than that observed in the control (Figure 12C). Slightly but statistically significant increase ($p \leq 0.05$) of the level was detected in the final day of the experiment.

In comparison, the TG in the T₃ treated 30-days old larvae was generally less than in the control (Figure 12; second row). The enzyme in the control gradually decreased from day 2 to day 3, then slightly increased on day 4 (Figure 12A; second row). In the T₃ treated larvae, the TG level was kept constant at low level throughout the challenging period at all examined concentrations of the thyroid hormone (Figure 12B, C, D; second row).

In the 45-days catfish, drastic changes in the synthesis level of TG was observed (Figure 3.13; third row). The enzyme in the control extensively decreased in day 2 and increased in day 3 and day 4 (Figure 12A; third row). When the fish obtained T₃ at 5 ppm, the enzyme level rapidly declined from day 1 through day 3 prior to ascended on day 4 (Figure 12B; third row). The difference of the enzyme synthesis level observed each day was statistically significant by Neuman-Keuls test at $p \leq 0.05$. An extensive ascending of the TG in the larvae that received T₃ at 20 ppm was detected on day 4 of the challenging to an energy exhaust. At 80 ppm of T₃,

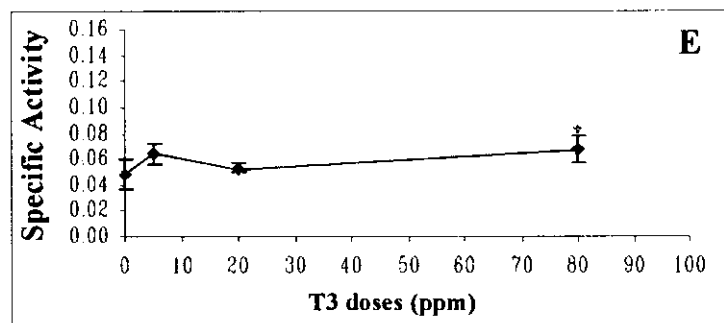
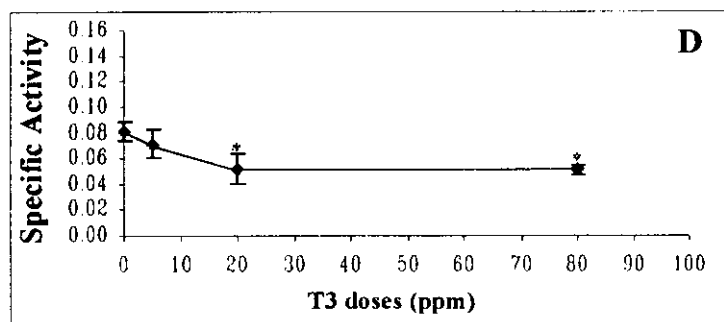
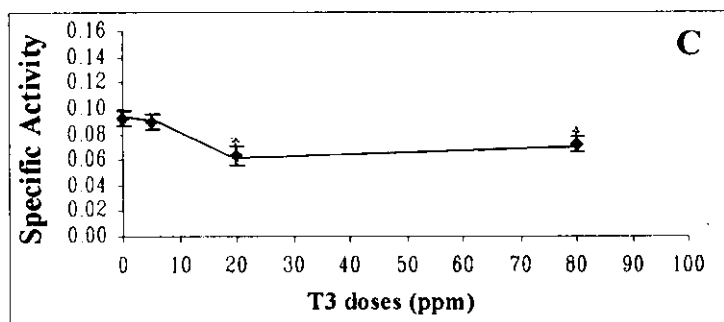
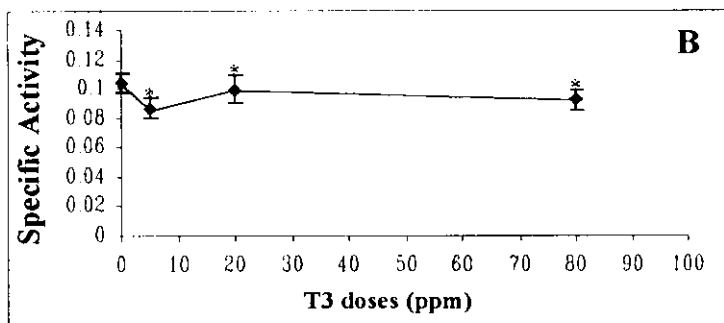
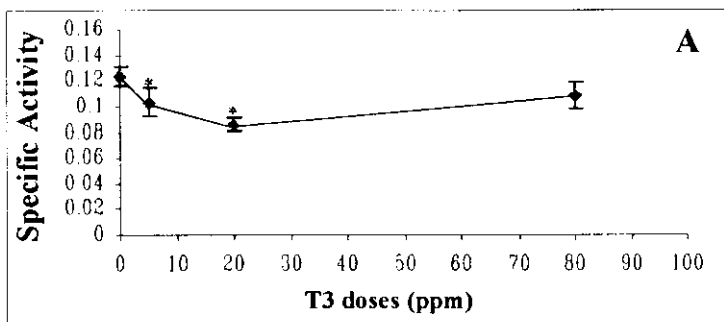


Figure 11 Effect of T₃ on TG. The larvae with age of 3(A), 7(B), 15(C), 30 (D) and 45 (E) days were reared in water contained 0, 5, 20, and 80 ppm of T₃. The specific activity of the enzyme was expressed in μ mole of hydroxamic acid/min/mg protein. Each enzyme assays was performed in triplicate. The vertical bar indicates the standard deviation (SD). An asterisk indicates statistical difference ($p \leq 0.05$) of the enzyme specific activity between the treatment and the control, determined by Dunnett test.

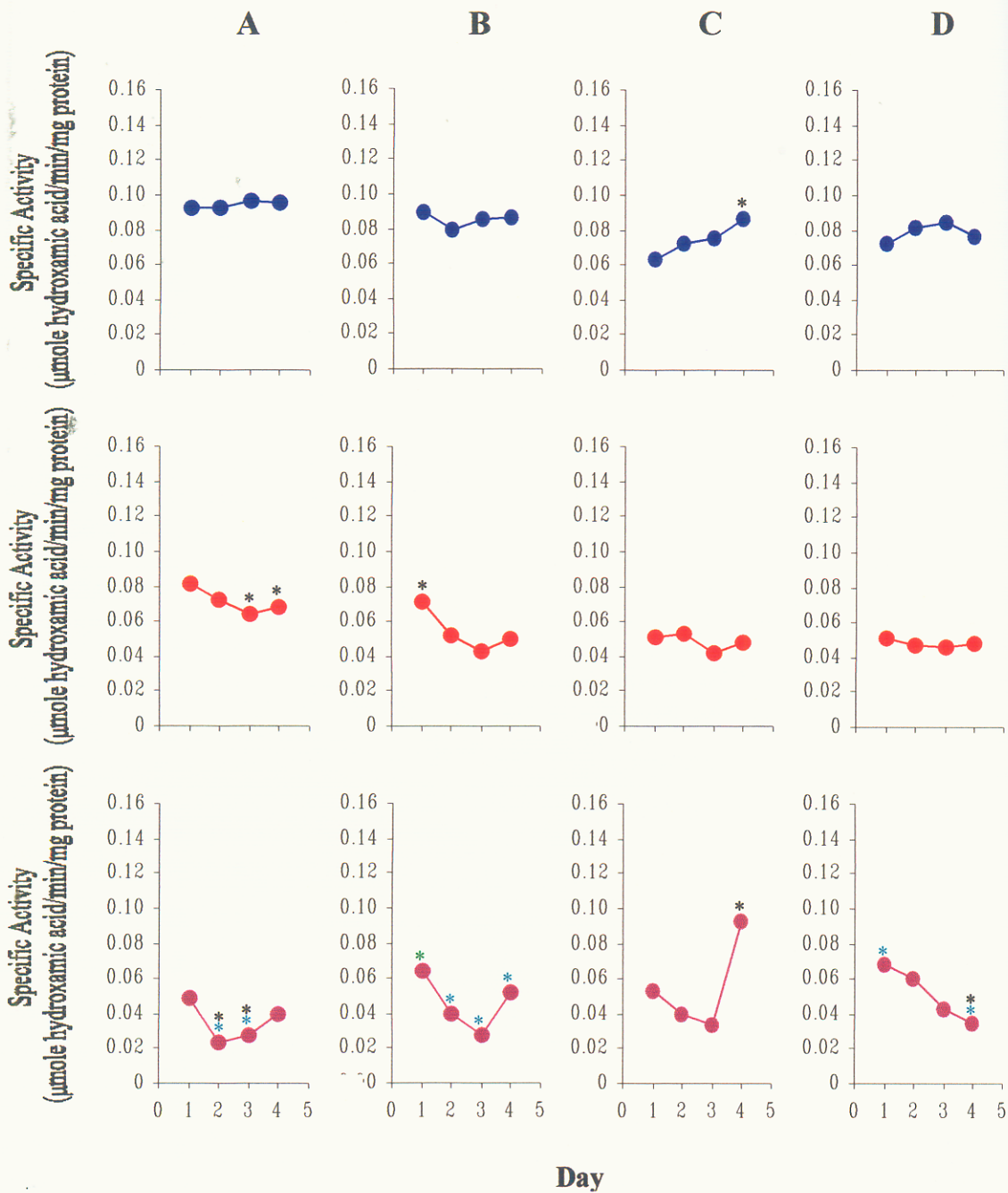


Figure 12

The changes profile of TG specific activity of the catfish exposed to T_3 . The larvae of 15 (●), 30 (●) and 45 (●) days old were exposed to T_3 for 4 days. **A**, **B**, **C**, and **D** indicate column of figures that the enzyme activity obtained from the larvae that exposed to T_3 at 0, 5, 20, and 80 ppm, respectively. Differences of the enzyme level within the same age and T_3 concentration on each day were considered statistically significant when $p \leq 0.05$ by Newman-Keuls test. Enzyme specific activities labeled with green asterisks are significantly different from each other. While the specific activity labeled with the black asterisk is significantly different from that without labeled.

however, persistent decrease of the TG was throughout the observation. The most remarkable reduction was on the day 4 of the treatment (Figure 12D; third row).

Although, the multifunctional enzyme, TG, was widely detected in animal including fish (Yasueda *et al.*, 1994; Sano *et al.*, 1996), the biological function in fish remains unknown. According to the knowledge obtained from studies in human, the gene expression of TG is directly mediated through the retinoic acid receptor (RAR) and its partner, retinoid X receptor. In which the latter is known as a central receptor that plays important role in the actions of the receptor of thyroid hormone (Mangelsdorf *et al.*, 1995). The observations obtained in this section clearly demonstrated that the level of TG was changed at several concentrations of T₃. It could, thus, indicate that thyroid hormone exerts an effect on synthesis of the TG in the green catfish. Although the mechanism of regulation could not be clarified by the results obtained here, the regulation mediated directly on function (nongenomic pathway) or through the competition to RXR between the receptors of thyroid hormone and retinoic acid (genomic pathways) is a possibility.

4. Effect of thyroid hormone regulation on the level of mRNAs

Thyroid hormone exerts a broad range of effects through two modes of actions, the nongenomic or extranuclear (for review see Davis *et al.*, 2002) and the genomic or intranuclear pathways (for review see Yen, 2001). The predominant mechanism, i.e. the genomic action, mediates at the expression of the gene targets by binding to the thyroid hormone receptor (TR) protein that acts in the nucleus as a transactivator. To obtain insight of the mechanism of thyroid hormone actions on metabolism of green catfish, gene expression level of thyroid hormone receptor and other involved in intermediary metabolism including G6PDH, TG, deiodinase was determined after the catfish larvae were treated with T₃. Semi-quantitative Reverse-Transcriptase Polymerase Chain Reaction (semi-quantitative RT-PCR) technique was adopted to quantitate the mRNAs, thus expression changes of the genes in the fish during development of the fish could be pursued.

4.1 Isolation of alpha thyroid hormone receptor (TR α), TG, and deiodinase III cDNA fragments of the green catfish

In order to amplify and quantitate the expression of the genes by RT-PCR, a pair of oligonucleotide primer that definitely recognizes the sequence of the genes is required. Specific primer design is known as a critical step for the success in the determination of the gene expression by RT-PCR. Normally, the specific primers could be synthesized based on an available nucleotide sequence, either full-length or partial fragment, of that gene. Unfortunately, neither nucleotide sequence nor amino acid sequence of TR α , G6PDH, TG and deiodinase III of the green catfish is available in the database. Moreover, even though nucleotide sequences of these genes have been reported in some fish species (Marchand *et al.*, 2001; Yamano *et al.*, 1994), they are quite low in similarity, in particular that of TR α , TG and deiodinase III. Thus, isolation of the genes was attempted in the green catfish to reveal the nucleotide sequences and use them for the specific primer design.

An amplification of fragment of the cDNAs from total RNA by 2 steps RT-PCR was first performed using a pair of degenerate primers, which designed according to the conserved regions of the genes reported in other vertebrates, in particular fish, species. The Consensus Degenerate Hybrid Oligonucleotide Primers program developed by Rose *et al.* (1998) was used to facilitate the generating of the primers. An identity of the translated amino acid sequence of the fragments was explored among those available in the GenBank databases using the BLAST algorithm (Altschul *et al.*, 1990, 1997). Then, the sequences were used in generating the new sets of primer for longer sequences by 5' and 3'RACE reaction.

The nucleotide together with its deduced amino acid sequence of the green catfish TR α , TG and deiodinase III cDNA fragments are given in Figure 13-15. By using Clustal W algorithm (Thomson *et al.*, 1994), the alignment of amino acid sequences of the genes against those of some teleosts and vertebrates was performed and identity (%) of the amino sequence was calculated as shown in Table 2.

4.2 The gene expression of TR α during growth of the green catfish larvae

To determine expression level of the TR α gene, a pair of specific primers was designed and used in the RT-PCR. The amount of the gene was then determined by

CGTATAACCTGCGAGGGCTGCAAGGGTTTCTTCCGGCGCACCATCCAGAAAAACCTGCAC	60
R I T C E G C K G F F R R T I Q K N L H	
CCGTCGTATTCTCTGCTAGTACGATGGCTGCTGCATCCTCGACAAGATCACACGCAACCAG	120
P S Y S C - Y D G C C I L D K I T R N Q	
TGCCAGCTGTGCCGCTTCAAGAAATGCATCGCAGTGGGCATGGCCCTGGACTTGGTTCTG	240
C Q L C R F K K C I A V G M A L D L V L	
GACGAGGCGAAGCGAGTGGCCAAGCGACGTCTCCTCGAGGAGAACCGGGAGAAGAGGAAG	300
D E A K R V A K R R L L E E N R E K R K	
AAGGAGGAGATGGTGAAGACGCTGCAGAACCGGCCGAGCCACGGGTACCGAATGGGAG	360
K E E M V K T L Q N R P E P T G T E W E	
CTGATCCGCATCGTGACCGAGGCTCACCGCCACACTAACGCCAGGGCTCACACTGGAAA	420
L I R I V T E A H R H T N A Q G S H W K	
CAGAAACGCAAGTTCTGCTGAAGACATTGGTCAGTCGCCTGTAGCCCCACGTCAGAC	480
Q K R K F L P E D I G Q S P V A P T S D	
GGGATAAAGTCGACCTGGAAGCCTTCAGCGAGTTTACCAAGATCATCACGCCAGCCATC	540
G D K V D L E A F S E F T <u>K I I T P A I</u>	
ACCCGCGTCGTCGACTTTGCCAAAAAAGTGCATGTTTTTCAGAGCTGCCTTGTGAGGAT	600
<u>T R V V D F A K K L P M F S E L P C E D</u>	
CAGATCATCCTGCTGAAGGGCTGCTGTATGGAAATAATGTCCTCGCGG	648
<u>Q I I L L K G C C M E I M S L R</u>	

Figure 13 Nucleotide and its deduced amino acid sequence of partial fragment of the green catfish thyroid hormone receptor alpha mRNA. The amino acid sequence derived from the cDNA sequence is placed underneath and written using the standard single-letter amino acid abbreviation. Nucleotide and amino acid numbers are at right. Ligand-binding domain (LBD) found in all TR(α) is underlined.

ATGGTGAACAGTAATGACAGAGATAATGGGGTACTGGAAGGCTGCTGGAATAAAAGTTTT	60
M V N S N D R D N G V L E G C W N K S F	
GAAGGTGGAATCAGTCCCATGGCATGGAAAGGGAGTGTGGAATCCTCAAAACTGGAGT	120
E G G I S P M A W K G S V E I L K T W S	
TCCGCTTCCTGCCTGCCGGTCCGCTATGGCCAGTGCTGGGTGTTTGCTGCTGTTGCCTGC	240
S A S C L P V R Y G Q C W V F A A V A C	
ACTGTGTCTCGAGCGCTAGGTATCCCCTGCAAAGTGGTGACTAACTACCTCTCGGCCCAT	300
T V S R A L G I P C K V V T N Y L S A H	
GACACCAACAGCAACATGGTGATTGAGCGCTATGTGGATGAAAATGGCAAGCTGCTGAAC	360
D T N S N M V I E R Y V D E N G K L L N	
ACCAGCAGAGACATGATCT	379
T S R D M I	

Figure 14 Nucleotide and its deduced amino acid sequence of partial fragment of the green catfish TG mRNA. The amino acid sequence derived from the cDNA sequence is placed underneath and written using the standard single-letter amino acid abbreviation. Nucleotide and amino acid numbers are at right.

AAAAACGCGCTGGTGTCCCTGATGCTGCTGCCGCGCTTTCTGTTGGCCGCGGTGTTGCTC	60
K N A L V S L M L L P R F L L A A V L L	
TGGCTTCTCGACTTGTCTGCATCCGGAGGAAGGTGCTGGTGAAAATGCGCGAACAGGAG	120
W L L D F V C I R R K V L V K M R E Q E	
GGCTCGACTCCCGACGACCCGCCGGTGACCGTGTCAAACCGAACCGGATGTTTCAGCTG	180
G S T P D D P P V T V S N S N R M F T L	
GACTCACTGCGCGCTGTGTGGTACAGCCAGAAGTTGGACTTCTGTAAGGCAGCGCACCTG	240
D S L R A V W Y S Q K L D F C K A A H L	
GGGCTGGCAGCGCCTAACAGCGAGGTTATGCCGCTCGGCCAACGAAACGCGCGGTATA	300
G L A A P N S E V M P L G E R K R A R I	
CTGGACTACGCCTGCGGCGGAGACCGTTCATCCTTAACCTCGGCAGCTGCTCATGACCG	360
L D Y A C G A R P F I L N F G S C S *	
CCGTTTCATGACGCGCTTGGCCGCGTTCGGGCGCGTTGCTGACCAGTACGCCGACATCGCC	420
GACTCGTTGCTCGTATACATCGAG	444

Figure 15 Nucleotide and its deduced amino acid sequence of partial fragment of the green catfish deiodinase III mRNA. The amino acid sequence derived from the cDNA sequence is placed underneath and written using the standard single-letter amino acid abbreviation. Nucleotide and amino acid numbers are at right.

Table 2 The identity comparison (%) of deduced amino acid sequence of the green catfish TR α , TG, and deiodinase III to those of other vertebrates.

Vertebrate specie	Green catfish		
	TR α	TG	Deiodinase III
Human	84	49	50
Mouse	84	50	50
Chicken	86	64	60
Frog	88	56	55
Fishes	90-91	69-77	68

relatively normalizing to the amount of the beta-actin gene, which is a house keeping and T₃-nonresponsive gene.

The whole body of the 3-, 7-, 15-, 30-, and 45-days larvae was extracted for the total RNAs. The amplification of the gene was carried out for 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The amount of the TR α was compared with that of beta-actin. Change in the level of the TR α mRNA was shown in Figure 16. The receptor gradually decreased from 3 days to 15 days after hatch. Thereafter, it rapidly increased and kept steady at the same level as found in the 3-days larvae when the fish was 30 to 45 days of age. Since the higher level of the receptor, the more capable of responding to thyroid hormone of the fish. It suggested that thyroid hormone exerts an important role in the catfish larvae at two stages, i.e. after newly hatch and during development. Moreover, numerous investigations have implicated the involvement of thyroid hormone in developmental and differentiation processes during the growth of fishes (see Jones *et al.*, 2002; Yamano and Miwa, 1998 for examples). It thus could be conceivable that thyroid hormones also exert their actions on development and organogenesis of the green catfish via TR.

4.3 Changes in the expression level of the TR α , G6PDH, TG and deiodinase III during actions of thyroid hormone

The 15-, 30-, and 45-days catfish larvae were treated with T₃ for 15 days, then the amount of TR α , G6PDH, TG and deiodinase III mRNAs was analyzed. Changes of the levels were shown in Figure 16. For TR α , it clearly showed that T₃ at 80 ppm increased the gene expression in all larvae ages (Figure 16A, B, C). In 15-days larvae, the 20 ppm of T₃, interestingly, decreased amount of the TR. While the gene level was little and no significant changes in 30- and 45-days fish, respectively, at the same dose of the hormone used (Figure 16B, C). However, the T₃ at 5 ppm showed only slightly effect on the TR gene of the 30-days larvae, but not to other ages.

For an alteration of the mRNA level of deiodinaseIII, T₃ supplementation at all examined doses significant increased the synthesis of the enzyme in 15- and 30-days catfish larvae (Figure 16A, B). However, only 20 ppm and 80 ppm of the hormone modulated the enzyme synthesis level in the 45-days fish (Figure 16C). Since deiodinase III has only inner ring deiodination (IRD) activity with preference for T₃ as the substrate and be involved in converting T₄ and T₃ to rT₃ and T₂. The result, thus, indicated higher responsiveness to T₃ of the younger fish.

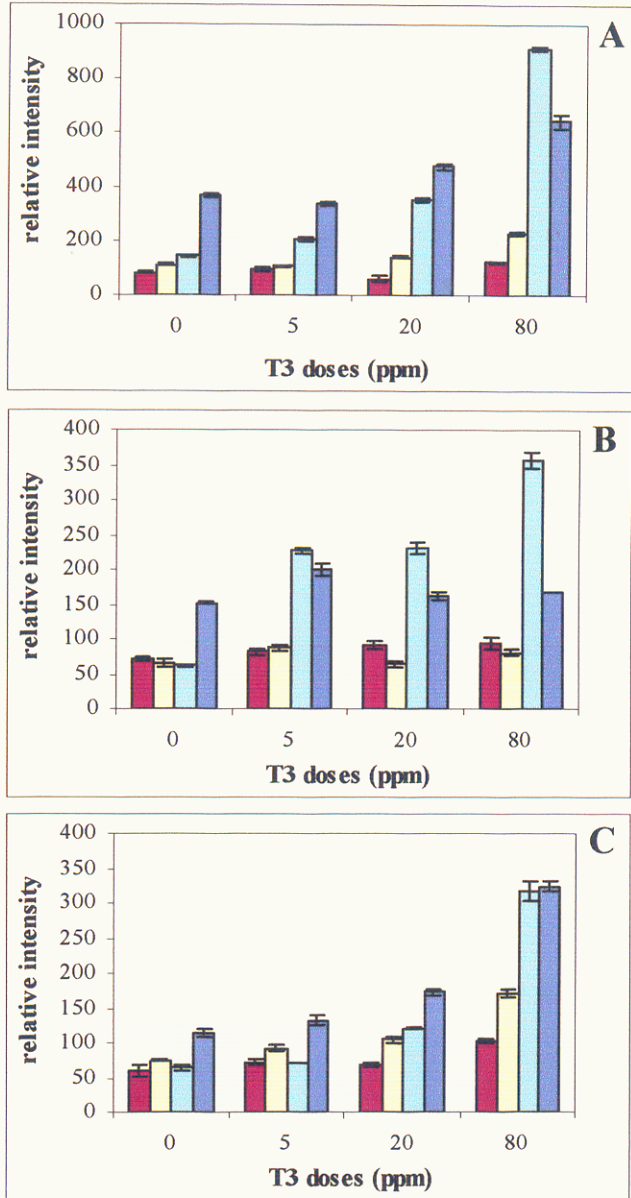


Figure 16

The effect of T₃ on gene expression of the green catfish. The catfish larvae at the age of 15 days (A), 30 days (B), and 45 days (C) were exposed to T₃ for 15 days prior to the amount of mRNAs of TR α (■), TG (□), Deiodinase III (□), and G6PDH (■) were determined and relatively compared to the beta-actin gene by semi-quantitative RT-PCR. Vertical bar indicates the standard deviation (SD).

The mRNA synthesis of G6PDH was significant increased when 15- and 45-days larvae obtained T₃ at 20 and 80 ppm (Figure 16A, C). The increase of the mRNA in 30-days fish was detected at only 5 ppm of the hormone (Figure 16B). G6PDH is known as the rate limiting step key enzyme of pentose phosphate pathway, and many reports showed both positive and negative response to thyroid hormone of the enzyme. The result obtained in this project showed that T₃ positively modulated the expression of G6PDH gene in the green catfish.

Increase of the TG mRNA can be observed in the catfish larvae that obtained T₃. All of the examined T₃ doses significant increased the synthesis level of the enzyme in 45-days fish (Figure 16C). T₃ at 20 and 80 ppm showed significant increase of the TG in the 15-days larvae (Figure 16A), and that of 5 and 80 ppm significant increased the enzyme level in the 30-days fish (Figure 16B). Although it could not definite conclude how thyroid hormone effects on the expression of TG, it here first showed an alteration of the TG expression by the thyroid hormone action.