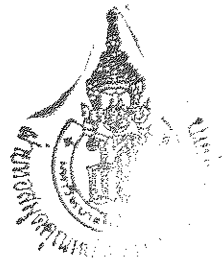

Effects of Thyroid Hormones on Early Development of Green Catfish (*Mystus nemurus*)

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ชื่อวิทยานิพนธ์	ผลของฮอร์โมนต่อม
	ปลากดเหลือง (*Mys*
ผู้เขียน	นางสาวลัดดา ลีละ
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บทค

เป็นที่ทราบกันดีกว่าฮอร์โมนต่อมไธร
พัฒนาการของสัตว์มีกระดูกสันหลังชนิดต่างๆ วทย
ของฮอร์โมนดังกล่าวในปลากดเหลือง (*Mystus ne*
อัตราการรอดตายของลูกปลา ระดับของเอนไซม์หลัก
glucose-6-phosphate dehydrogenase (G6PDH)
จากนี้ยังได้ทำการศึกษาความสัมพันธ์ระหว่างฮอร์โ
พิจารณาจากการเปลี่ยนแปลงของระดับเอนไซม์ tran
ฤทธิ์ในนิวเคลียสของฮอร์โมนโดยอาศัย thyroid horm

ผลการศึกษาพบว่าฮอร์โมนต่อมไธรอยด์ทั้ง
6.25 ppm ถึง 300 ppm และระยะเวลาที่ไข่ปลาได้
เป็นตัวอ่อนของไข่ปลากดเหลือง และพบว่าลูกปลา
ช่วงระยะสั้น มีอัตราการรอดที่แตกต่างกัน ขึ้นอยู่กั
อัตราการรอดอย่างมีนัยสำคัญทางสถิติในลูกปลาอายุ
ฮอร์โมนเป็นช่วงระยะเวลานาน มีอัตราการรอดที่เพิ่
ของฮอร์โมนที่ออกฤทธิ์กับอายุของลูกปลาเป็นแบบผ

ฮอร์โมนมีผลต่อระดับการสร้างเอนไซม์ G6P
การศึกษามีการเปลี่ยนแปลงระดับเอนไซม์ทั้งสองชนิ
ระยะเวลาสั้น ส่วนลูกปลาที่ได้รับฮอร์โมนเป็นช่วง
ชนิดลดลงอย่างต่อเนื่อง โดยอาจจะทำให้กลูโคสใน
มีประสิทธิภาพมากขึ้น ส่งผลให้ลูกปลาสามารถดำรง
เอนไซม์ TG ที่เกิดขึ้นหลังจากที่ลูกปลาได้รับฮอร์โม

ของฮอร์โมนต่อมไธรอยด์ต่อวิถีการทำงานของวิตามินเอ ซึ่งอาจเกิดขึ้นแบบ genomic คืออาศัย thyroid hormone receptor หรือ แบบ non-genomic

นอกจากนี้ยังทำการแยกและศึกษาลำดับเบสของชิ้นส่วน thyroid hormone receptor ชนิดแอลฟา (TRα) พบว่าระดับการแสดงออกของยีน TRα มีการเปลี่ยนแปลงในระหว่างการพัฒนาของลูกปลากดเหลือง รวมทั้งระหว่างที่ปลาได้รับฮอร์โมนต่อมไธรอยด์

Thesis Title	Effects of Thyroid Hormones on Early Development of Green Catfish (*Mystus nemurus*)
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Abstract

It is well known to date that thyroid hormones play an important role on growth and early development of vertebrates. In this thesis, the green catfish (*Mystus nemurus*) was investigated for the effect of thyroid hormones on hatching, survival, and two key enzymes of the carbohydrate metabolism, i.e. glucose-6-phosphate dehydrogenase (G6PDH) and lactate dehydrogenase (LDH). Intercommunication between thyroid hormone and retinoic acid was observed via an expression level of transglutaminase (TG). In addition, the hormone action mediated via thyroid hormone receptor (TR) was also determined through the expression level of the receptor.

Thyroid hormones in both T_4 and T_3 forms at concentrations of 6.25 to 300 ppm did not show effect on hatchability of the catfish. Neither short nor long exposure changed the hatchability of the fish eggs. Effect of short-term treatment of T_3 on survival of the green catfish varied according to the age of larvae. Significant increases of survival were detected in the larvae of 15, 30, and 45 days of age. Reverse correlation of thyroid hormone concentration and age of fish was found in the long-term treatment.

The G6PDH and LDH expressions were affected by the thyroid hormone action. Immediate responses occurred in most ages of green catfish larvae, and at particular concentrations of thyroid hormone. Increase of the capability of the larvae in lowering the expression level of the two key enzymes of carbohydrate metabolism was demonstrated in the long-term treatment. Lengthening the larvae viability by redirection of the glucose breakdown by thyroid hormone to the more effective pathway of energy production was suggested. The possible effect of thyroid hormone

on retinoid pathway was demonstrated by an alteration of TG level, Non-genomic effect of the hormone on TG was also suggested.

Finally, a partial fragment of thyroid hormone receptor α–isoform was successfully cloned and its sequence was analyzed. The expression level of the TRα varied during the growth of green catfish. The receptor level was affected by thyroid hormone with a typical manner.

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Contents

	pages
Abstract (Thai)	(3)
Abstract (English)	(5)
Acknowledgement	(7)
Content	(8)
List of tables	(9)
List of figures	(10)
List of abbreviations	(12)
Chapter	
1. Introduction	
Introduction	1
Review of literature	3
Objective	27
2. Materials and methods	
Materials	28
Methods	31
3. Results	41
4. Discussion	79
5. Conclusion	86
References	88
Appendix	102
Vitae	118

List of Tables

Table		pages
4.1	Comparison of identity (%) of nucleotide sequence and deduced amino acid sequences of green catfish TRα	73
6.1	The alteration of G6PDH, LDH and TG activity during growth of the green catfish	108
6.2	Effect of short-term treatment with different doses of T_3 on G6PDH activity in fish of each age	109
6.3	Changes of G6PDH specific activity of green catfish at 15, 30 and 45 days of age exposed to T_3 at different concentrations for 4 days	110
6.4	Effect of short-term treatment with different doses of T_3 on LDH activity in fish of each age	111
6.5	Changes of LDH specific activity of green catfish at 15, 30 and 45 days of age exposed to T_3 at different concentrations for 4 days	112
6.6	Effect of short-term treatment with different doses of T_3 on TG activity in fish of each age	113
6.7	Changes of TG specific activity of green catfish at 15, 30 and 45 days of age exposed to T_3 at different concentrations for 4 days	114
6.8	Changes in TRα gene expression level during development of the green catfish	115
6.9	Effect of short-term treatment with T_3 on the expression level of TRα of green catfish larvae at 3, 7 and 15 days of age	115
6.10	Effect of long-term treatment with T_3 on the expression level of TRα of green catfish larvae at 30 and 45 days of age	116

List of figures

Figure		pages
1.1	Structure of thyroid hormones	4
1.2	The negative feedback control of thyroid hormone synthesis and secretion	5
1.3	Comparison of amino acid homologies and their function properties among TR isoforms	12
1.4	Action of thyroid hormone in cell nuclei	15
1.5	Multiple sequence alignment of TR from mammals, amphibians and teleost fish	23
3.1	Effect of T_3 doses and exposure time on hatching percentage of the green catfish	42
3.2	Effect of T_3 doses on hatching (%) of the green catfish	44
3.3	Effect of T_4 on hatching of green catfish	46
3.4	Effect of DMSO and methanol on survival (%) of green catfish	48
3.5	The doses effect of T_3 on survival of green catfish	49
3.6	Effect of thyroid hormone on survival of the catfish	52
3.7	Specific activity profile of Glucose-6-phosphate dehydrogenase, Lactate dehydrogenase and Transglutaminase during development of green catfish	55
3.8	Effect of T_3 on G6PDH	56
3.9	Profile of dynamic change of G6PDH activity	58
3.10	Effect of T_3 on LDH	61
3.11	Profile of dynamic change of LDH activity	62
3.12	Effect of T_3 on TG	65

List of figures (continue)

Figure		pages
3.13	Dynamic change of transglutaminase activity of catfish exposed to T_3	67
3.14	Partial nucleotide sequence and its deduced amino acids of green catfish thyroid hormone receptor alpha isoform (TRα)	71
3.15	Comparison of amino acid sequence of green catfish thyroid hormone receptor fragment to those from the other fish and vertebrate species	72
3.16	Change in the level of TRα genes during development of green catfish	75
3.17	Effect of T_3 on the expression level of TRα gene	78
6.1	Formaldehyde agarose gel of total RNA isolated from whole body of green catfish larvae age of 45 days	106
6.2	Small hatchery set for the hatching study	107
6.3	RT-PCR analysis of TRα mRNA	117

List of Abbreviations

AMV	=	avian myeloblastic virus
bp	=	base pair
DMSO	=	dimethyl sulfoxide
DNA	=	deoxyribonucleic acid
DNase	=	deoxynucleotidase
dNTP	=	deoxynucleotide triphosphate
EDTA	=	ethylenediaminetetraacetate
G6PDH	=	Glucose-6-Phosphate Dehydrogenase
h	=	hour
LDH	=	Lactate Dehydrogenase
$MgCl_2$	=	magnesium chloride
mM	=	millimolar
mg	=	milligram
ml	=	millilitre
μmole	=	micromole
NADH	=	reduce nicotinamide adenine dinucleotide
NADPH	=	reduced nicotinamide adenine dinucleotide phosphate
OD.	=	optical density
°C	=	degree Celcius
PCR	=	polymerase chain reaction
ppm	=	part per million
RAR	=	retinoic receptor
RNase	=	ribonuclease
rpm	=	revolutions per minute
RT-PCR	=	reverse transcriptase polymerase chain reaction
RXR	=	retinoid X receptor
T_2	=	3,3′ diiodothyronine
T_3	=	3, 5, 3′ triiodothyronine
T_4	=	3, 5, 3′, 5′ tetraiodothyronine (Thyroxine)

List of Abbreviations (continue)

TAE	=	Tris-acetate-EDTA buffer
TG	=	Transglutaminase
TR	=	thyroid hormone receptor
TREs	=	thyroid hormone responsive elements

Chapter 1

INTRODUCTION

Introduction

Thyroid hormones have a wide range of biological effect on numerous vertebrate species including mammalians (for reviews see Bernal and Nunez, 1995, Bettendorf, 2002), avians (for review see McNabb, 1989), amphibians (for reviews see Tata, 1999, Shi *et al.,* 2001), and fish (for review see Power *et al.*, 2001). The major role involves growth and developmental regulations of young animals, as well as regulation of the basal metabolism of macromolecules, such as carbohydrate and lipid (Brent, 1994), in adults. Thyroid hormones predominantly elicit their action via binding to a specific nuclear receptor named thyroid hormone receptor (TR) which is a member of the nuclear receptor superfamily.

According to its role on numerous aspects of vertebrate development and metabolism, investigations to reveal the functions of thyroid hormone have been emphasized in particular the higher vertebrate species. In the lower vertebrates, particularly in fishes, studies have been attempted to reveal the biological role of the thyroid hormones in these species. These include the observations on changes of the thyroid hormone level during developmental stages (Tanaka *et al.*, 1995), effects of thyroid hormones administration on growth and development of fish, and the influence of the hormones on metabolism. The role of thyroid hormones have been demonstrated in some teleosts including carp (Lam and Shama, 1985), tilapia (Lam, 1980; Nacario, 1983), and zebrafish (Brown, 1997). Carbohydrates and lipids are the macromolecules in fish that their metabolisms has been reported being regulated by thyroid hormones (Tripathi and Verma, 2003). In addition, several efforts have been attempted on thyroid hormone receptor (TR) in order to elucidate the mechanism of thyroid hormone actions. Thyroid hormone receptors from several fish species, including Japanese flounder (Yamano *et al.*, 1994), zebrafish (*Danio rerio*)(Essner *et al.*, 1997), Atlantic salmon (*Salmo salar*)(Marchan *et al.*, 2001; Jone *et al.*, 2002),

have been cloned and sequenced. Moreover, subtypes of TR and distribution of its mRNA in various tissues during development have been reported in fish including Japanese flounder (Yamano and Miwa, 1998), rainbow trout (Jones *et al.*, 2002), zebrafish (Essner *et al.*, 1997, 1999), and seabream (Nowell *et al.*, 2001). However, in comparison with the knowledge in mammals, the effect of the thyroid hormone, in particular that on the metabolic pathways, has been little known in fish.

The *Mytus nemurus* or green catfish or yellow catfish (Suvatti, 1950) is one of the most important freshwater fish cultured in Thailand and other countries in Southeast Asia. Mass production of the catfish juveniles was achieved by artificial breeding (Amatyakul *et al.*, 1995), and hatchery for fries distribution throughout the country (Leesa-Nga, *et al.*, 2000). Because of the fish's palatability and high nutritive values, the business farming is preferred and the consumer demand is relatively high (personal communications). As the return of the investment in catfish farming is engaged in the production outcome, several efforts thus have been accomplished in order to seek for the optimal growth and survival of the juveniles. These include reproductive traits (Khan *et al.*, 1990; Hardjamulia and Suhenda, 2000), digestive process and capacity (Kamarudin *et al.*, 2001), feeding behavior (Amornsakun *et al.*, 1997, 1998), types of diet (Khan *et al.*, 1993; Eguia *et al.*, 2000), and the effect of stocking density (Khan, 1994). However, the outcome of the fishery is still inconsistent. The mortality rate is still high, in particular during the first week after hatch (Amatyakul *et al.*, 1995; personal communication with the Songkhla Inland Fisheries Station).

Thus, based on the documentation of hatchability and survival enhancement in fish species, efforts were attempted in this thesis to improve the hatching rate and survival of juvenile *M. nemurus* by using thyroid hormones. In addition, more attempts were contributed to understand the effect of thyroid hormone on metabolism during early development, as well as the molecular mechanism of the thyroid hormone actions, in this catfish.

Review of literature

1. Thyroid hormone structure, synthesis, and secretion

Thyroid hormones are the amino acid derivative molecules secreted from thyroid gland which is characteristic in each vertebrate species. In mammals, the thyroid gland is composed of two lobes connected by an isthmus across the ventral surface of the trachea. Whereas in lower vertebrates such as fish, no organized gland is formed or developed. The thyroid synthesizing cells are singled out in a small groups in the loose connective tissue of the pharynx (for review see Power *et al.*, 2000).

The basic molecular structure of thyroid hormone can be divided into three regions: a hydrophilic 4′-hydroxyl group, two hydrophobic phenyl rings linked together by an ether bond, and an amino acid side chain on the tyrosine ring. Iodide substitutions to hydrogen atoms on the phenyl rings generate several forms of thyroid hormone include L-3,5,3′,5′-tetraiodothyronine (L-thyroxine, T_4) and L-3,5,3′-triiodothyronine (L-triiodothyronine, T_3) (Figure 1.1)

The synthesis and secretion of thyroid hormones are regulated by a negative-feed back of the hypothalamus, pituitary and thyroid gland network. At the beginning, thyroid-releasing hormone (TRH) is secreted by hypothalamic neuron and transported to the anterior pituitary where the receptor for TRH is located. The thyroid stimulating hormone (TSH) is secreted thereafter to regulate the thyroid gland in synthesis and secretion of thyroid hormones (for review see Yen, 2001). High level of thyroid hormones in the bloodstream, inversely, inhibits the secretion of TRH and, then, TSH (Figure 1.2).

Synthesis of thyroid hormones in the thyroid gland is located in the highly vascularized spheroidal follicles, consisting of a layer of epithelial cells surrounding a lumen filled with proteinaceous colloid. The thyroid hormones requires iodide ion (I^-) and an iodoperoxidase (or thyroid peroxidase). Iodides are trapped from the bloodstream, then iodinated and coupled by iodoperoxidase to tyrosine residues in a protein called thyroglobulin to form the iodinated thyroglobulin (Ohmiya *et al.*, 1990;

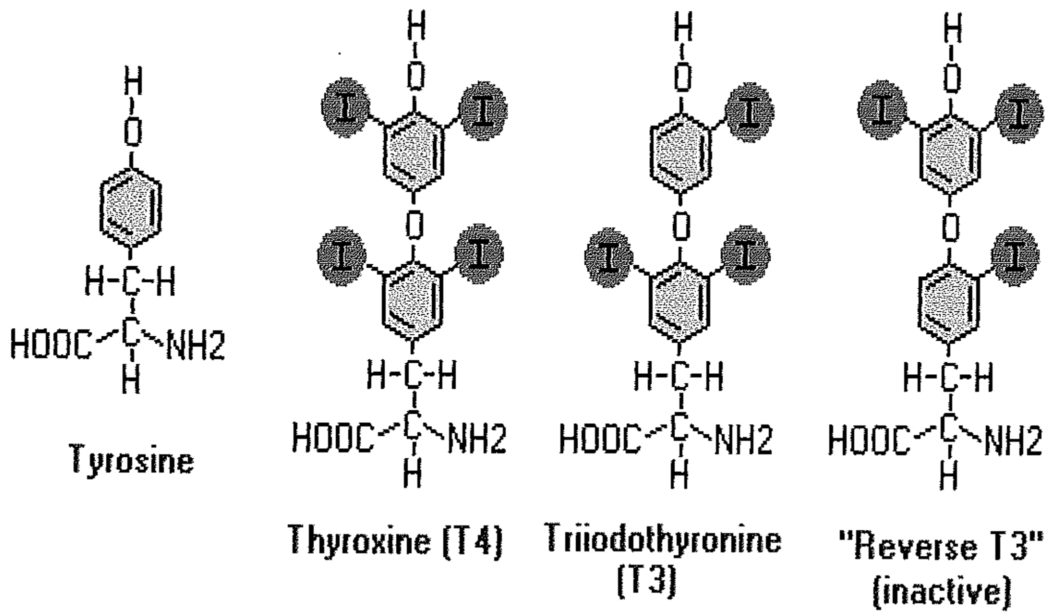

Figure 1.1 Structure of thyroid hormones

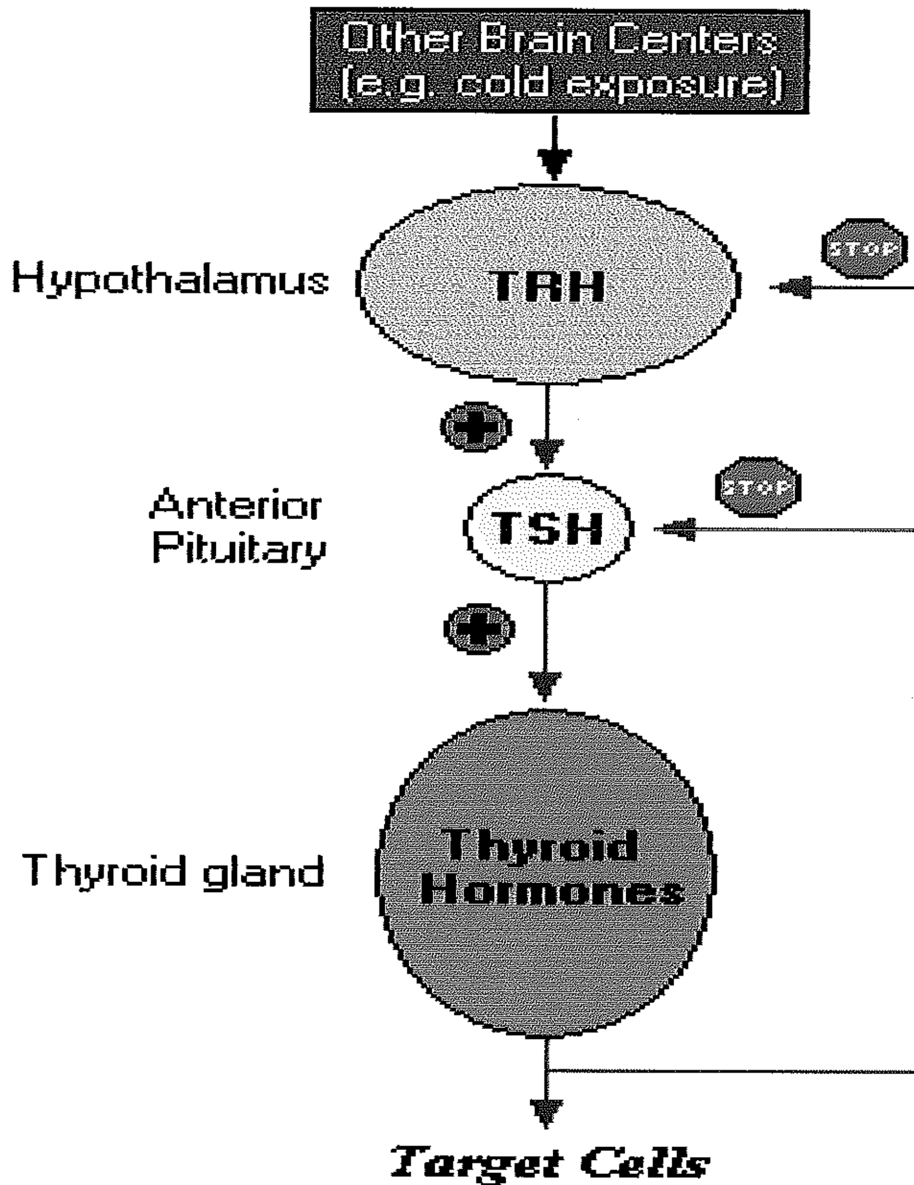

Figure 1.2 The negative feedback control of thyroid hormones synthesis and secretion

Palumbo *et al.*, 1990; Hayashi *et al.*, 1991; Xiao *et al.*, 1995). Formation of T_4 and T_3 occurs within the thyroglobulin molecule, involves an oxidative coupling mechanism of diiodothyronine and/or monoiodothyronine to a free radical and, then, interaction of two radicals (Harington and Rivers, 1945). Hydrolysis to release T_4 and T_3 from the thyroid gland into the bloodstream simultaneously takes place as the thyroid follicles incorporate to lyzosomes. Then the T_4 and T_3 in the blood are distributed to the peripheral tissues via thyroid hormone binding proteins.

2. Thyroid hormone distribution

Thyroid hormone action and metabolism occur in the intracellular compartment. However, because of their highly hydrophobic phenyl structure, thyroid hormones have a strong tendency to partition into lipid membranes (Hillier, 1970; Dickson *et al.*, 1987) that can result in marked depletion of thyroid hormones from blood by permeation into cells (Mendel *et al.*, 1987). Thus, a mediator for moving the hormones across the plasma membrane is requested to ensure distribution to the peripheral tissues located far from the thyroid gland.

There are three thyroxine-binding proteins found in the bloodstream of vertebrates, i.e. thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin. All vertebrate species possess at least one of these thyroid hormone-binding proteins (for review see Schreiber and Richardson, 1997; Schreiber *et al.*, 1998). In human, TBG have the highest affinity for T_4, and 75% of T_4 in the blood circulation bound to this protein (Robbin and Edelhoch, 1986). In the lower vertebrates such as amphibians and fish, little is still known about these proteins, however functions of albumin as the principal T_4-binding protein and TTR as the T_3 principal binding protein have been suggested (for review see Power *et al.*, 2000). Furthermore, plasma lipoproteins function in transporting thyroid hormones has been proposed in fish (Babin, 1992).

3. Thyroid hormone deiodination

The metabolism of thyroid hormones in the extra thyroidal tissues occurs via a complex series of enzymatic process, involves deiodination, conjugation, oxidation, and/or deamination of the iodothyronine compounds (Eale, 1984). Deiodination is a mechanism of iodide removal from the iodothyronine molecules. It is a very

important mechanism of controlling the thyroid hormones level in blood (Eale *et al.*, 1997). Thyroid gland secretes both T_4 and T_3 to the blood circulation. However, T_4 is the major form of the hormone being secreted, and functions as a prohormone. The conversion into the active molecule, T_3, occurs in the peripheral tissue by deiodination so that one of iodide atom is removed from the outer ring of T_4 (Eale, 1984).

There are two major deiodination processes, ORD (outer ring deiodination) and IRD (Inner ring deiodination), found in vertebrates, which are catalyzed by deiodinase (Orozco *et al.*, 1997). Deiodination is the pathway that produces not only the active metabolite, i.e. T_3, but also the less or inactive metabolites. For example, it is the mechanism of converting T_4 and T_3 to 3,5,5′-triiodothyronine (rT_3) and 3,3′-diiodothyronine (T_2), respectively. In mammals, 3 types of deiodinase have been identified (Leonard and Visser, 1986). The type I deiodinase (DI) has both ORD and IRD activities and locates in liver, kidney and thyroid gland. The type II deiodinase (DII) possesses only the ORD activity and is expressed in pituitary, brain and brown adipose tissue. Whereas, the type III deiodinase (DIII) catalyzes only IRD and found located in the central nervous system and the placenta (St. Germain, 1994). In comparison to in mammalians, studies of the deiodinases in fish are much less. However, 3 types of the enzyme with different tissues of expression, similarly to those found in mammalians, have been reported in tilapia, *Oreochromis aureus*, and other teleosts (Mol *et al.*, 1997; for review see Power *et al.*, 2001). DI of tilapia possesses similar properties to that in higher vertebrates, in spite of its predominant expression in kidney, but not in the liver.

4. Physiological role of thyroid hormones

Thyroid hormones exert many effects on growth, development and metabolism of higher vertebrates (e.g. mammals and birds) and lower vertebrates (e.g. amphibians, reptiles and fish). The influences of thyroid hormones on growth mediated through regulation of the growth hormone synthesis have been documented in children and young animals (Brent, 1994). The stimulating effect of thyroid hormones on growth resulting in increasing body length and weight was demonstrated in many fish species (Lam *et al.*, 1985; Reddy and Lam, 1992). The effect of thyroid hormones on developmental processes, on the other hand, is very important during the life of non-

mammalian vertebrates. In amphibians, the ontogenic transformation involving changes in specificity and remodeling of tissues and organs (e.g. neuron system, gastrointestinal tract) that occur during the transition from larvae to juveniles and to adult, is induced and regulated by thyroid hormones (Tata 1993; for reviews see Hourdry 1993; Su *et al.*, 1999; Shi *et al.*, 2001). Thyroid hormones also play an important role in development and differentiation during metamorphosis (smoltification) in fish (Yamano *et al.*, 1991; Miwa *et al.*, 1992). The bony fish that has less dramatic development than in amphibians, acceleration of pectoral fin and scale differentiation were detected in zebrafish and goldfish, respectively (Brown, 1997; Reddy and Lam, 1992).

For higher vertebrates, in particular human, a deficiency of the hormones (hypothyroidism) results in severe impairment of mental development and growth in childhood (cretinism). While thyroid hormone deficiency in adults leads to a decrease in basal metabolic rate, lowers body temperature and heart rate, results in weight gains and a series of symptoms called "myxedema". Inversely, an increase in thyroid hormone levels (hyperthyroidism) leads to an increased metabolic rate and body temperature, and weight loss. In the lower vertebrates, gluconeogenesis and glycolysis as well as lipogenesis and lipolysis are the major metabolic pathways affected by thyroid hormone actions. Short-term effect of T_3 on the level of malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH) and β-hydroxy-β-methylglutaryl CoA reductase (HMGCoAR) was detected in the Southeast Asian climbing perch, *Anabas testudineus*, (Varghese, 1999). Though, a biphasic effect of the T_3 was shown on cholesterol content. In addition, the hormonal control of T_4 on metabolism was demonstrated in a walking catfish, *Clarias batrachus* (Tripathi and Verma, 2003). Administration of T_4 in the presence of an deiodinase inhibitor reduced LDH and G6PDH activities, and T_4-dependent inhibition in anaerobic power and selective anabolic activities of the hexose monophosphate (HMP) pathway was suggested (Tripathi and Verma, 2003).

5. Thyroid hormone receptor (TR)

5.1 Multiple isoforms and structure of TR

Thyroid hormones have multiple levels of control but their predominant action exerts via the transcription pathway (Glass and Holloway, 1990). The action at this level is mediated through thyroid hormone receptor (TR) and its isoforms which functions as the hormone-activated transcription factor, and consequently controls the expression of the hormone target genes (Zhang and Lazar, 2000).

TR is a protein member of the nuclear receptor (NR) superfamily, which consists of a large number of special transcription factors including retinoid receptors (retinoic receptor or RAR and retinoid X receptors or RXR), vitamin D_3 receptor (VDR), and the steriod receptors. The thyroid hormone receptor has higher affinity for T_3 than T_4 (Oppenheimer *et al.*, 1987; Samuels *et al.*, 1988). The activities of TR are regulated by its cognate ligands, in particular T_3 (Mangelsdorf *et al.*, 1995). However, in the absence of T_3, TR is able to heterodimerize with RXR and binds to DNA. This characteristic is different from those members of the steroid receptors (for review see Yen, 2001; Harvey and Williams, 2002).

In most vertebrates, TRα and TRβ isoforms are encoded by two separate genes, THRA (NR1A1) and THRB (NR1A2), respectively. The THRA is transcribed as TRα_1 and TRα_2 (or C-erbA α_2) mRNAs which differ by alternative splicing of 3′ exon (for review see Harvey and Williams, 2002). Furthermore, two additional TRα isoforms, $\Delta\alpha_1$ and $\Delta\alpha_2$, transcribed from a novel promoter in TRα_1 and TRα_2 mRNAs were identified. In contrast, THRB is transcribed as TRβ_1 and TRβ_2 mRNAs by separate promoters, and alternative splicing at 5′region caused the TRβ_1 and TRβ_2 proteins to have distinct N-termini. In rat, TRβ_3 mRNA is found transcribed from the third promoter of the THRB gene. In addition, translation from the next ATG codon, inside the TRβ_3 mRNA, generated another TRβ_3 isoform, $\Delta\beta_3$, which is a repressor *in vitro* (Williams, 2002; for reviews see Brent, 1994; Yen, 2001; Harvey and Williams, 2002).

Similarly in all nuclear hormone receptors, the structure of the TRs in general contains six (A-F) domains that are well-organized into functional domains and regions. These functional domains and regions include the N-terminal domain (or A/B

domian), the conserved DNA-binding domain (DBD), a variable hinge region (D domain), and the ligand-binding domain (LBD).

The A/B domain is highly variable in amino acid sequences and in length. While, human and rat are 97% and 99% identical in their DBD and LBD sequences, respectively. They are only 88% identical in their A/B domain (Koenig *et al.*, 1988). This domain contains a transactivation function, i.e. regulates gene expression by interacting with the core transcriptional machinery, coactivators, or other transactivators (for reviews see Tsai and O'Malley, 1994; Mangelsdorf *et al.*, 1995).

Downstream from the A/B domain, the DBD is located in the central core (C domain) of the protein. This domain is responsible for DNA recognition and dimerization (for reviews see Carson-Jurica *et al.*, 1990; Tsai and O'Malley, 1994; Mangelsdorf *et al.*, 1995). The DBD has two zinc fingers constructed from the coordination of four cysteines and one zinc ion. TRs predominantly bind DNA as heterodimer with the retinoid X receptor (RXR), a receptor of 9-cis retinoic acid. The ability to heterodimerize with RXR increases binding affinity and specificity to target genes of the TRs (Wu and Koenig, 2000; Zhang and Lazar, 2000). Binding of TRs to the target genes occurs via the binding of this domain to the thyroid hormone responsive element (TRE) that locates at the regulatory region of the target gene. The first zinc finger of the DBD recognizes the specific sequence and bind to the major groove of TRE (for review see Yen, 2001). While, the second zinc finger recognizes and binds to the minor groove of TRE. The RXR dimerization surfaces are located in this second zinc finger.

Distal to the DNA-binding domain is a variable hinge region (D domain), containing a nuclear localization signal (for review see Evans, 1988). Findings suggested that this region allows the receptor to bend or alter its conformation (for review see Tsai and O'Malley, 1994), as well as serving as a contact surface with corepressor proteins or having allosteric effetcs on the interaction between TRs and corepressors (for review see Yen, 2001).

The LBD, located in the C-terminal region of the TR protein, is relatively large and functionally complex. The domain is believed to contain regions important for several activities including ligand-binding, receptor dimerization, hormone induced-transcriptional activation (transactivation), and intramolecular repression by

unliganded TRs (for reviews see Tsai and O'Malley, 1994; Mangelsdorf *et al.*, 1995; Wu and Koenig, 2000). The X-ray crystallographic analysis of liganded nuclear receptors including TRα_1 (Wagner *et al.*, 1995) revealed more understandings in the role of this region. The ligands were found deeply located within the hydrophobic ligand-binding cavity organized from 12 helices of LBD.

The DBD and LBD regions of TR isoforms are very conserved but diverge at the amino terminal domain (Figure 1.3), implying to the difference in the transcriptional regulation role among TR subtypes, as well as that of TRs from different animal species.

5.2 Expression and distribution of TR isoforms

TRα_1 and TRα_2 mRNAs are expressed in most tissues, however the expression varies according to developmental stage and specific tissues. For example, the expression of TR subtypes α_1, α_2 and β_1 in mammals are widely distributed in tissues including heart, liver and brain, whereas the TRβ_2 expresses only in the brain. Moreover, while TRα_1 is the major TR isoform in the fetal brain, TRβ_1 expression is markedly increased in brain at birth and maintained during adult life. More examples of developmental specific expression of TRs are in amphibians in which TRβ mRNA showed a dramatic expression and an immediate increase of the TRβ level before the stage of metamorphosis, the time that thyroid hormone function and thyroid hormone specific gene are first observed (for review see Brent, 1994). In fish, the TRs expression is tissue and developmental specific that is varying among fish species.

5.3 Molecular mechanism of TR action

Different from other members in the nuclear hormone receptor superfamily, thyroid hormone receptor can bind to TREs both in the absence and in the presence of specific ligand, i.e. T_3. In the absence of T_3, the heterodimer of TR-RXR, which constitutive bound to TREs, interacts with the corepressor proteins such as nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid receptors (SMRT) which both, in turn, interact with the histone deacytylase (HDAC). This interaction results in inhibition of the histone deacylation. In addition,

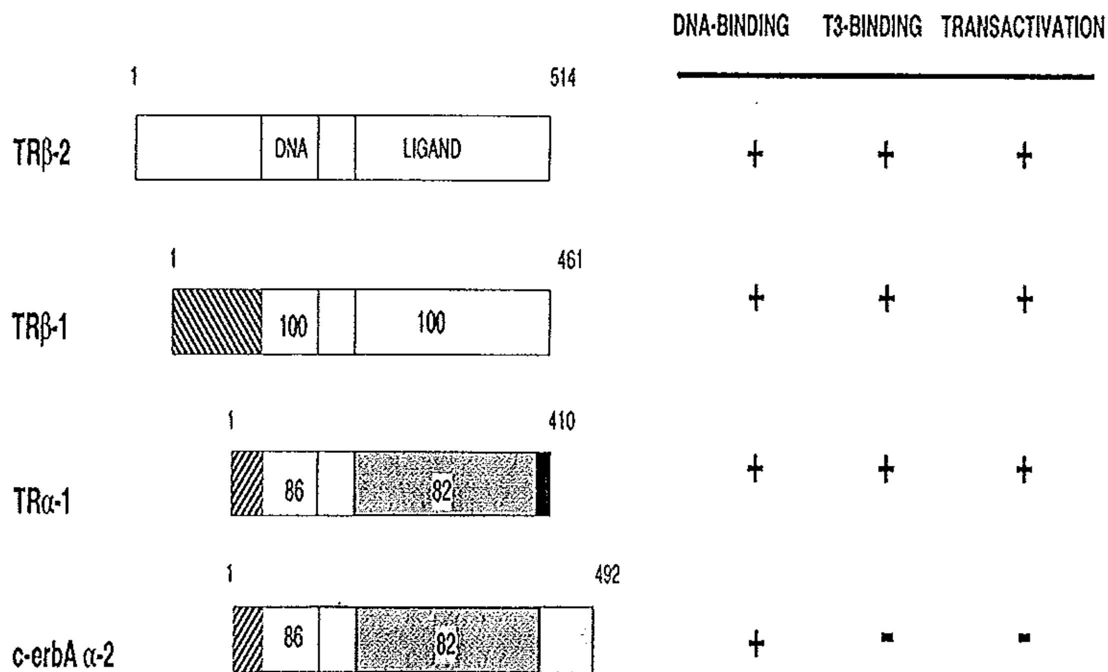

Figure 1.3 Comparison of amino acid homologies and their function properties among TR isoforms. The length of receptor is indicated above the receptor diagram, and the percent amino acid homology with TRβ_2 is included in the receptor diagram (From Yen, 2001).

direct communication of TR with transcription factor (TFIIB) and SMRT can interact to other transcription factors including TAFII32 and TAFII70. So, it is suggested that TR links to both histone modulating factors and the general transcription machinery. This binding results in suppressing the basal target gene transcription as the complex blocks the entry of the promoter (TATA) binding proteins (for reviews see Yen, 2001; Harvey and Williams, 2002).

On the other hand, in the presence of T_3, the TR-RXR interaction with the ligand leads to the replacement of the corepressor complex with the coactivator complex that possess histone acetyltransferase (HAT) activity. This interaction leads to modification of histones around the TRE promoters and opens chromatin conformation, facilitating the access of key transcription factors to the promoter resulting in the increase of transcription rate (for review see Harvey and Williams, 2002).

The summary of the mechanism process of thyroid hormone genomic action via thyroid hormone nuclear receptor in target cells is as shown in Figure 1.4. The hormone T_4 and T_3 enter the cell by both passive and facilitate diffusions. T_4 is, then, deiodinized to an active metabolite, T_3, in the cytoplasm of the target cell. T_3 binds to TRs that are constitutive bound to TREs of target gene as heterodimer or possibly homodimer form. The T_3-receptor complex further interacts with other specific coactivators at DNA regulatory region resulted in increase of gene expression and products of the target gene (Brent, 1994).

5.4 Cross-communication of thyriod hormone with retinoic acid receptor (RAR) via TR

Receptors in the nuclear hormone receptor superfamily including TR can communicate and modulate transcriptional activities of each other. In theory, this cross-talk can multiply the possible modes of gene regulation, leading to a greater and more flexible arrangement of transcriptional responses to environmental changes. This intercommunication can occur via several mechanisms including promiscuous binding to hormone receptor elements such as TREs, formation of heterodimers and competition for cofactors (Schule and Evans, 1991; Juge-Aubry et al., 1995). Non-restrict binding to one partner and heterodimer formation are the widely spread

mechanisms of cross talking among the members of the nuclear hormone receptor superfamily.

Cross-communication and inter-regulation of TR have been demonstrated in several nuclear hormone receptors including estrogen receptor (for review see Vasudevan *et al.*, 2002), vitamin D receptor (Garcia-Villalba *et al.*, 1996; Takeshita *et al.*, 2000) and retinoic receptor (RAR) (Lee *et al.*, 1994). The heterodimer of TR and retinoid X receptor (RXR) have been shown to mediate dual-ligand regulation of transcription and block ligand-dependent transcription of other members via RXR during the action of thyroid hormones (for reviews see Yen, 2001; Harvey and Williams, 2002). Similar mechanisms were suggested to occur for TR blockade of RAR-mediated transcription. In the absence of thyroid hormone, TR can form heterodimer with RAR and RXR on a retinoic acid response element, and T_3 but not retinoic acid decreased TR/RAR heterodimer binding to the DNA target (Yen *et al.*, 1992). Interaction between TR and RAR was more evidenced when the co-transfection experiments were used to demonstrate the inhibition of the RA response mediated by $TR\alpha_1$ that resulted in an alteration of retinoic acid-stimulated neural differentiation in mouse embryonic stem cells (Lee *et al.*, 1994).

Since thyroid hormone (predominantly T_3) and retinoic acid (RA) are essential for normal vertebrate differentiation and development and are known to co-regulate several genes in particular the gene of thyroid stimulating hormone (for review see Wolf, 2002). In addition, the early development of animals is known to be sensitive to retinoic acid (Brockes, 1996). Co-regulation effect of thyroid hormone and RA have been intensively investigated with transglutaminase as a candidate marker for the RA action (Enderlin *et al.*, 1997; Westergaard *et al.*, 2001).

Transglutaminase (TG) is a group of Ca^{2+} dependent enzymes involving in post-translational modification of protein. This enzyme catalyzes acyl-transfer reaction between the γ-carboxy amide group to peptide bond of glutamate and a variety of primary amine. In the presence of the ε amino group of lysine that serves as the acyl acceptor, the cross-linking of protein molecules occurs through the formation of ε(γ-glutamyl) lysine linkage. This covalent cross-linking is highly stable and resistant to proteolytic breakdown and physiological degradation (for reviews see

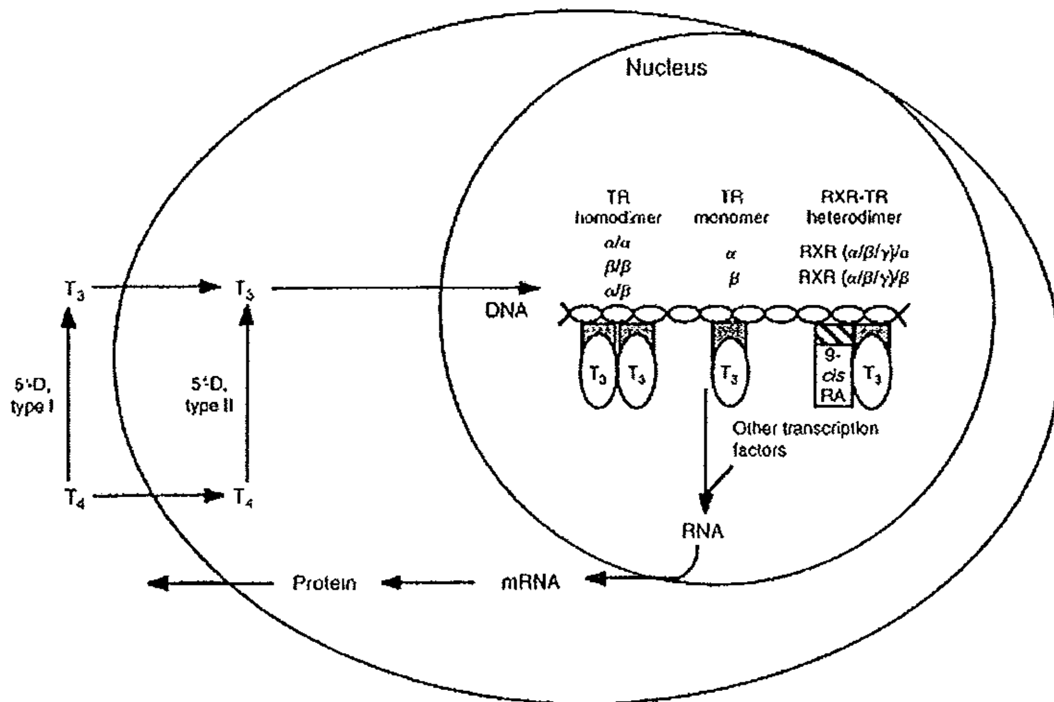

Figure 1.4 Action of thyroid hormone in cell nuclei (Brent, 1994)

Chandrashekar and Mehta, 2000, Lorand and Graham, 2003).

TG is expressed in various organisms, ranging from unicellular organisms to vertebrates, and in varieties of cells and tissues such as heart, kidney, lung and spleen. The enzyme comprises many forms which are produced from 6 distinct genes, and possesses multi-functions including that in blood clotting process, wound healing, keratinization of skin, program of cell death, and signal transduction. The expression of TG in tissues is regulated by retinoids through the RAR and RXR at the transcriptional level and the protein turnover process (Chen and Metha, 1999). Thus the expression level of TG is considered as a good indicator for retinoid action (Chiocca *et al.*, 1989; Enderlin *et al.*, 1997).

6. Thyroid hormones in growth and development of fish

The effects of thyroid hormones have been well established not only on developmental, metabolic, and homeostatic processes in higher vertebrates such as mammals and avians, but their influences on early development and metamorphosis also have been documented in amphibians and fish. Many efforts to reveal the potential roles as well as the mechanism of actions of thyroid hormones in fish larvae have been attempted (for review see Power *et al.*, 2001). The studies have not been restricted only on the hatchability, survival and metamorphosis of the animals. The effects of the hormones on expression level of enzymes in metabolic pathways and TRs also have been demonstrated in some fish species (Liu *et al.*, 2000; Tripathi and Verma, 2003).

6.1 Role of thyroid hormones on fish eggs

Thyroid hormones first detected in fish eggs are transferred from maternal circulation. This was supported by many experiments such as the detection of T_4 in the unfertilized eggs of coho salmon (*Oncorhynchus kisutch*) (Kobuke *et al.*, 1987) and stripe bass (*Morone saxatilis*) (Brown *et al.*, 1987). These findings were agreed with the observations of Ayson and Lam in 1993. After 24 h from the time they injected female rabbitfish (*Siganus guttatus*) with various dosages of T_4, the levels of both T_4 and T_3 in unfertilized eggs of the treatments were found significantly higher than the control. Whereas, those in both fertilized and unfertilized eggs of the control

did not significantly differ. This demonstrated and confirmed the maternal transportation of thyroid hormones to fish eggs, and implied to the importance of thyroid hormones on embryonic and, maybe, early larval stage of development, which led to several trials on the effect of exogenous thyroid hormones on the hatchability in fish. However, up to now both positive and negative responses on egg viability have been reported. Treatment of both non-buoyant and buoyant eggs of grouper (*Epinephelus* sp.) and Asian seabass (*Lates calcarifer*) with T_3 significantly improved the egg viability and larval survival (Lam, 1995). On the other hand, injection of T_4 to rabbitfish could not improve hatchability of the fish (Ayson and Lam, 1993). Takawa and Hirano (1991) also reported no effect of thyroid hormones on hatching rate. Hatchability and hatching time detected in the thiouracil treated fish did not significantly differ from those in the control. Fish species, thyroid hormones dosage, and hormone administration modes possibly caused these arguable observations. More investigations on the effects and mechanism of thyroid hormones action in egg of fish are required prior to have a definite conclusion, leading to success of the hormone application in improvement of mass production for aquaculture.

6.2 Thyroid hormones effect on fish larval survival

The function of the endocrine system during early ontogeny and metamorphosis was examined in several marine fish. The decrease of thyroid hormone level in tissues occurred during embryonic development to the stage of completion of yolk absorption. However, the hormone level was gradually increased during early larval life, and was significantly elevated during developmental changes to juveniles (Tanaka *et al.*, 1995). The thyroid hormone increased to a maximum around the metamorphic climax, followed by a mark decrease after metamorphosis of the fish. Treatment of fish larvae with thyroid hormone apparently stimulated the uptake of protein and fat in digestive tract. This suggested the benefit of using exogenous thyroid hormones in growth enhancement of fish larvae (Tanaka *et al.*, 1995).

The existence of the hypothalamus-pituitary gland-thyroid tissue (HPT) in fish larvae is divergent among teleosts. In chinook salmon (*O. tschawytscha*), coho salmon (*O. kisutch*), Atlatic salmon (*S. salar*), and fathead minnow (*Pimephales promelas*),

the thyroid follicles were found in embryos before hatch (Hoar, 1939; Leatherland and Lin, 1975; Wabuke-Bunoti and Firling, 1983; Greenblatt, 1989). Whereas in *Tilapia nilotica*, the follicles active only after yolk absorption (Nacario, 1983). Thus, it suggested the non-essential function of thyroid during the embryonic development of tilapia. However, it was demonstrated that supplementation with high dose of T_4 (0.5 ppm) is effective to enhance yolk resorption while with low dose (0.1 ppm) elevated growth of the tilapia larvae (Nacario, 1983). The observation indicated the enhancement of growth and development of fish larvae with appropriate concentrations of exogenous thyroid hormone. More studies reported the effect of exogenous thyroid hormones on survival, growth and development of fish larvae. The improvement of larval survival by exogenous T_3, or with the combination to cortisol reported in stripe bass (*M. saxatilis*) and threadfin (*Polydactylus sexfilis*), respectively (Brown *et al.*, 1988; Brown and Kim, 1995). Increase in the larval survival by size of fish after immersion in T_4-treated water was detected in carp (Lam and Sharma, 1985), tilapia (Lam, 1980; Nacario, 1983) and milkfish (Lam *et al.*, 1985). Although, no effect of exogenous T_4 on growth of larvae was reported in rabbitfish (*S. guttatus*) (Ayson and Lam, 1993), the larvae from the spawners treated with thyroid hormone at the appropriate amount showed better survival compared to the control. The finding demonstrated an important role during early larval development of the rabbitfish. In addition, researches on exogenous thyroid hormones also attended the effect of the hormones on metamorphosis, besides on growth and early development of fish. The administration of exogenous thyroid hormone to zebrafish induced pectoral fin differentiation and also enhanced growth of the pelvic fins. Whereas the inhibition of thyroid hormone synthesis, suppress the transformation from larvae to the juveniles (Brown, 1997). In summer flounder (*Paralichthys dentatus*), thyroid hormone (T_4) was found necessary for the completion of metamorphosis (Schreiber and Specker, 1998). For example, an alteration of the thyroid status of the larvae at late premetamorphic stage accelerated the rate of the larval development to prometamorphosis, early and mid metamorphic climax stages. Whereas the larvae treated with thiourea at this stage showed static development in early metamorphic climax. Thiourea treatment, though, could inhibit completion of metamorphosis in early and mid, but not in late metamorphic climax stage.

6.3 Thyroid hormone and metabolism in fish

The researches on effects of thyroid hormones have been emphasized on not only growth and development but also on metabolism of animals. Several evidences pronounced this effect in vertebrates, and it has been well known till now that their metabolisms are regulated by thyroid hormones (for review see Brent, 1994).

Thyroid hormones play an important role on metabolism, especially of carbohydrate, of fish (Tripathi and Verma, 2003). However there are still not many studies reported on mechanism of this action. Many specific enzymes of the carbohydrate metabolic pathways including glucose-6-phosphate dehydrogenase (G6PDH) and lactate dehydrogenase (LDH) have been demonstrated influenced by thyroid hormones.

Functions of these two key enzymes of carbohydrate metabolism are conserved among vertebrates. G6PDH is well known as the rate limiting step key enzyme of pentose phosphate pathway. This enzyme catalyzes the hydrogenation of glucose-6-phosphate to 6-phosphogluconate, and produces reducing equivalent (NADPH) that is necessary for the biosynthesis of fatty acid and a pentose (i.e. ribose) which the latter is a very important raw material for nucleic acid synthesis. The G6PDH is present in most tissues of multicellular organisms (Kletzien *et al.*, 1994). LDH is an enzyme with multi-isoforms. It is a key enzyme that catalyzes the conversion of pyruvate to lactate with the regeneration of NAD^+. This reaction occurs in cells in which oxygen is limited, such as in the muscle cells during an intensive exercise (Stryer, 1981). It also involves in pyruvate regeneration for synthesis of oxaloacetate in gluconeogenesis (Tripathi and Verma, 2003).

In fish, G6PDH and LDH usually are the candidate markers in observations on effects of thyroid hormones on carbohydrate metabolic pathways. The activities of these two enzymes have been shown altered by thyroid hormone action in many fish species. Leary *et al.* (1997) demonstrated that after 3 h of injection with high and low doses of thyroid hormone (0.65 μg and 0.065 μg T_3/50 g body weight, respectively), the hepatic G6PDH activity of hagfish (*Myxine glutinosa*) increased significantly compared to the control. In brook charr (*Salvelinus fontinalis*), long term supplement with T_3 enhanced G6PDH activity in the liver of the animals those kept in the high-density broodstock. High T_3-administration induced significant lowering of

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hepatic glycogen content (Scott-Thomas *et al.*, 1992). For the effect of thyroid hormone on activity of LDH, Tripathi and Shukla (1989) documented that the administration of T_3 to thiouracil treated fish increased the activities of hepatic and skeletal muscle LDHs to 7 and 5 folds, respectively.

The negative response to thyroid hormones of G6PDH and LDH was also demonstrated. Varghese and Ommen (1992) reported the reduction of G6PDH activity in climbing perch (*A. testudineus*) after the administration of thyroid hormones. The enzyme activity decreased after the thiouracil treated fish received T_3 (1 μg) for 1 day. Whereas, T_2 (0.5 and 1 μg) stimulated the activity of the enzyme, comparing with the control. This lowering activity of G6PDH was in agreement with the observation in *C. batrachus*. The exogenous thyroxine administration decreased G6PDH and LDH synthesis of the thiourea exposed animals, suggested to the thyroid hormone-dependent inhibition in anaerobic energy production and selective anabolic activities of the hexose monophosphate pathway (Tripathi and Verma, 2003).

7. Thyroid hormone receptor in fish

In comparison with that in mammals, little is known at molecular level of structure and function of thyroid hormone receptor in fish during development. Limitation was on size of fish eggs and larvae that made it difficult to be studied with some techniques such as ligand binding assay. However, during the recent period of the DNA technology development, study of TR in teleost fish, so far, has been much in progress.

In teleosts, the first successful cloning of TR cDNA was reported in Japanese flounder (*Paralichthys olivaceus*) (Yamano and Inui, 1995). The receptor was found in four isoforms, i.e. two isoforms of TRα and the other two isoforms of TRβ. The TRβ subtypes were from an alternative splicing of a single gene whereas the TRα subtypes are synthesized from two distinct genes at the level of transcription (Yamano *et al.*, 1994 and Yamano and Inui, 1995). This characteristic is in reverse to that found in other vertebrates, in particular human, in which TRα subtypes are generated from an alternative splicing of one mRNA and the subtypes of TRβ are synthesized from two distinct gene promoters (see section 5.1 in this chapter for details). The existence of fish TR and TR subtypes have been reported in other

species of fish including zebrafish (*D. rerio*) (Esser, 1997; Liu *et al.*, 2000), Atlantic salmon (*S. salar*) (Marchan *et al.*, 2001; Jones *et* al., 2002), Atlantic halibut (*Hippoglossus hippoglossus*) (Llewellyn *et al.*, 1999). In addition, Kawakami *et al.* (2003) recently reported the presence of TRβ subtypes as well as TRαA and TRαB (Kawakami *et al.*, 2003 (in press)) in conger eel (*Conger myriaster*) and proposed that the TRβ subtypes are the products of two distinct genes.

So far, the structure of fish TRs is highly conserved (see Figure 1.5 for an example of amino acid sequences alignment among TRs from fish species and other vertebrates). Functional domains including the DBD and LBD are conserved, but high variable at the amino acid terminal or A/B domain. Structural conservation of the DBD and LBD of TRα among mammals, birds and fish imply that this vertebrate TR isoform binds to the same TREs and thus produces the transcription activation in the similar way to that have been characterized in mammalian receptor.

In spite of the study and isolation the TR isoforms in many fish species, the insights of TRs level and specific tissues of the gene expression at each stage of fish was also important. These would lead to the understanding of thyroid hormone and TRs relationship and insight of thyroid hormone action on the development of fish. Though there were a few studies on the TRs expression in metamorphosing fish, the evidence obtained until now can provide an idea about the TRs action in fish development.

Yamano and Miwa (1998) demonstrated the expression of two isoforms of TR (TRα and TRβ) from the period of hatching to juvenile stages of Japanese flounder. They observed that the low level of TRs were present in fertilized eggs. The receptor level increased after hatching and maintained during premetamorphic stage. The rapid increase to the highest level of TRαA occurred at metamorphic climax whereas the TRβ subtypes reached the maximum level at the post-metamorphic climax stage. The increase of TRβs to the maximum level was 5 days later than of TRαA. An interesting observation was while the level of TRαA dropped at the post-metamorphic climax stage of the juvenile the TRβs level remained on the maximum, though, after the metamorphosis was completed. In addition, in comparison to TRβ, the level of TRαA was shown higher in every stages determined. The specific tissue localization revealed strong expression level of TRαA in the skeletal muscle and epithelial cells of

digestive tract whereas TRβ showed a strong expression in the cartilage cell and osteoblasts surrounding cartilages and bones. So, taken from the observations, it demonstrated that the development of these tissues is regulated by thyroid hormone action mediated via thyroid hormone receptor at different timing and by different TR isoforms.

The investigation of TRs in zebrafish was reported in 2002 by Lui *et al.* They showed that TRα expression occurred before the expression of TRβ, and at higher level. In addition, these TRα and TRβ possess different activities. The transactivation activity of the $TR\beta_1$ was dependent on absence or presence of T_3, i.e. the activity of the receptor was repressed when T_3 absent. While that of TRα still being functioned even in the absence of T_3. Therefore developmental regulation by thyroid hormone in zebrafish may be involved in this complexity and more researches are needed for more understanding. The expression of TR during development also was investigated in salmonid species including rainbow trout and Atlantic salmon (Jones *et al.*, 2002). Monitoring the expression level of the TRα by semi-quantitative RT-PCR at select stages during embryonic and larval development revealed that the level of the receptor reached a peak at hatch. Then, gradually increased during larval development and reached the maximum at the stage of first feeding. These findings led to the suggestion about the functioning of thyroid axis and responding to thyroid hormone of this fish species during embryonic and larval stages.

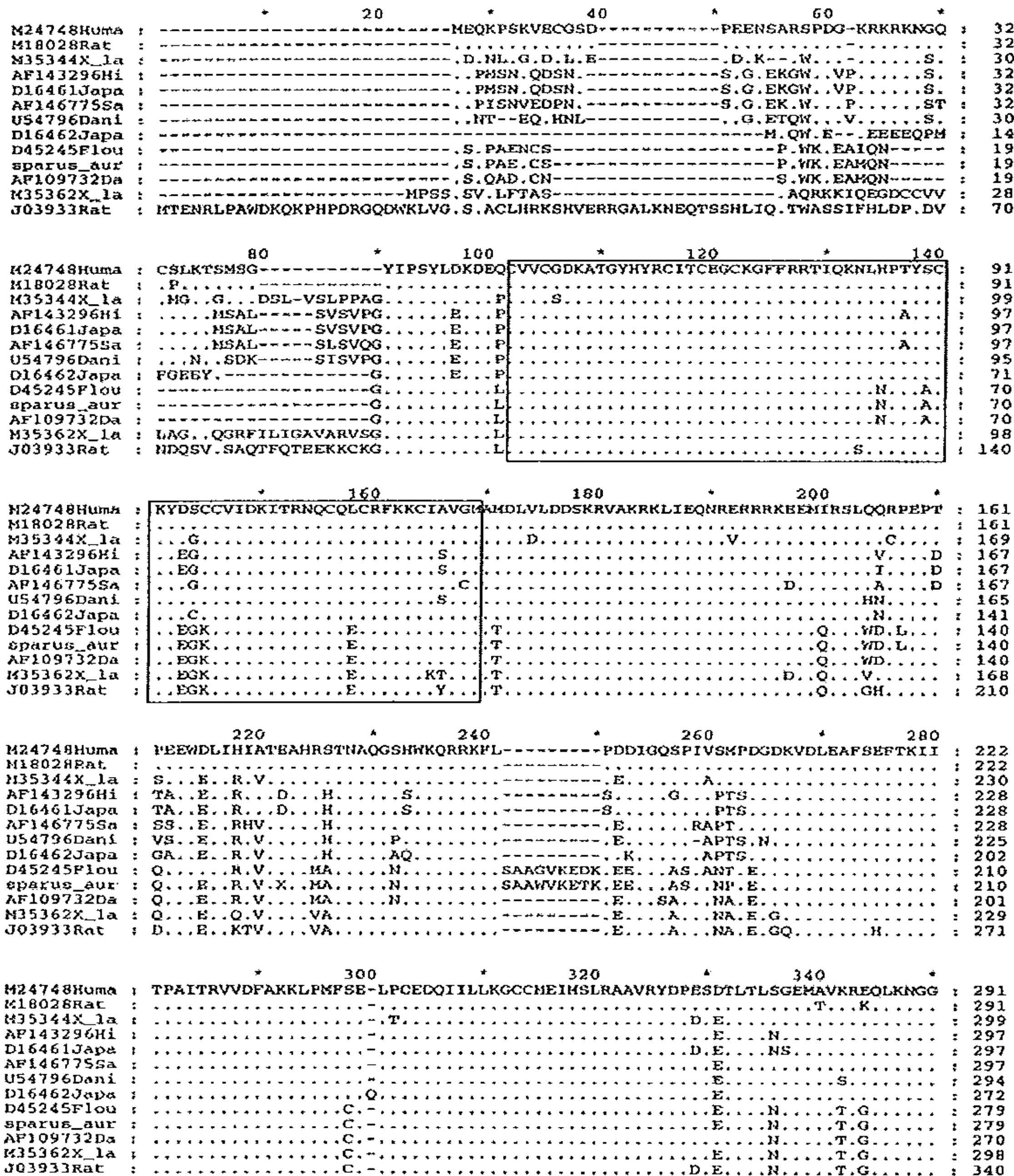

Figure 1.5 Multiple sequence alignment of TR from mammals, amphibian and teleost fish (the genebank sequence accession number is indicated) using Clustal W; human TR1 (M24748); rat TR (M18028); *Xenopus laevis* TRαB (M35344); halibut TRα (AF143296); Japanese flounder TRα (D16461); *Salmo salar* TRα (AF 146775); zebrafish TRα (U54796); Japanese flounder TRβ (D16462 and D45245); sea bream TRβ (*Sparus aurus*); zebrafish TRβ (AF 109732); *Xenopus* $TR\beta_2$ (M35362), and rat TRβ (J03933). Spacer (-) have been introduced to give maximum homology and (•) in the alignment denote identical amino acid residues. The DNA binding domain recognizes thyroid hormone response elements (TREs) is boxed. (from Wu and Koenig, 2000).

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                  360         *        380         *        400         *        420
M24748Huma : LGVVSDAIFELGKSLSAFNLDDTEVALLQAVLLMSTDRSGLLCVDKIEKSQEAYLLAFEHYVNHRKHNIP : 361
M18028Rat  : ...................................................................... : 361
M35344X_la : .........D.....A...........................T.....C..T................. : 369
AF143296Hi : .........D.....AQ........................TS.E...QC.............Y...... : 367
D16461Japa : .........D...E.GQ.......................HQ..E...QC.............Y...... : 367
AF146775Sa : .........D.....AQ........................TL......C..T................. : 367
U54796Dani : .........D......Q........................T..E....C..M................S : 364
D16462Japa : .........D.....AQ........................T.......C..T..........Y...... : 342
D45245Flou : .........D..V...S.....................P..SS.E...PC..E..........Y...K.A : 349
sparus_aur : .........D..V...S........................SS.E....C..E..........Y...K.A : 349
AF109732Da : .........D..V...S.....................P..TS.E....C..E..........Y...K.A : 340
M35362X_la : .........D..V...S.S...................P..SS.E....C..G..........Y.....A : 368
J03933Rat  : .........D..K...S.....................P...A.E....Y.DS..........Y...H.T : 410

                    *         440         *        460         *        480
M24748Huma : HFWPKLLMKVTDLRMIGACHASRFLHMKVECPTELPPPLFLEVFEDQEV-------------- : 410
M18028Rat  : .................................................-------------- : 410
M35344X_la : ......................C..........................-------------- : 418
AF143296Hi : ...............................S.................-------------- : 416
D16461Japa : .................................................-------------- : 416
AF146775Sa : ................................N................-------------- : 416
U54796Dani : ................................................GSTGVAAQEDGSCLR : 427
D16462Japa : ................................N................-------------- : 391
D45245Flou : ..............................................----------------- : 395
sparus_aur : ...............................S..............----------------- : 395
AF109732Da : ..............................................----------------- : 386
M35362X_la : ..............................................----------------- : 414
J03933Rat  : ..............................................----------------- : 456
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Figure 1.5 continue

8. The green catfish (*Mystus nemurus*)

M. nemurus (Cuvier and Valenciennes) belongs to Order Siluriformes, Suborder Siluroidei, and Family Bagridae. It is one of the most important freshwater catfish cultured in Southeast Asia and has high potential for commercial fisheries (Smith, 1945). The green catfish possesses similar characteristics to other members in the Siluriformes. They are scaless and have 3 types of whisker or barbel around their mouth. These are mandibular, maxillary, and nasal barbels (Mesomya *et al.*, 2002). *M. nemurus* from each region are varied in color and size. They are typically found in a variety of habitats including rivers (e.g. from brackish water at the mouths of rivers to upstream water), ponds, swamps, and other water bodies (Wongrat and Krudphan, 1994; Amatyakul *et al.*, 1995). In Thailand, the catfish are yellowish, so this fish has a common name as yellow or green catfish (Suvatti, 1950). Moreover, their body sizes are much larger compared to those found in Malaysia. In natural water, the average size of the green catfish is 20-25 cm. Mass production of this catfish seeds was successfully achieved using artificial breeding (Amatyakul *et al.*, 1995), and the fries are typically distributed by the Thai Fisheries Department to fish farmers. The green catfish can live and grow in variety of environments, but generally prefers clear water stream with hard soil or rocky bottom at the depth of water 2 to 40 meters. The optimum density of fish population in the rearing reservoir was demonstrated ranging from 285 to 375 fish/m^3 (Khan, 1994). They are generally omnivorous that feed primarily on crustaceans (crab and prawns), aquatic and terrestrial insects, fish and vegetation (Mesomya *et al.*, 2002).

The green catfish is one of the most popular and economically important foods in Thailand that has gained popularity among consumers due to its palatability and nutritional value. The analysis of dried green catfish indicated that the fish was high in omega-3 fatty acids, and the level was higher than that found in marine fish such as sardine (Mesomya *et al.*, 2002). As the mass production of the green catfish is not constant, several exertions have been made to provide information leading to the improvement the production. These include larval digestive capacity (Kamarudin *et al.*, 2001), feeding behavior (Amornsakun *et al.*, 1997, 1998), types of diet (Kamarudin *et al.*, 2001), and effect of stocking density on growth (Khan, 1994). However, no observation on thyroid hormones has been documented in *M. nemurus*.

The green catfish larvae were found hatching out with yolksac that were completely absorbed within 72 h after hatching (Amornsakun *et al.*, 1997). The newly-hatch larvae were shown to survive for several days without feeding. The survival rate and growth of the 2 days old larvae with 4 days delay in feeding were not significantly different from those with immediate start of feeding (Amornsakun *et al.*, 1997). Feeding time for the green catfish, in particular those in the stages of 3 to 15 days, was shown to be able to accomplish in both day and night time (Amornsakun *et al.*, 1998). The importance of exogenous digestive enzymes during early exogenous feeding stage was demonstrated (Kamarudin *et al.*, 2001). The activities of digestive enzymes include pepsin, chymotrypsin, trypsin, and amylase were investigated in the fish larvae fed with live and artificial diets. Pepsin was shown to appear and be active earlier than other examined digestive enzymes (Kamarudin *et al.*, 2001).

Objectives

The experimental researches were conducted to elucidate the role, effects, and actions of thyroid hormones in the green catfish (*M. nemurus*), in order to use thyroid hormones as the exogenous supplements for the improvement of mass production of the catfish.

Chapter 2

MATERIALS AND METHODS

1. Materials

1.1 Instruments

Instrument	Model	Company
Autoclave	HA-300 MTI	Hirayama
Centrifuge	Micro centrifuge 4210	ALC
Centrifuge	5804R	Eppendorf
Gel Document (Program Labworks 4.0)	C-80	UVP
Incubator	27	Thelco
Micro-pipettes		Gilson, Nichipet EX Biopipett, Corning
PH meter	pH meter 240	Corning
Power supply	Power Pac 3000	Bio RAD
Spectrophotometer	8453	Hewlett Packard
Submarine electrophoresis	AE-6100	ATTO Corparation
Stirrer	PC-410	Corning
Thermocycler	5333	Eppendorf
UV visible		Vilber Lourmat
Vortex-mixer	VX 100	Labnet
Water bath	Wb22	Memmert

1.2 Chemicals

1.2.1 Analytical grade

Chemical	Company
Absolute ethanol	Merck
Acetic acid	BDH
Ammonium sulfate	Univar (AJAX chemicals)
Bovine serum albumin	Sigma
Calcium chloride	Merck
Copper sulfate(Cupric sulfate)	J.T. Baker
Dimethylsulfoxide (DMSO)	Lab Scan
Ethylene diamine tetraacetic acid (EDTA)	Carlo Erba
Ferric chloride (Iron (III) chloride anhydrous)	Merck
Formaldehyde	Sigma
Formamide	Sigma
D-glucose-6-phosphate	Sigma
Glutathione	Sigma
Hydroxylamine	Univar
DL-lactic acid sodium salt	Fluka
L-Glutamic acid-γ-hydroxamate	Sigma
Methanol	Lab Scan
N-Carbobenzyloxy-glutaminyl-glycine	Sigma
β-Nicotinamide adenine dinucleotide (NAD)	Sigma
β-Nicotinamide adenine dinucleotide, reduced form (NADH)	Sigma
β-Nicotinamide adenine dinucleotide phosphate (NADP)	Sigma

Chemical	Company
Sodium hydroxide	BDH
Trichloro acetic acid	Carlo Erba
3,3′,5,5′-Tetraiodo-L-thyronine, sodium salt (T_4)	Sigma
3,3′,5-Triiodo-L-thyronine, sodium salt (T_3)	Sigma

1.2.2 Molecular biology grade

Chemicals and bacterial cells	Company
Agarose	Promega
AMV reverse transcriptase	Promega
Deoxy nucleotide triphosphate	Promega
100 bp DNA ladder	Biolabs
DNase I	Promega
Ethidium bromide	Promega
E. coli JM 109	Promega
Guanidine isothiocyanate	Promega
MMLV-Reverse transcriptase	Promega
MOPs	Sigma
pGEM-Teasy vector	Promega
Ramdom primer	Promega
RNA easy kit	QIAGEN
Rnasin Ribonuclease Inhibitor	Promega
Taq DNA polymerase	Promega

1.3 Animals

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

2. Methods

2.1 Animal breeding and larval care

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

2.1.1 Animal breeding

Mature male and female green catfish (*M. nemurus*) weighing 0.5 to 1 kg were netted from the brood stock pond and transferred to a rearing pond. The females were injected twice with a combination of Lutinizing hormone releasing hormone analogue, LHRHa (Suprefact, Hoechst AG, Germany), and the dopamine antagonist (Motilium, Janssen). The first dosage was injected into the epaxial muscle with 10 μg of LHRHA and 10 mg of dopamine antagonist/kg body weight. Thereafter 6 h, the second dosage of injection was conducted with 20-25 μg of LHRHa and 10 mg of dopamine antagonist/kg body weight. After 6-10 h from the second hormone injection, eggs were stripped. In the mean time, the male was sacrificed to get the testis. The testis was crushed in a net and consequently washed with appropriate volume of water to achieve the milt solution. The milt was then mixed to the eggs with feather for 2-3 min in a chamber prior to being placed evenly onto the nylon net supplied with the flow-through water system and continuous aeration. After hatching (about 36-48 h), larvae were transferred to a nursery pond.

2.1.2 Animal care

Catfish (*M. nemurus*) 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 days (after hatch) to 7 days old. Then, they were 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 of age. They were fed with powder feed 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. Fish wastes and debris were removed every other day, in the morning, by suction. Every week, 75 % of water in the pond was replaced in order to maintain the water quality. Fish were collected from the pond at the ages of 3, 7, 15, 30 and 45 days old for further treatments.

2.2 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 were prepared by diluting the 10 mg/ml stock solution with water to reach a nominal concentration. Control of each experiment was treated with DMSO or methanol at the highest volume of hormone used in the experiment. For the long-term hormone treatment studies, working hormone solutions were prepared and kept in 20 L-gallon containers and dispensed into the treatment containers.

2.3 Animal collection for experimental treatments

General procedures will be stated in this section. More specific points for each experiment will be detailed in sub-section of each result. To obtain a conclusion that is applicable to all of green catfish, more than 1 separate experiments with replicates were conducted for each hormone treatment. All catfish, whole body or organ tissues, were kept at –20 °C until further analysis.

2.3.1 For the hatching rate study

All of experimental hatcheries were conducted in a plastic box (12.5 cm x 15 cm x 5 cm). A rectangular wire-frame with nylon net was placed and attached to the box so that the frame was 1 cm above the bottom, leaving space for the aeration pipe to fit in (see picture in the appendix). In each box, 500 ml of water or water

containing thyroid hormone was added, 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. At least 3 separated experiments with 3 replicates were conducted for each hormone treatment.

2.3.2 For the survival rate study

In short-term treatment study, green catfish at specific ages were randomly collected from the nursing pond and placed in buckets containing 8 L of T_3 at the concentrations of 5, 10, 20, 80, 160 and 320 ppm. The control group was in water containing 16 ppm of DMSO. Continuous aeration was supplied through out the treatment. After the animals were treated for 24 h, they were transferred to 1 L of fresh water in a plastic container. Number of dead fish was recorded and the dead was removed everyday. The survival rate was calculated by subtraction of the dead from the total number at beginning of the experiment. At least 2 individual experiments with 2 replicates were conducted for each treatment. 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.

For the long-term treatment of T_3, fish at the ages of 15, 30 and 45 days were exposed to T_3 for 15 days. The animals were sampled out from the nursing pond at specific ages and were reared in plastic containers containing 1 L of the hormone solution. The controls were reared in water containing 8 ppm of DMSO. Each chamber was continuously aerated and fish were fed with powder feed twice a day. Containers were cleaned daily. Water or water containing hormone in each chamber was renewed by three-forth substitution with fresh water or solution every 2 days. Dead fish were counted and removed everyday. At least 2 individual experiments with 3 (for fish 15 and 45 days of age) and 2 (for fish 30 days of age) replicates were conducted for each treatment. Groups of 20 (for those at 15 and 30 days of age) and 10 (for those at 45 days of age) fish were assigned in each replicate.

2.3.3 For the enzyme assays

Fish were transferred from the nursing pond at specific ages into buckets containing 8 L of T_3 solution (5, 20 and 80 ppm). Controls were in water containing 8 ppm of DMSO. After 24 h, the animals were transferred to separate plastic containers containing 1 L of fresh water or hormone solutions. They were further exposed to the hormone for another 3 days with continuous aeration but without feeding. In each experiment for each age of fish, groups of fish (2000, 1500, 300, 40, and 30 for 3, 7, 15, 30, and 45 days of age, respectively) were assigned to four containers. One container for the control, and another three for T_3 treatment at concentrations of 5, 20, and 80 ppm, respectively. Fish were collected from at least 3 separate experiments, and up to 10 % of stocking fish in each container were collected daily and were immediately stored frozen at -80 °C until analysis.

2.3.4 For TR assay

For the short-term treatment effect, fish with 3, 7 and 15 days of age were treated, and groups of fish were assigned the same as described in section 2.2.3. The animals were collected after exposed to T_3 for 24 h, and immediate frozen and stored at -80 °C until the TR assay was performed.

For the long-term treatment effect, fish of 30 and 45 days were treated, and groups of fish were assigned the same as described in section 2.3.2 of the T_3 long-term treatment study on the survival of fish. The animals were exposed to the hormone for 15 days, then they were collected and immediately stored frozen at -80 ° C until analysis.

2.4 Enzymatic activity assays

2.4.1 Protein preparation for enzyme assays

Whole body of catfish 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. Then, cell debris was removed from the homogenate by centrifugation at 14,000 rpm for 90 min. The supernatant was collected and kept at -20 °C until use. For preparing of crude protein for the enzymatic activity assays of G6PDH and LDH, the

supernatant was aliquoted (300 μl) in 1.5 ml tube. Saturated ammonium sulphate solution was added to get the final concentration of 85% in the protein solution. The protein precipitate was collected by centrifugation at 10,000 rpm for 15 min. The protein was kept as pellet at -20 °C. The protein pellet was re-dissolved in 300 μl of 50 mM Tris-HCl, pH 7.4 prior to the enzymatic activity assays. All steps were carried out at 4 °C.

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

The enzyme 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 (see appendix for more details). The change in absorption was measured for 5 min (see appendix for more details). The activity units of the enzyme was calculated on the basis that one unit (U) of enzyme activity is the amount of enzyme required to reduce one μmole of NADP under the assay condition. The specific activity of the enzyme is defined as the activity units per mg of protein. All enzymatic activity determinations were carried out in 3 replicates.

2.4.3 Lactate dehydrogenase (LDH) activity assay

Enzymatic 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 (see appendix for more details). Standard curve was constructed at 0.00125, 0.0025, 0.005, 0.010, 0.020 and 0.040 μ moles of NADH, respectively. One unit (U) of the enzyme is defined as the amount of enzyme required to reduce one μmole of NAD^+ per min. Specific activity of the enzyme is defined as units per mg of protein. All enzymatic activity determinations were carried out in 3 replicates.

2.4.4 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 allowed to occur 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 for the absorption 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 units per mg of protein. All enzymatic activity determinations were carried out in 3 replicates.

2.5 RNA extraction

Total RNA from the whole fish 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 into a new tube, and another centrifuged was performed at 12,000 to 14,000 rpm for 3 min. The lysate was carefully transferred into a new tube, and 70% of ethanol was added to precipitate the DNA. The lysate mixture, including DNA precipitate, was apply 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 solutions 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.

2.6 Removal of contaminated DNA with deoxynuleotidase (DNase)

Although the company claims that silica-membrane technology of the RNeasy kit efficiently removed most of the DNA, further DNA removal is necessary for certain RNA application such as RT-PCR which is sensitive to very small amount of the DNA. 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. The 20 μl reaction mixture was composed of 2 μg of RNA, reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 50 mm $MgCl_2$, 2.5 mM spermidine, and 50 mM DTT), 20 units of ribonuclease inhibitor (RNase inhibitor), and 2 units of DNase I. Digestion of the DNA was allowed to occur at 37 °C for 30 min prior the 20 mM EGTA solution was added and the mixture was incubated 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.

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

2.7.1 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 AMV transcriptase buffer (250 mM Tris-HCl pH 8.3, 250 mM KCl, 50 mM $MgCl_2$ 2.5 mM spermidine and 50 mM DTT), 20 units of RNase inhibitor, 1 mM of dNTPs mixture (1 mM each of dATP, dGTP, dTTP and dCTP),0.75 μg of random primers, and 20 units of AMV reverse transcriptase. All components were added prior to incubation at 42 °C for 30 min. The reaction mixture then was kept at -20 °C, or immediately proceeded for the specific synthesis of the second strand cDNA and amplification of the DNA fragment by polymerase chain reaction (PCR).

2.7.2 Specific DNA amplification by polymerase chain reaction (PCR)

PCR was used to synthesize the second strand of the cDNAs and to amplify for thyroid hormone receptor and beta actin gene fragments using first strand cDNAs, obtained from reverse transcription of total RNA, as templates. A pair of degenerated oligonucleotide primers specifically designed for thyroid hormone receptor (forward primer, 5′-TGGCCAAGCGGAAGYTNATHGAGA-3′; backward primer, 5′-GCCCGCAGGGACATDATYTCATRCA-3′) and a pair of specific primers for beta-actin (Promega) were used in amplification of the thyroid hormone receptor and beta-actin 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 (10 mM Tris-HCl (pH 9.2), KCl (50 mM), $MgCl_2$ (3.75 mM for TR, or 1.87 mM for beta-actin), dNTPs (250 μM), 5' and 3' specific primers (0.5 μM of each), and Taq DNA polymerase (1 unit). The synthesis and amplification were started with an initial denaturation of cDNAs at 94 °C for 6 min. This was followed by cycles (35 to 40 for thyroid hormone receptor, or 25 to 30 cycles for beta-actin) of 94 °C for 30 s, 58 °C for 30 s 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 % agarose gel containing 0.5 μg/ml ethidium bromide, and gels were visualized under ultra-violet illumination. Each RT-PCR analysis was repeated at least three times for each set of RNA samples.

2.8 Isolation and purification of DNA from agarose

The PCR reaction products were separated on 1.5% low melting agarose gel containing 0.5 μg/ml 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 microcentrifuge tube. Then, 5 volumes of solution containing 10 mM Tris and 1 mM EDTA, pH 8.0 were added and 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.

2.9 Cloning of DNA fragment

Purified PCR product was cloned into pGEM-T easyVector (Promega) with 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 1h at room temperature. Thereafter, half of ligation product was transformed into JM109 High Efficiency Competent Cells (Promega). The bacterial cells were heat-shocked for 90 s at 42 °C, and immediately cooled down on ice for 2 min. LB medium was added prior to the cell mixture was incubated at 37 °C for 1h. All transformation culture was plated onto LB/amplicillin/X-Gal agar. Plates were incubated overnight at 37 °C. White colonies that generally contain the DNA insert plasmid were selected.

2.10 Purification of plasmids

Escherichia coli plasmids were prepared by the alkaline lysis procedure published by Birnboim and Doly (1979), using the Perfectprep plasmid mini kit (Eppendorf, Germany). In brief, an overnight culture of plasmid-containing *E. coli* was prepared in the presence of 100 μg/ml culture of ampicillin. The cells were collected by centrifugation, and suspended in a buffer solution containing RNase A. The bacterial membrane was then lysed with 0.1 M NaOH and 0.5 % SDS within 5 min at room temperature. Thereafter, the lysate was neutralized and adjusted to be in a chaotropic salt (1.6 M guanidine hydrochloride) for binding to silica material. The precipitated chromosomal DNA and cell debris were removed by centrifugation. The supernatant containing the plasmid was purified by adsorption of the DNA to the silica matrix suspension. Bound plasmids were then freed from salts, proteins and other cellular impurities by washing with 20 mM NaCl and 2 mM Tris-HCl, pH 7.5 in 80 % methanol. The plasmid was finally eluted with 10 mM Tris-HCl and 1 mM EDTA, pH 8.5.

2.11 DNA sequencing (cycle sequencing method)

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.

2.12 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 (see appendix for more details).

2.13 Determination of RNA concentration

The concentration of RNA was determined by measuring the absorbance at wavelengh of 260 mm. An absorbance of 1 unit at 260 nm corresponds to 40 μg/ml of RNA (Sambrook *et al.*, 1989). The purity of RNA obtained was also determined from the ratio of the absorption reading at 260 nm and 280 nm (A_{260}/A_{280}).

2.14 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 which was more appropriate, to achieve normality and analyzed by the test of regression (at level of 95% confidence) prior to multiple comparison test.

Chapter 3

RESULT

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

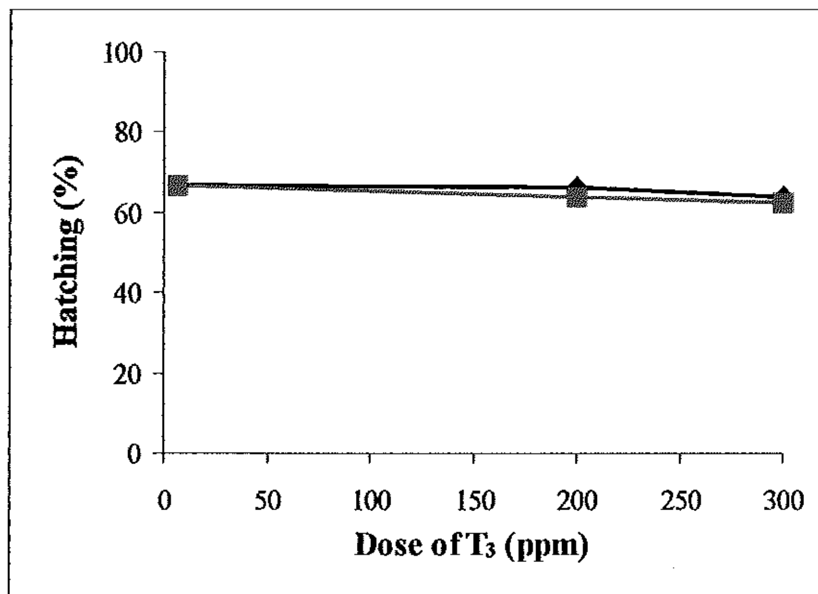
It has been widely known that thyroid hormones are important for early development of the vertebrates including fish. Although the effect of thyroid hormones on the hatching ability also has been studied in several fish. The response to the hormone involvement in hatching rate of the animals were still un-patterned, i.e. it could be ranged from positive to negative responsiveness from one to another species (Ayson and Lam, 1993; Lam and Sharma, 1985; Takawa and Hirano, 1991). Thus, thyroid hormones stimulate the embryonic development and/or hatching mechanism only in some fishes has been proposed (Lam and Sharma, 1985). In this thesis, the effect of thyroid hormones on hatching rate was attempted in the green catfish (*M. nemurus*) 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 developed 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 The effect of T_3 dosage and exposure period on the percentage of hatching

The experiment was performed in the 3 x 2 factorial design using concentrations of T_3 at 6.25, 200 and 300 ppm and the period of 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 %. The fertilized eggs incubated in the water contained T_3 until hatch did not show difference in the percentage of hatching from those exposed to T_3 for 1h (Figure 3.1). Neither the hatching rate of fish was significantly changed when eggs



	Hatching (% ± SD)	
Dose of T_3 (ppm)	**1 h**	**Until hatch**
6.25	66.45 ± 21.51	66.59 ± 21.37
200	63.88 ± 24.42	66.05 ± 24.47
300	62.35 ± 28.37	63.95 ± 30.05

Figure 3.1 Effect of T_3 doses and exposure time on hatching percentage of the green catfish. Fertilized eggs were exposed to T_3 (6.25, 200 and 300 ppm) for 1 h (■) or until hatched (36-48 h) (◆). Number of fish larvae hatched out was counted and the percentage of hatching was calculated as described in the method section.

were exposed to different concentrations of T_3 (Figure 3.1). No significant difference was confirmed by the two-way analysis of variance (ANOVA) at $p > 0.05$. The hatching percentage, with SD, at the concentrations of T_3 at 6.25, 200, and 300 ppm is shown in the table below the figure.

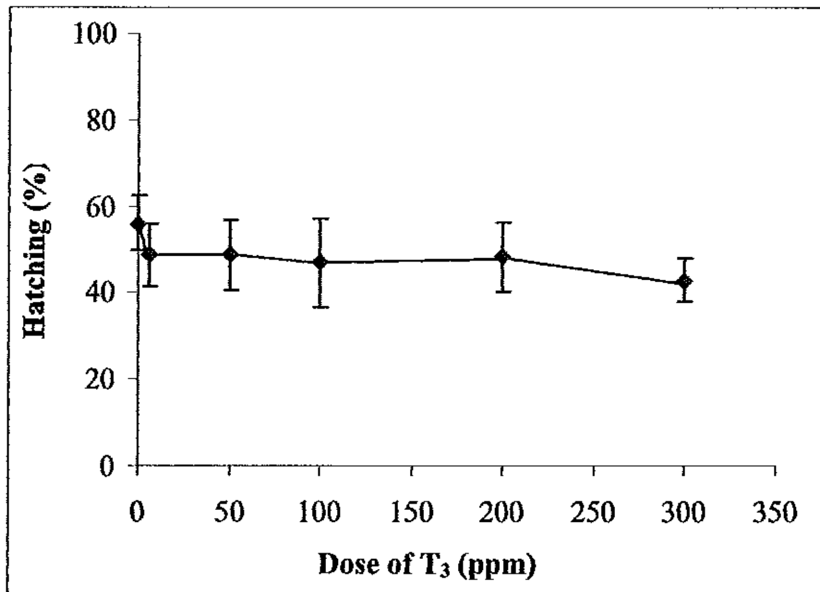
1.2 The effect of T_3 dosage on the hatching rate

Since dosage of T_3 examined, i.e. 6.25, 200, and 300 ppm, in studying the period of exposure to the hormone of fish eggs showed no significant difference in hatching rates between the treatments and the control. The question arose whether the appropriate dosage of T_3 could be in somewhere between 6.25 ppm to 300 ppm. So, next attempt was performed with a few more doses of T_3. Fertilized green catfish eggs were exposed to T_3 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 3.2 showed the hatching percentages of treated eggs which ranged from 42 to 49 %, while that of the control was around 56 % (see table below the figure for more details). However, by one-way analysis of variance, it revealed none of significant difference ($p > 0.05$) between the treatments and the control. Thus, this could definitely be concluded that T_3 exerts non-effect on the hatching of the green catfish.

1.3 The effect of T_4 doses and exposure time on hatching of green catfish eggs

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 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.



	Dose of T_3 (ppm)					
	0	6.5	50	100	200	300
Hatching (% ± SD)	55.93 ± 6.312	48.85 ± 7.245	48.69 ± 7.961	47.13 ± 10.26	48.27 ± 8.016	42.83 ± 5.029

Figure 3.2 Effect of T_3 doses on hatching (%) of the green catfish. Fish eggs were exposed to T_3 (6.25, 50, 100 and 300 ppm) until hatched. The control (0 ppm of T_3) was hatched in water containing 30 ppm of DMSO. Vertical bar indicates the standard deviation (SD).

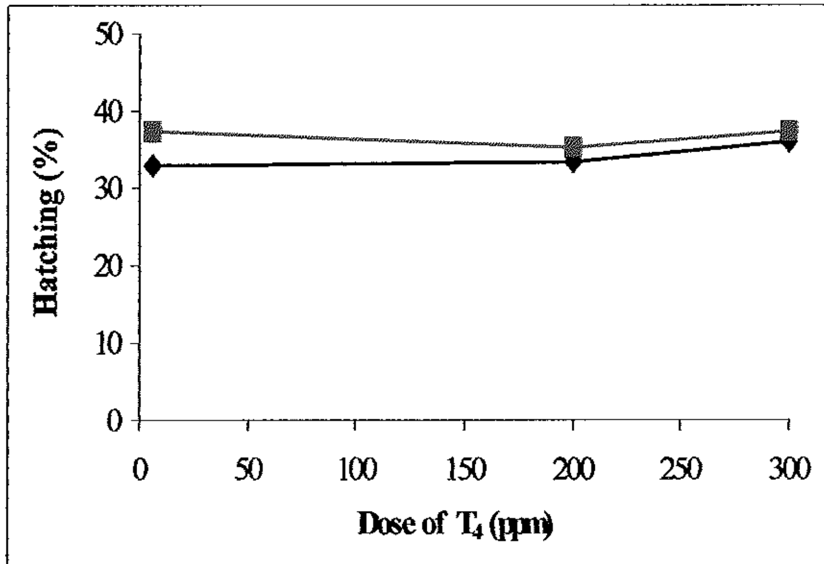
The percentages of hatching obtained in this experiment ranged from 33 to 38% as shown in Figure 3.3 (see table below the figure for more details). The hatching rates of eggs exposed to T_4 for 1 h were slightly higher than those longer exposed to the hormone. However, the two-way analysis of variance at $p > 0.05$ revealed no statistical difference between the treatments exposed to T_4 for 1 h and those exposed to the hormone until hatch. The hatching rates among of all treatments exposed to different concentrations of the hormone (Figure 3.3) 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 by immersing fish larvae into thyroid hormone containing water for both short and long periods was observed in several fish species (see review of literature for details). Thus, in this thesis, experiments were performed to reveal the effects of T_3 on survival of the green catfish. In order to define the developmental periods that thyroid hormone is a necessity for life, the observation was thus performed at several post-hatched ages of the catfish, i.e. 3, 7, 15, 30 and 45 days. The experiments were also developed to reveal both short and long treatment effects and to search for the effective doses of the hormone in enhancing the survival rate of the fish.

2.1 The effect of T_3 dissolving agent on green catfish survival rate

Because of the aromatic phenyl-structure that causes the molecule to be a hydrophobic compound, T_3 has to be dissolved in an organic solvent to form a solution. Normally, two organic solvents, i.e. methanol and dimethylsulfoxide (DMSO), have been used for *in vitro* experiments of T_3. However, no observation was reported on the possible effect of these solvents on fish larvae. Thus, this experiment was conducted to select the most appropriate solvent for use in the survival studies of the catfish. The catfish larvae at 3, 7, 15, 30, and 45 days of age were reared in water containing 2 ppm of methanol or DMSO for 1 day. The larvae were then transferred to normal water and reared without feeding, the numbers of fish survived was recorded daily.



	Hatching (% ± SD)	
Dose of T_4 (ppm)	1 h	Until hatch
0	-	34.37 ± 15.92
6.25	37.34 ± 8.37	32.99 ± 14.04
200	35.37 ± 9.64	33.45 ± 8.42
300	37.41 ± 9.75	36.17 ± 6.13

Figure 3.3 Effect of T_4 on hatching of the green catfish. Fish eggs were fertilized and immersed in water containing 6.25, 200 and 300 ppm of T_4. Number of the fish larvae hatched from eggs exposed to T_4 for 1 h (■) or until hatched (36-48 h) (◆) was determined and the percentage of hatching was calculated.

Survival profile of the fish at each age was shown in Figure 3.4. In the larvae of 3 and 7 days old, the profile obtained from the methanol treatments was not different from that of the DMSO treatments (Figure 3.4**A**, **B**). The larvae at 3 days after hatching gradually increased in mortality rate during the first couple of days after being exposed to the solvent, then the rate increased rapidly thereafter (Figure 3.4**A**). Similar survival profile was observed in fish larvae of 7 days after hatch. However, the decrease of survival was steeper and occurred immediately after the day the animal were exposed to the solvent (Figure 3.4**B**).

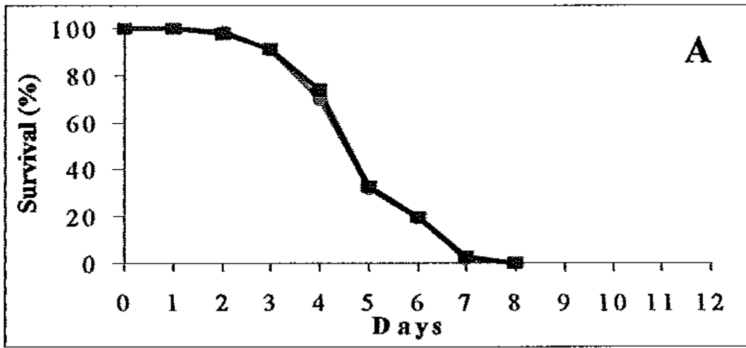
Some differences were observed in the profile obtained from fish larvae of 15, 30, and 45 days after hatch. That is, while the survival in fish larvae of 15 and 30 days of age exposed to DMSO was higher than those exposed to methanol (Figure 3.4**C**, **D**). The mortality rate of the larvae at age of 45 days old exposed to DMSO was a little bit higher than that found in the animals exposed to methanol (Figure 3 .4**E**). However, survival rates between the DMSO and the methanol treatments, were not statistically different ($p > 0.05$).

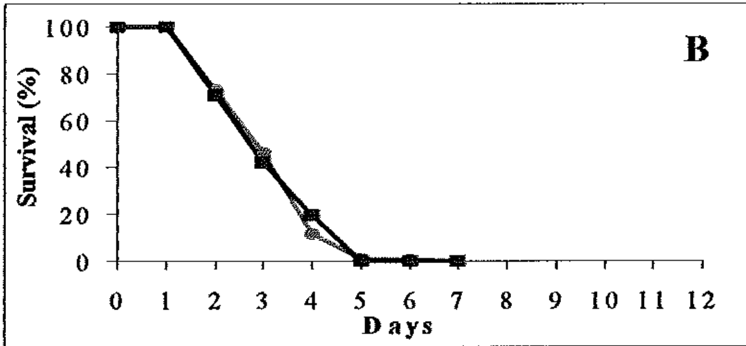
From the results obtained above as well as because of the fact that DMSO has higher boiling point (189 °C) than methanol (65 °C), thus it is less volatile and risk of vapor inhalation. The DMSO was accordingly chosen to be a solvent for dissolving T_3 in further experiments.

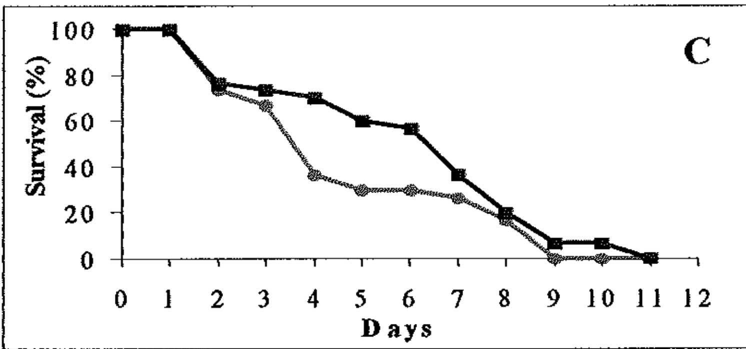
2.2 Effect of short-term treatment of T_3 on survival profile of green catfish

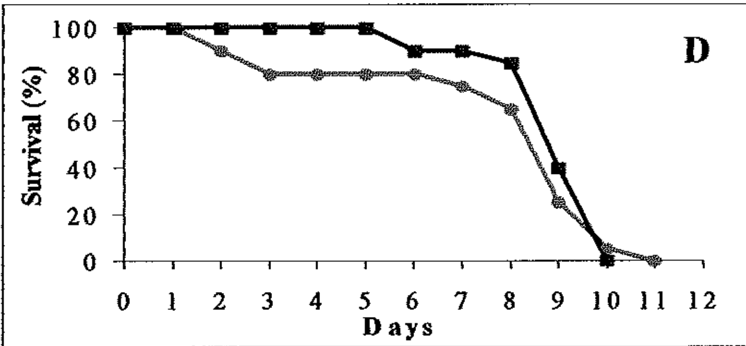
To determine the effect of short-term treatment of T_3 on the survival profile, the catfish larvae of 3, 7, 15, 30, and 45 days after hatch were exposed to T_3 at various concentrations for 24 h. They, then were transferred and maintained in normal water without feeding. The numbers of viable fish were recorded as described in Methods.

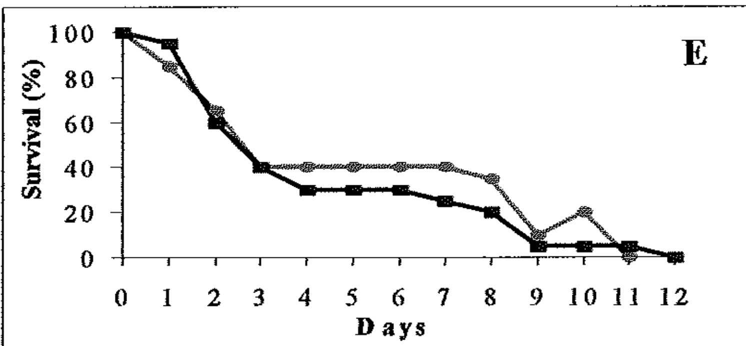
Survival profiles obtained from the animals of each age at different concentrations of T_3 were shown in Figure 3.5. The experiments revealed that green catfish at different ages were varied in responsiveness to T_3. In all of the catfish at 3 and 7 days after hatch, the mortality rates of the treated groups were higher than in the control at all concentrations of T_3. The mortality of the fish occurred immediately after the day they were exposed to the hormone (Figure 3.5**I**). The mortality rate reached maximum on day 5 and day 3 (after exposed to T_3) observed in the larvae of


A
Survival (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Days

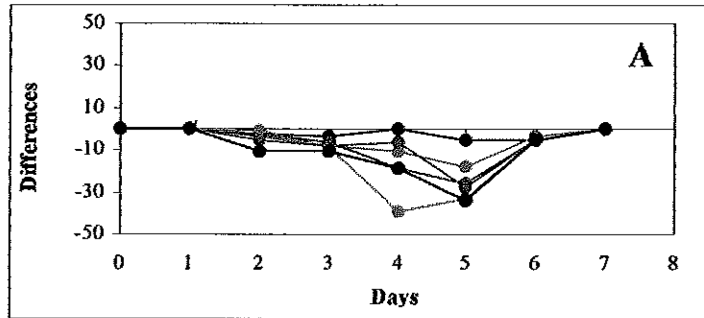

B
Survival (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Days

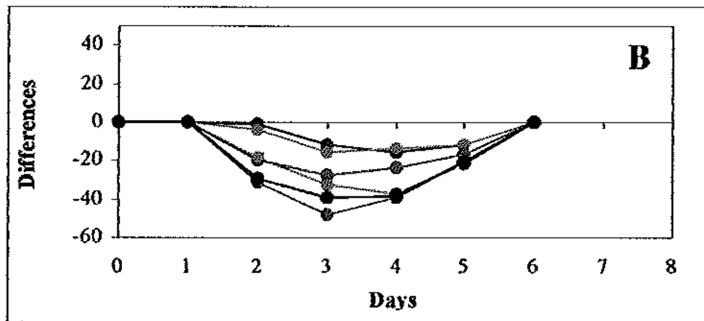

C
Survival (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Days

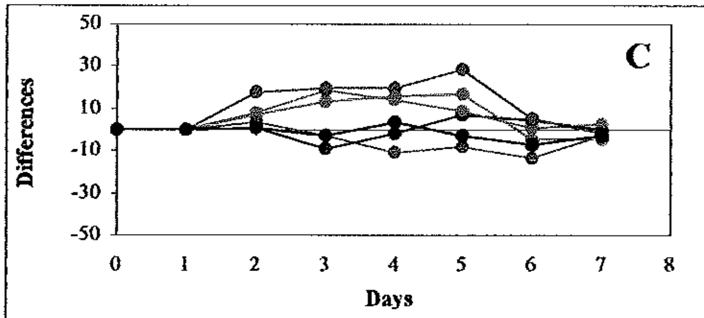

D
Survival (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Days

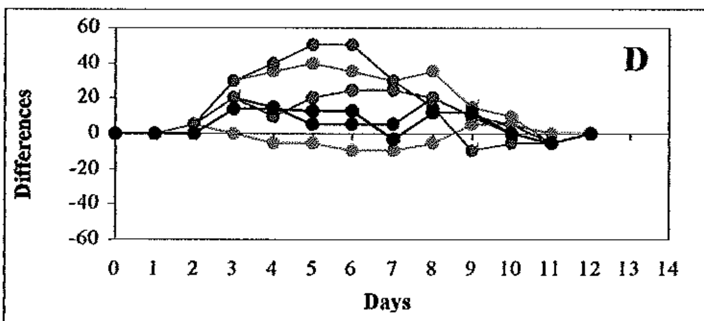

E
Survival (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Days

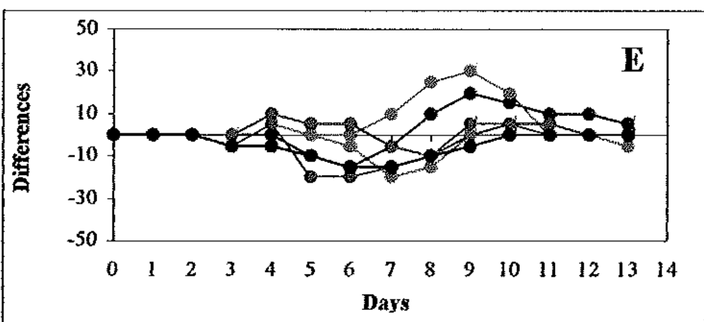
II











3 and 7 days old, respectively (Figure 3.5**II A, B**). Moreover, the higher concentration of T_3 the animals were exposed to, the higher mortality rate occurred.

On the contrary, the experiments in fish ages of 15, 30 and 45 days after hatch showed the effect of T_3 at a definite dosage on the survival of the larvae. At 15 days old of age, the exposure to T_3 at the concentrations of 10 to 80 ppm could decrease the mortality rate of the treatments more than that of the control (Figure 3.5**I C, 5II C**). Whereas, higher mortality rate was observed in the animals exposed to the hormone at 5, 160, and 320 ppm. For the green catfish larvae at 30 days after hatch that exposed to T_3 at all concentrations, except at 80 ppm, showed higher survival than the control. At 80 ppm of T_3, the mortality rate was slightly higher in the treatments (Figure 3.5**II D**).

Specific change of the survival profile was observed in fish of 45 days old. Higher and lower rates of mortality, compared with the control, could be observed within the same concentration of the hormone (Figure 3.5**I E**, and 3.5**II E**). T_3 at 5 ppm gradually increased the mortality rate to reach the maximum after the animals were exposed to the hormone for 6 days. Then, the mortality rate decreased to the maximum peak at day 9 of exposure to the hormone (Figure 3.5**II E**). At 10 ppm of T_3, the mortality rate of the fish was entirely decreased and the survival of the animals was higher than in the control. However, at the concentration of 20 ppm, change of the survival profile occurred in the opposite direction to that detected at 5 ppm of the hormone. That is, the survival increased during the first period after the larvae were exposed to the hormone. The mortality rate decreased in a gradual manner to reach minimum at day 4. Thereafter, the rate increased, thus bringing the survival down to the level below than that detected in the control (Figure 3.5**II E**). At the hormone higher than 80 ppm, the mortality rate was greater than that of the control (Figure 3.5**II E**).

Taken all the results obtained in this section could direct to the conclusion that T_3 exerted an involvement in mortality rate of the green catfish larvae. The enhancement effect on survival of the fish at each age occurred at only definite concentrations of the hormone, e.g. 10 to 80 ppm for the 15 days old fish, 10 to 20 ppm for the 45 days old fish. Moreover, within 5 to 320 ppm of T_3, only the larvae at

15 days after hatch and that with an older age could conduct the positive capable of responding to the hormone, i.e. decrease in the mortality rate..

2.3 Effect of long-term treatment of the T_3 on survival rate of green catfish

According to the results obtained in the result section 2.2, it was clearly shown that short-term treatment with T_3 enhanced the survival of the green catfish larvae. The effective dosages of the T_3 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, thus, arose whether and how the survival of the fish larvae would be if the animals obtain the hormone for a longer period. To answer this question, the green catfish larvae at 15, 30, and 45 days after hatch were reared in water containing 10, 20, and 80 ppm of T_3 for 15 days as described in Methods.

As shown in the Figure 3.6**A**, the survival of the 15 days old fish, after exposure to T_3 for 15 days, at all examined concentrations of the hormone, was lower than that found in the control. At 10 ppm and 20 ppm of the hormone, the mortality rate increased in a gradual manner from the first day after exposure and persisted throughout the experiment. Whereas, the larvae exposed to 80 ppm of T_3 showed rapidly declining survival and no animal was viable on the day before the end of the experiment. The differences of survival between the 15 days old green catfish larvae exposed to the 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 treatment at 20 ppm of T_3 showed higher survival level of the animals than that in the control at shorter period, i.e. within the first week of the treatment.

For fish 30 days of age, the significant enhancement of survival ($p \leq 0.05$) was observed at 10 and 80 ppm of T_3 (Figure 3.6**B**). The mortality rate occurred, however, at lower speed than that found in the control. At 80 ppm of the hormone, the mortality rate of the animals was steady after day 11 till throughout the experiment. However, at T_3 of 20 ppm, increase of the mortality rate was remarkable after the larvae were exposed to the hormone longer than 5 days (Figure 3.6**B**). The survival between the treatment and the control was statistically different by Dunnett test at $p \leq 0.05$.

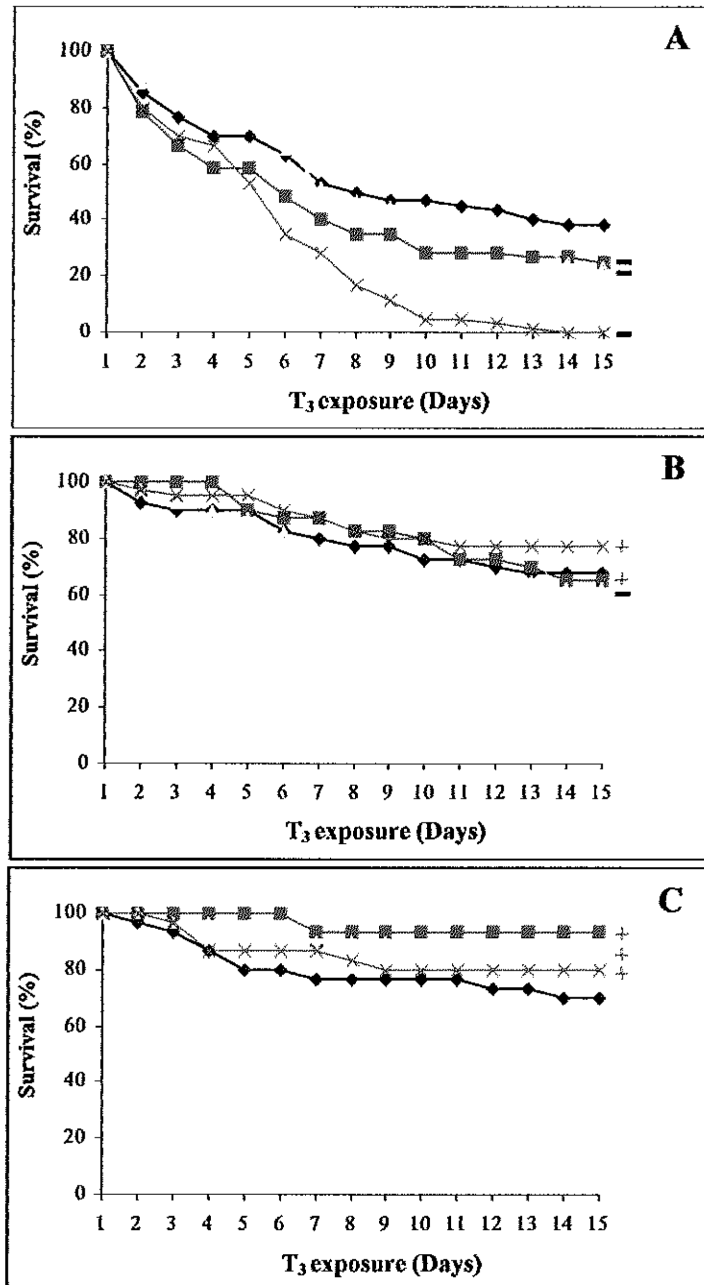


Figure 3.6 Effect of thyroid hormone on survival of the catfish. The larvae at 15 (**A**), 30(**B**) and 45(**C**) days of age were reared in the presence of 10 ppm (■), 20 ppm ( ), and 80 ppm (×) of T_3, while fish in the control (◆) was reared in the normal water. Fish was feed, and water was aerated and changed as described in the Methods. The survival was recorded everyday for 15 days. Statistical differences ($p \leq 0.05$), by Dunnett test, of the higher survival percentage of the treated fish than that of the control is indicated by positive symbol (in red), while that lower than the control is indicated by negative symbol (in black).

The experiment of the fish larvae at 45 days of age indicated that the hormone at all examined concentrations enhanced the survival of the fish larvae. At the day 15 of the treatments, more than 80% of survival was obtained (Figure 3.6C). However, the enhancement of the survival had a reverse correlation to the concentration of the hormone, i.e. the higher survival occurred at the lower T_3 concentration.

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

In vertebrates, thyroid hormones influence the metabolism of the 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 (see review of literature for details). However, the effect was so far different from one to another fish species. In this thesis, with an attempt to demonstrate the influence of T_3 on metabolism of the catfish, expression level of two key enzymes of the carbohydrate pathway, G6PDH and LDH, was determined. Moreover, as it recently has been shown the cross-influence among nuclear hormone receptors signaling pathways (Bonilla *et al.*, 2001), the expression of TG, the retinoic acid-dependent multifunctional enzyme, was thus examined in order to reveal a possible cross-influence of thyroid hormone on the retinoic metabolism in the catfish.

To determine how T_3 exerts on the expression level of these three enzymes, three main investigations were undertaken. These include profiling for the enzyme expressions during fish growth, after short-term exposure to T_3, and after exposure to the hormone and challenge to stresses. The experiments were emphasized on the fish larvae at 3, 7, 15, 30, and 45 days after hatch, except the observation of an alteration in the expression level under pressure was carried out in the larvae of 15, 30, and 45 days of age.

3.1 Effect of T_3 on G6PDH

To determine the expression level of G6PDH during the growth of catfish, 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 from fish of each age is shown in Figure 3.7**A**. The protein expression pattern of G6PDH was non-linear correlated to the age of the larvae, but quite stable in range of 20 to 30 μmole NADPH/min/mg protein throughout the growth period of observation of the green catfish. The enzyme level increased in a gradual manner during the growth from 3 to 15 days after hatching, then gradually declined thereafter. The minimal level of the enzyme was observed at 30 days of age. The expression of G6PDH, then, increased once. At 45 days of age, the expression level of the enzyme was slightly higher than found in other ages (Figure 3.7**A**).

The effect of the short-term treatment with T_3 on the level of G6PDH was determined at concentrations of 5, 20, and 80 ppm of the hormone, and the larvae were exposed for 24 h. The alteration of the G6PDH expression levels was demonstrated in Figure 3.8. In the fish at 3 days of age, the enzyme level of the treatments was significantly higher than the control ($p \leq 0.05$) after the animals were exposed to 20 ppm of the hormone (Figure 3.8**A**). Whereas, the G6PDH level of the larvae at 7 days of age was significantly higher in every treatment than in the control (Figure 3.8**B**).

Similar observation was experienced in fish larvae of 15 days of age. The G6PDH expression level in the treatments increased to the higher level than in the control after the larvae obtained 20 and 80 ppm of T_3 (Figure 3.8**C**). The difference between the enzyme level of the treatments and that of the control was statistically significant by Dunnett test at $p \leq 0.05$. Hence, it is conceivable that at 20 ppm, T_3 exerted the most enhancements on the protein expression level of G6PDH in the green catfish during 3-15 days of growth. The opposite effect was observed in the catfish larvae at 30 days after hatch. The G6PDH level of the treatment was significantly lower ($p \leq 0.05$) than that in the control after the animals were exposed to T_3 at the concentration of 5 ppm (Figure 3.8**D**). However, at the same concentration of T_3 used, the expression level of the enzyme in the treatment was significantly increased to the

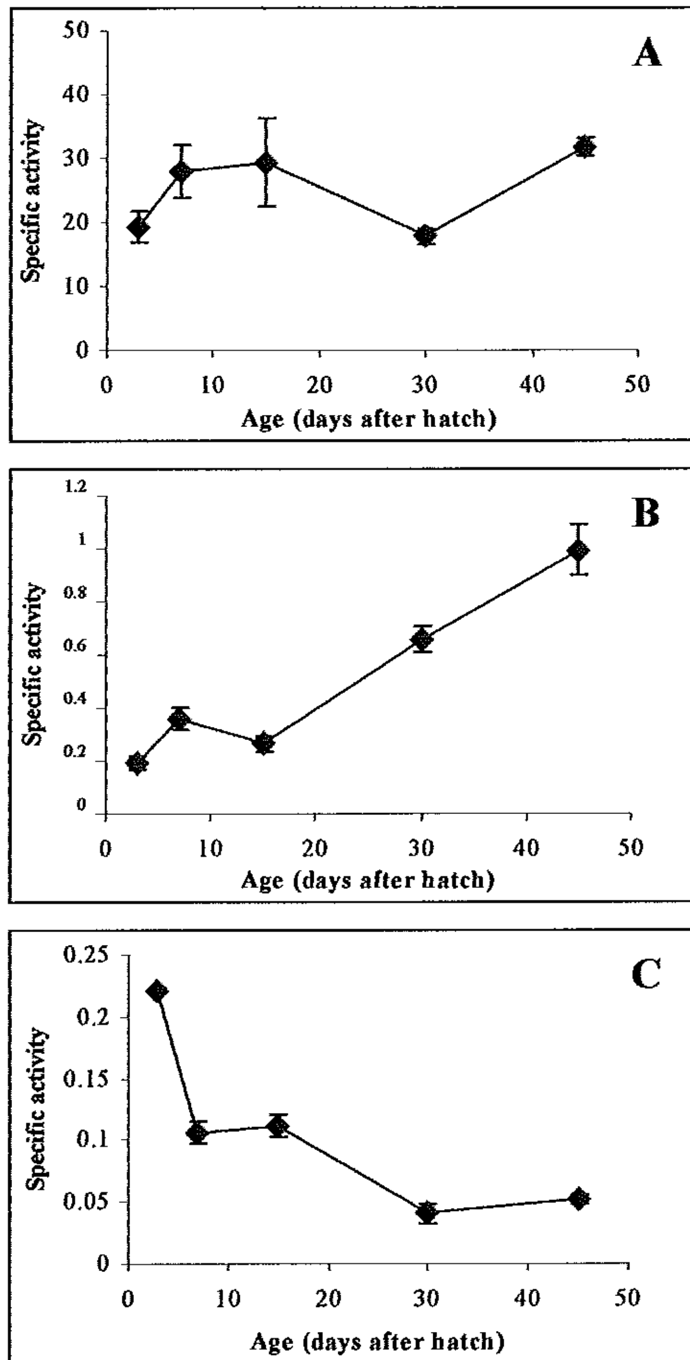
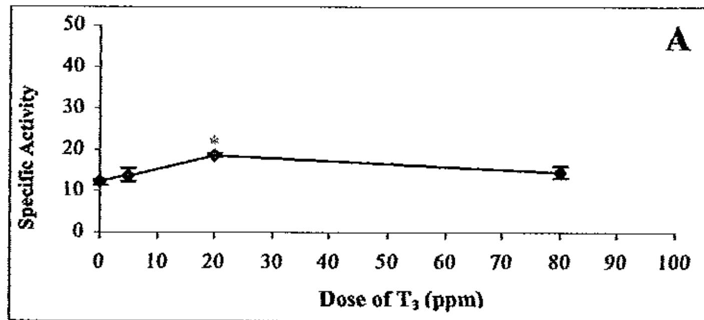
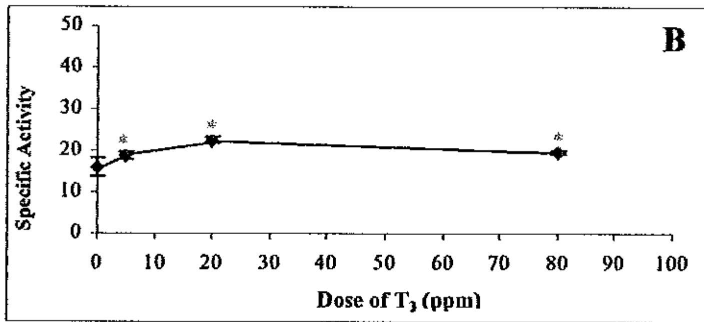
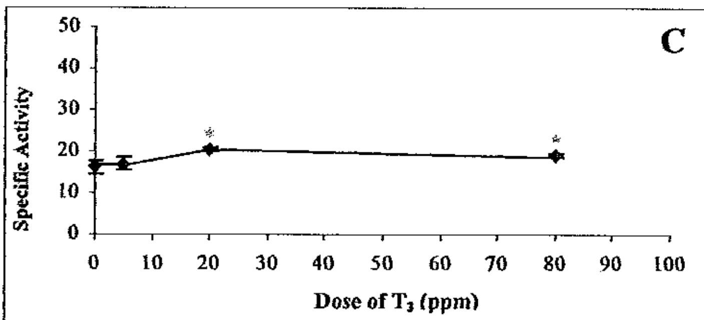
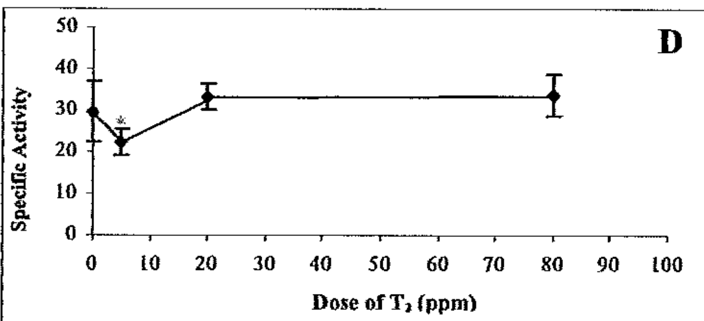


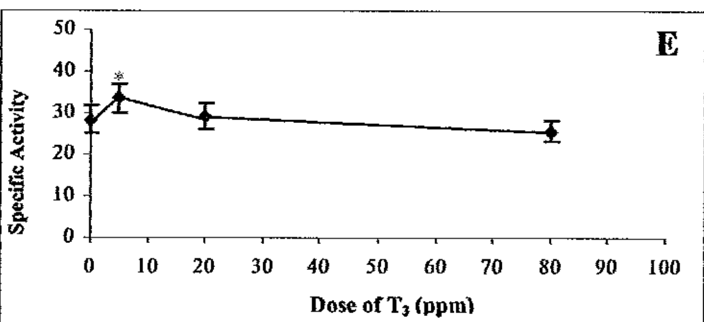
Figure 3.7 Specific activity profile of Glucose-6-Phosphate Dehydrogenase (**A**), Lactate Dehydrogenase (**B**), and Transglutaminase (**C**) during development of green catfish. Activity of enzyme was analyzed using the crude protein from 100-150 of the larvae 3 or 7 days of age, 25-40 of that 15 days of age, 3-5 of that 30 or 45 days of age. The fish from 3 separate experiments were extracted and determined for the enzymes. For each protein extracts, the enzymes were assayed in tripicate. Vertical bar indicates the standard deviation (SD).


A
Specific Activity
0
10
20
30
40
50
0 10 20 30 40 50 60 70 80 90 100
Dose of T3 (ppm)


B
Specific Activity
0
10
20
30
40
50
0 10 20 30 40 50 60 70 80 90 100
Dose of T3 (ppm)


C
Specific Activity
0
10
20
30
40
50
0 10 20 30 40 50 60 70 80 90 100
Dose of T3 (ppm)

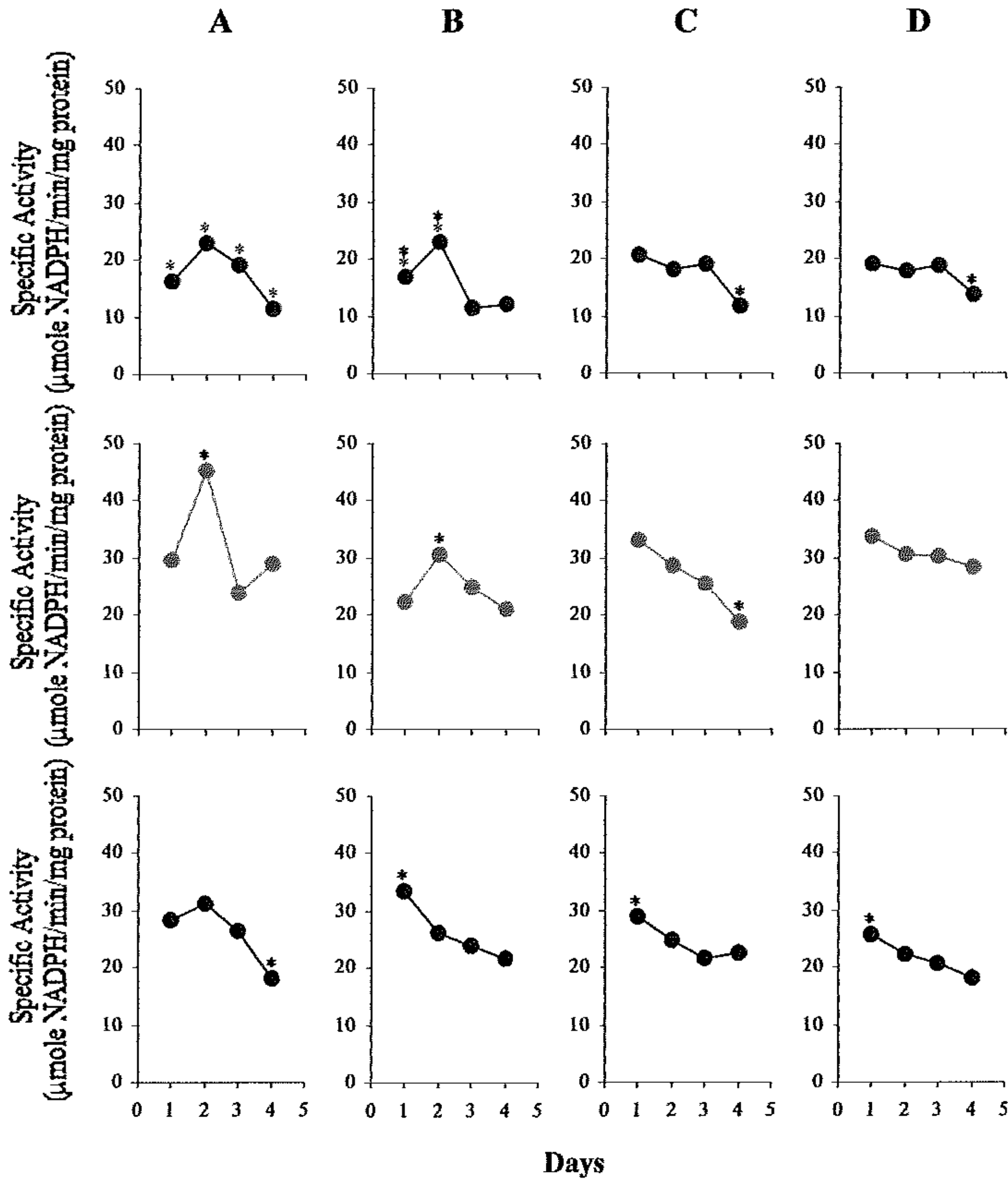

D
Specific Activity
0
10
20
30
40
50
0 10 20 30 40 50 60 70 80 90 100
Dose of T3 (ppm)


E
Specific Activity
0
10
20
30
40
50
0 10 20 30 40 50 60 70 80 90 100
Dose of T3 (ppm)

higher level than that in the control, was detected in the fish larvae at 45 days of age (Figure 3.8**E**)

In the investigation for an alteration of the G6PDH level in T_3 treated catfish during being challenged to the depletion of an energy supply, the 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. Fish were collected each day, and G6PDH was determined as described in Methods. An alteration of the enzyme expression level was shown in Figure 3.9. In the control fish at 15 days of age, the gradual increase of the enzyme level occurred from day 1 to day 2, then decreased in day 3 and was persistent through the day 4 (Figure 3.9A; first row). An alteration of the enzyme level similar to that of the control was found in the larvae exposed to T_3 at 5 ppm. The increase of the level occurred from day 1 to day 2 after exposure to the hormone (Figure 3.9B; first row). However, the rapid decline of the level took place on day 3 then the level had been steady till day 4. The increase of G6PDH level that occurred in the fish of control and those exposed to T_3 at 5 ppm would indicate the raise in consumption of the blood glucose in these animals during the first few days after exposure to the hormone and were challenged to pressure. The different alteration of the G6PDH level was observed in the larvae exposed to 20 and 80 ppm of the hormone. The enzyme level was not raised up as experienced in the control and the T_3-treatments, at 5 ppm. Significantly unchanged ($p > 0.05$) was detected during day 1 to day 3 of the treatment. However, the expression level significantly decreased ($p \leq 0.05$) in day 4 of the challenging period (Figure 3.9C, D; first row). It obviously showed that the expression of G6PDH in the larvae obtained T_3 at high dosage kept steadily, i.e. no sudden raise up or fall down, at the low level during the first few days the fish faced to the energy lacking.

In the larvae 30 days of age, the extremely increase of the G6PDH level was detected from day 1 to day 2 in the control (Figure 3.9**A**; second row). The similar elevation of the enzyme level, but with less degree, also observed in the larvae those obtained T_3 at concentration of 5 ppm (Figure 3.9**B**; second row). The expression levels of G6PDH in the catfish exposed to 20 and 80 ppm of the hormone, however, were not altered significantly during the first 3 days. Moreover, the enzyme level was


A
B
C
D
Specific Activity (µmole NADPH/min/mg protein)
Specific Activity (µmole NADPH/min/mg protein)
Specific Activity (µmole NADPH/min/mg protein)
Days

continuously unchanged through the day 4 in the 80 ppm treatment (Figure 3.9**C**, **D**; second row). A siginificant ($p \leq 0.05$) decrease of the enzyme level was detected in the 20 ppm treatment on day 4 (Figure 3.9**C**; second row). In comparison, though the enzyme detected in both 20 ppm and 80 ppm T_3 treatments was unchanged in its expression levels, the enzyme in the 80 ppm treatment existed at higher level (Figure 3.9**C**, **D**; second row).

Comparing to that observed in 30 days of age, the control of 45 days of age showed less, or none, in changes of the G6PDH level (Figure 3.9**A**; third row). The significant decrease of the enzyme occurred on the last day of the observation. In the comparison to the larvae at 15 and 30 days of age, different alteration of the enzyme expression was found in all T_3 treated larvae of this 45 days of age. The steadiness of the enzyme level during the last final days, rather than in the first few days was experienced.

In compilation of all observations generated in this section, it clearly demonstrated to the same necessity of the G6PDH at all ages of the green catfish. Thyroid hormone (T_3), particularly at 20 ppm, increased the expression level of the enzyme after the catfish obtained the hormone for a short period. However, the expression of the enzyme was regulated to be at lower level during the animals were challenged with the pressure, i.e. lacking of an energy, for a long period. The thyroid hormone at specific amount increased the ability of the animals in down regulating the expression of the G6PDH. The lowering the enzyme level is probably a mechanism to redirect the utility of glucose for biosynthesis, e.g. fat, into energy production. This 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.

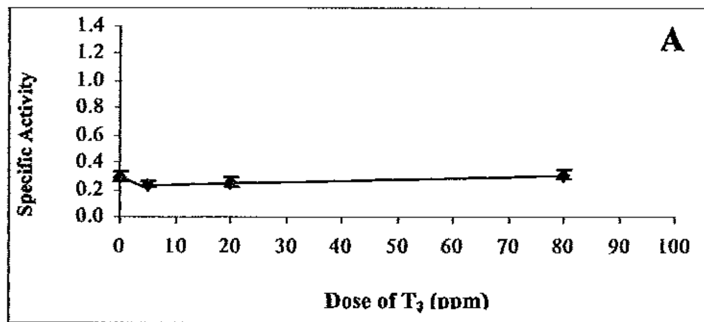
3.2 Effect of T_3 on LDH

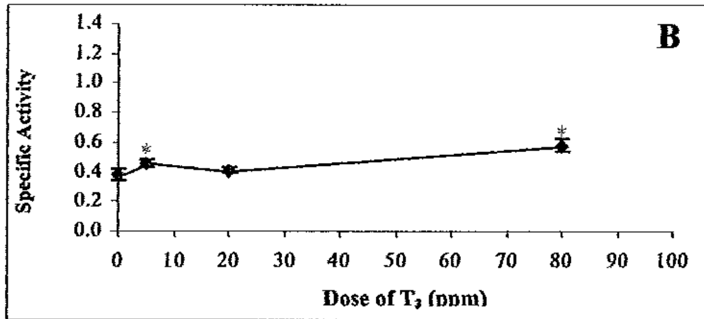
Different from the function of G6PDH, LDH is widely known as the key enzyme of an anaerobic energy-production of glucose breakdown. This energy production pathway is very important for cells those are sensitive to rapid ATP needs, or the oxygen depletion is experienced. In the green catfish, the expression of LDH observed during the growth of the animals was shown in Figure 3.7**B**. The enzyme

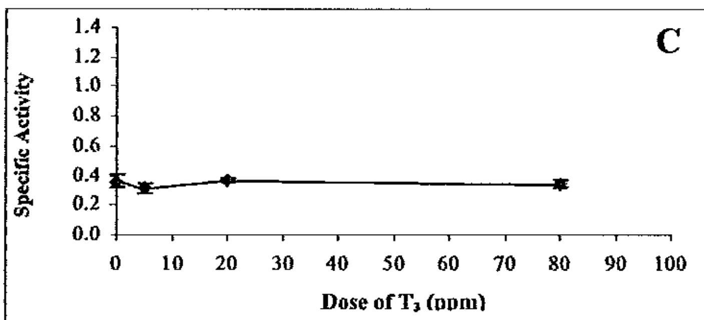
level was almost linear correlated to the age of the fish. An obvious decrease of the expression level of LDH occurred at 15 days of age. However, a drastic increase of the enzyme immediate occurred since there.

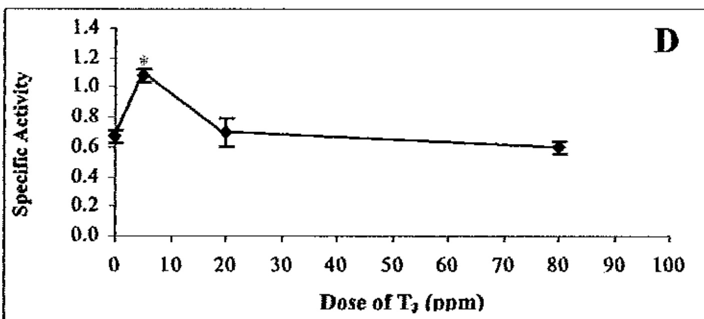
The short-term treatment with T_3 affected the LDH expression level of the green catfish larvae of each age, after they received the hormone for 24 h, in different direction. The enzyme levels in the larvae at 3 and 15 days of age were not significant changed ($p > 0.05$) from the control at every concentrations of the T_3 used (Figure 3.10**A**, **C**). The significant raising up of LDH expressions, however, at different concentrations of the hormone was observed in fish of 7, 30, and 45 days of age. In the larvae at 7 days of age, the expression of the enzyme in the animals exposed to 5 and 80 ppm of T_3 were significantly higher than that of the control (Figure 3.10**B**). While, T_3 at 5 ppm extremely enhanced the level of LDH in the fish at 30 days of age (Figure 3.10 **D**). The level of the enzyme was raised up to nearly twice of the level detected in the control. At two concentrations, i.e. 5 and 20 ppm, T_3 increased the LDH levels of the 45 days old catfish significantly (Figure 3.10**E**).

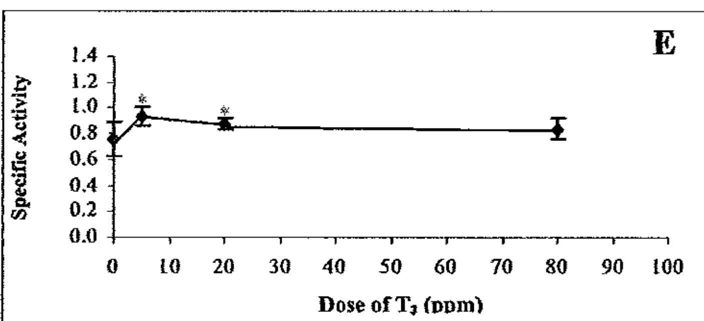
An alteration of the LDH expression profile, while the green catfish experienced the stress condition, i.e. exhausted of energy supply, was shown in Figure 3.11. In the catfish larvae at 15 days of age, the expression of the enzyme in the control gradually increased from day 2 to day 4 (Figure 3.11**A**; first row). An increase of the LDH level from day 2 to day 3 also was detected in the larvae treated with T_3 at 5 ppm (Figure 3.11**B**; first row). However, the level remained constant after day 3. Interestingly, at T_3 of 20 and 80 ppm, the expression level of LDH was unchanged throughout the period of being challenged to the pressure (Figure 3.11**C**, **D**; first row). It was maintained approximately at 0.3 µmole NADH/min/mg protein at both concentrations of the hormone.

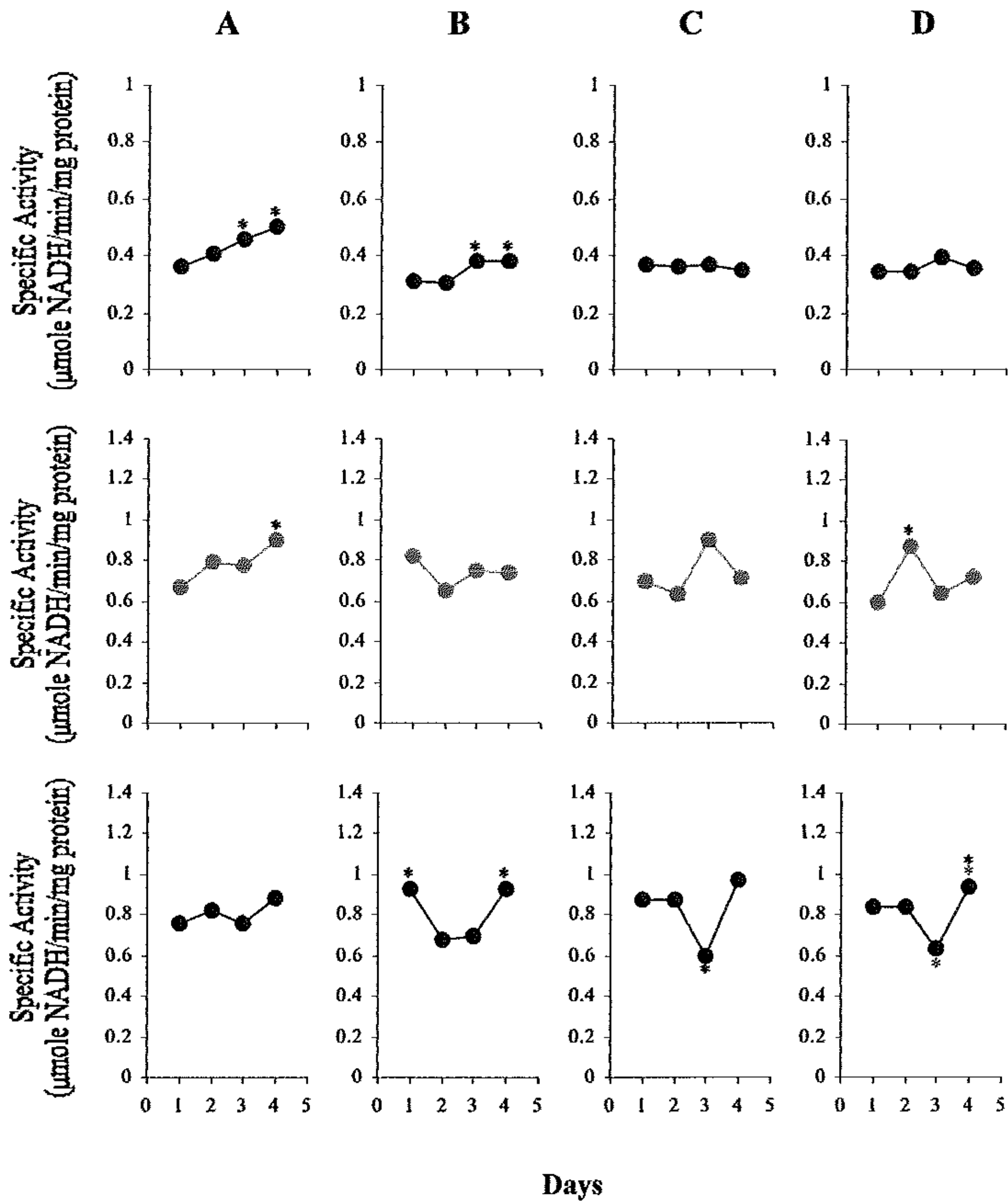

A
Specific Activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60 70 80 90 100
Dose of T3 (ppm)


B
Specific Activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60 70 80 90 100
Dose of T3 (ppm)


C
Specific Activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60 70 80 90 100
Dose of T3 (ppm)


D
Specific Activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60 70 80 90 100
Dose of T3 (ppm)


E
Specific Activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60 70 80 90 100
Dose of T3 (ppm)


A
B
C
D
Specific Activity (µmole NADH/min/mg protein)
Days

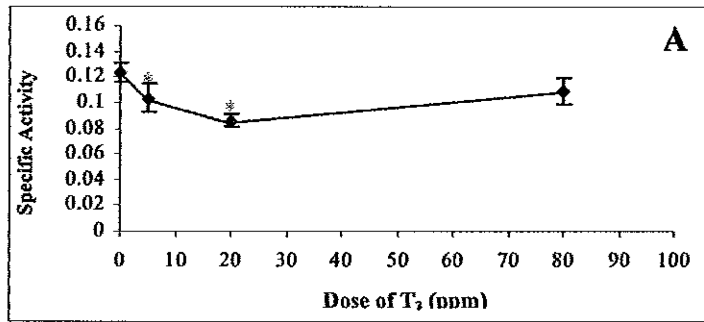
Additional steadiness of the LDH level was remarked in the control catfish larvae with an older age. At 30 days after hatching, the enzyme level of the control was only slightly increased in the day 4 of observation (Figure 3.11**A**; second row), whereas the enzyme expression was entirely unchanged in the fish control at 45 days of age (Figure 3.11**A**; third row). The constant enzyme level also occurred in the larvae 30 days of age exposed to T_3 at all concentrations (Figure 3.11**B**, **C**, **D**; second row). An exception, however, was at the 80 ppm of the hormone. The level slightly climbed up in day 2, but then fell down to the previous level and remained low in the following days (Figure 3.11**D**; second row). The decrease of the
LDH expression was significant in the T_3-treated catfish larvae at 45 days of age. The decrease occurred on day 2 or day 3 or both, and this apparently depended on the concentration of T_3. At the hormone of 5 ppm, expression of the enzyme significantly declined ($p \leq 0.05$) on day 2 (Figure 3.11**B**; third row). The level unchanged through day 3 prior ascended on day 4 to the same level as detected in day 1. Brief descending of the enzyme expression occurred in day 3, at 20 and 80 ppm of the thyroid hormone (Figure 3.11 **C**, **D**; third row). Declining of the enzyme expression to the previous level and to the slightly higher level were detected in the fish exposed to 20 ppm and 80 ppm of T_3, respectively.

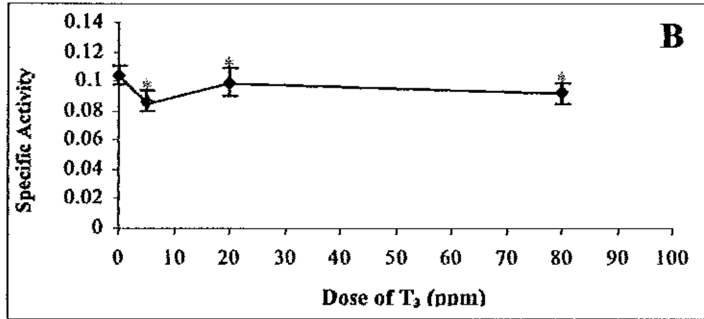
The observations obtained in this section could indicate the needs of LDH during the growth of the green catfish. The animals with an older age require higher level of the enzyme for their activities. Thyroid hormone at specific concentrations altered the expression level of the enzyme at specific ages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

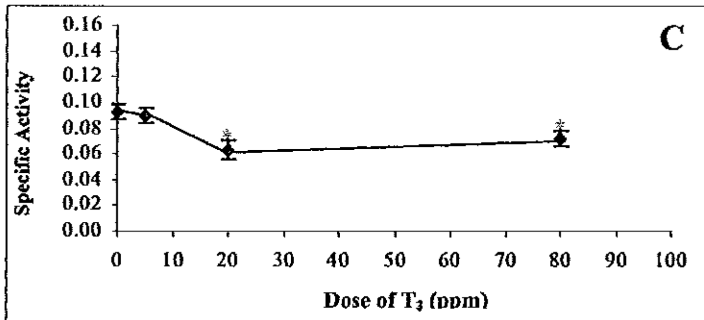
3.3 Effect of T_3 on TG

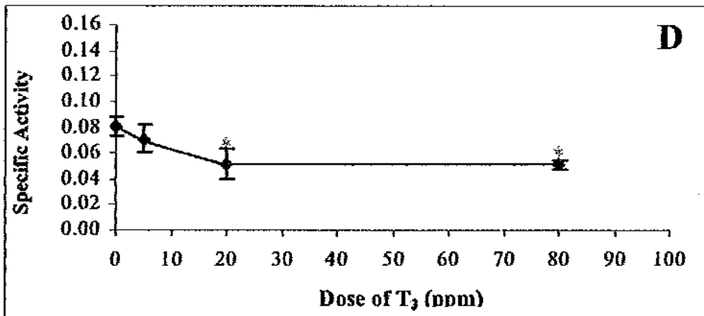
To determine the expression level of TG during the growth of the catfish, the larvae at 3, 7, 15, 30, and 45 days of age were collected and the activity of the TG was determined as described in Methods. The TG expression level of the animals was shown in Figure 3.7**C**. A reverse expression of the enzyme was detected comparing with that of LDH (Figure 3.7**B**). The decrease of the enzyme level took place during the 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 steady from 7 to 15 days of age. The decline of the TG level occurred once from 15 to 30 days after hatching, however in more gradual manner. The enzyme level, hitherto, was kept constant, though slightly higher expression of TG was observed in the 45 days old catfish larvae. Thus, it clearly demonstrated in this thesis that TG has less function during the early developmental period of the green catfish.

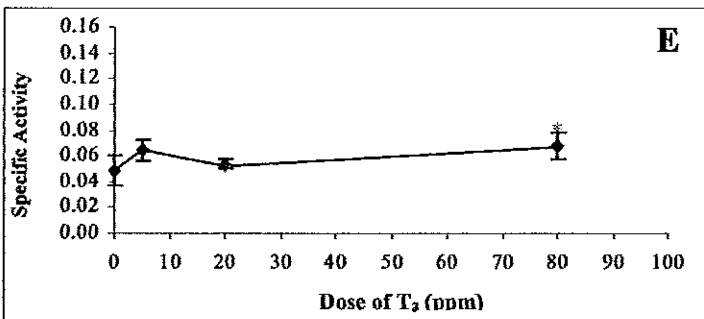
The thyroid hormone exertion on the expression level of TG after the catfish larvae obtained T_3 for a short period was demonstrated in Figure 3.12. The significant decrease of the TG level was observed in the 3 days old larvae, after the animals obtained T_3 at 5 and 20 ppm (Figure 3.12**A**). Whereas in the catfish larvae at 7 days of age, the enzyme level of the treated fish was significantly below that of the control at all examined concentrations of the hormone (Figure 3.12**B**). The similar regulation of the TG expression by the thyroid hormone was detected in the catfish at 15 and 30 days (Figure 3.12**C**, **D**). The decrease of the level occurred at 20 and 80 ppm, but not 5 ppm, of the T_3. Interestingly, a considerable increase of the TG level was observed in the green catfish larvae 45 days of age after the animals received 80 ppm of the thyroid hormone (Figure 3.12**E**). Thus, taken all results obtained, down regulation of the enzyme expression is applicable to all observations in which the larvae were treated with the thyroid hormone for 24 h.


A
Specific Activity
Dose of T_3 (ppm)


B
Specific Activity
Dose of T_3 (ppm)


C
Specific Activity
Dose of T_3 (ppm)

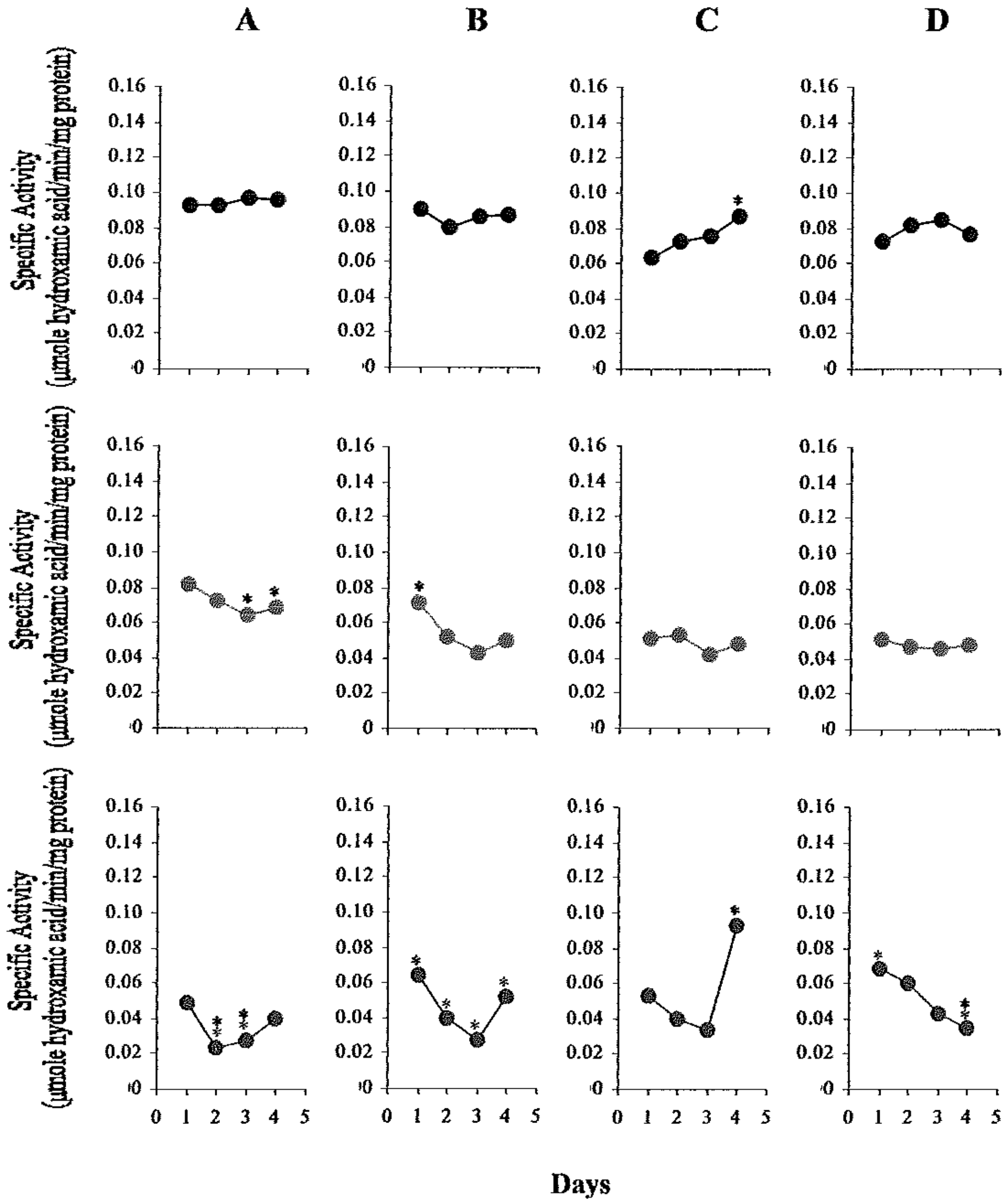

D
Specific Activity
Dose of T_3 (ppm)


E
Specific Activity
Dose of T_3 (ppm)

The alteration of the TG level during the 4 days period that the catfish were challenged to stress was shown in Figure 3.13. In the larvae at 15 days of age, the enzyme level was unchanged in the control, as well as in those exposed to 5 and 80 ppm of the T_3 (Figure 3.13**A**, **B**, **D**; first row). At the concentration of T_3 of 20 ppm, the expression of TG was steady during the first few days, however, at lower level than that observed in the control (Figure 3.13**C**). Slightly, but statistically significant increase ($p \leq 0.05$) to a higher level was revealed in the final day of the observation.

In comparison with the observations in the younger catfish, the TG expression in the larvae at 30 days old after the T_3 treatment, generally, was less than that in the control (Figure 3.13; second row). The enzyme level in the control gradually decreased from day 2 to day 3, then slightly increased on day 4 (Figure 3.13**A**; second row). However, the level on day 4 still was significant lower ($p \leq 0.05$) than that on the first day. In the T_3 treated larvae, the TG level was kept constant at low level throughout the challenging period at all concentrations of the thyroid hormone used (Figure 3.13 **B**, **C**, **D**; second row). An exception was detected at T_3 of 5 ppm that the TG expression significantly declined from day 1 to day 2. Then, the entire level was kept steady (Figure 3.13**B**).

In the catfish at 45 days of age, drastic changes in the expression level of TG was revealed (Figure 3.13; third row). The enzyme level in the control extensively decreased in day 2 and, then, increased in day 3 and day 4 (Figure 3.13**A**; third row). Extreme change in the level of the TG was found after the catfish larvae obtained thyroid hormone at 5 ppm (Figure 3.13**B**; third row). The enzyme level rapidly declined from day 1 through day 3 prior to a raise up on day 4. The difference in the enzyme expression level observed on each day was statistically significant by Newman-Keuls test at $p \leq 0.05$. An extensive raise of the TG in the animals being received T_3 at 20 ppm, was experienced on day 4 of challenging to the energy exhaust. At T_3 of 80 ppm, on the other hand, persistent decrease of the TG expression was detected throughout the observation. The most remarkable reduction was on the day 4 (Figure 3.13**D**; third row).


A
B
C
D
Specific Activity
(μmole hydroxamic acid/min/mg protein)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
0 1 2 3 4 5
Days

Although, the multifunctional enzyme, TG, was widely detected in animal including fish (Yasueda *et al.*, 1994; Sano *et al.*, 1996), however the biological function in fish remains unknown. According to the knowledge obtained from studies in human, the expression of TG is directly mediated through the retinoic acid receptor (RAR) and its partner, retinoid X receptor. The latter is known as a central receptor that plays role in the actions of the receptor of thyroid hormone (Mangelsdorf *et al.*, 1995). The observations obtained in this section clearly demonstrated that the expression level of TG was changed at several concentrations of T_3. This could 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 in this thesis, 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 on expression level of thyroid hormone receptor

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 more insight of the mechanism of thyroid hormone actions on development, survival, and metabolism of fish, study of thyroid hormone receptor is a must. In this thesis, the gene expression level of thyroid hormone receptor after short-term and long-term treatments with T_3 were investigated in the catfish larvae. According to the knowledge generated from the effect of thyroid hormone on the survival of the catfish (section 2.2 and 2.3), the TR level under the short-term exposure to the hormone was investigated in fish larvae at 3, 7, and 15 days of age. On the other hand, the expression level of the TR regulated by long-term treatment with the thyroid hormone was determined in the catfish larvae at 30 and 45 days of age. The Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) technique was adopted to amplify the TR mRNA, thus the expression of the gene during development of the

fish as well as an alteration of the expression of the TR occurring during the action of thyroid hormone could be pursued.

4.1 Isolation of partial fragment of green catfish thyroid hormone receptor (TR) cDNA fragment

In order to amplify and determine the expression level of the gene of interest by RT-PCR, a pair, forward and backward, of oligonucleotide primer that definitely recognizes the sequence of the gene is required. Specific primer design is known as a critical step for the success in the determination of the gene expression level by RT-PCR. Normally, the specific primers could be synthesized based on the available nucleotide sequence, full-length or partial fragment, of that gene. However, neither nucleotide sequence nor amino acid sequence of TR of catfish is available in the database. Even though, nucleotide sequences of the TR have been reported in some fish species (Marchand *et al.*, 2001; Yamano *et al.*, 1994), those sequences are quite low in similarity. Thus, in this thesis, isolation of the partial fragment of the TR gene was attempted in the green catfish with two major purposes. These are to reveal the nucleotide sequence of the gene of the green catfish and to obtain the nucleotide sequence of the gene for the specific primer design.

To isolate the partial fragment of TR gene from the green catfish, an amplification of the TR cDNA by RT-PCR was performed using the forward and backward degenerate primers. The pair of primers was designed from the conserved regions in the TR genes reported in other fish species (Marchand *et al.*, 2001), using the Consensus Degenerate Hybrid Oligonucleotide Primers program developed by Rose *et al.* (1998). The cDNAs were denatured at 94 °C for 6 min, and followed by 50 cycles at 94 °C (30 s), 55 °C (2 min), and 72 °C (1 min), then ending with 5 min of extension at 72 °C. The PCR product was separated and analyzed on 1.5 % agarose gel. The cDNA fragment of desired size (~ 400 bp) was obtained. The cDNA was extracted and purified from the gel according to the protocol described in section 2.7, and the purified cDNA was inserted into the pGEM-T easy vector as described in the section 2.8. The nucleotide sequence of the inserted cDNA fragment was determined by the cycle sequencing method described in section 2.10. The identity of the translated amino acid sequence of the cDNA was explored among those available in

the GenBank databases, using the BLAST algorithm (Altschul *et al.*, 1990, 1997). The catfish sequence showed highest identity to the α - isoform of TRs reported in fish and other vertebrate species. The lower identity of the amino acid was exhibited toward to the β-isoform of the TRs. Further comparison of the sequence to the conserved domain database revealed the presence of motifs which are ligand binding domain (LBD) found in all vertebrate TRs. It was, therefore, concluded that the isolated fragment of cDNA clone is the TRα of the green catfish.

The nucleotide sequence together with its deduced amino acid sequence of the green catfish TRα cDNA fragment are given in Figure 3.14. The green catfish TR fragment clone obtained from this thesis is 369 nucleotides in length. This is equivalent to 123 amino acid residues which is approximately 30% of the entire TRα polypeptide strand reported in other vertebrate species (Marchand *et al.*, 2001; Essner *et al.*, 1997; Yamano *et al.*, 1994; Lazar *et al.*, 1988). To fully establish the identity of the isolated TR sequence of the catfish, it was aligned against some teleosts and other vertebrates TR proteins using Clustal W algorithm (Thomson *et al.*, 1994). The alignment of the amino acid sequence from the catfish TRα against those from other vertebrates is shown in Figure 3.15. Identity, calculated by the Clustal W algorithm, of the green catfish TRα at both the nucleotide and the protein levels with other animals is shown in Table 4.1. The green catfish TR exhibited the highest identity of both nucleotide and amino acid sequences to rainbow trout (*O. mykiss*) TR α-isoform.

To determine the expression levels of the gene of interest by PCR, a specific primer pair is usually designed from nucleotide sequence of that gene. With the TRα sequence obtained from previous section, a pair of primers for specific amplification of the TRα could be designed so that the expression levels of the TRα during developmental changes as well as during the action of thyroid hormone can be determined by RT-PCR. However, as the degenerate primers used in obtaining the nucleotide sequence extremely primed to a specific nucleotide region of the TRα gene. This was confirmed by not only a single band of the cDNA fragment was obtained from several independent reactions, but also these fragments all had the same nucleotide sequence (data not shown). Thus, the degenerate primers can be adopted in the investigation of the gene expression level of the TRα during the growth

```
AAC CGG GAG AAG AGG AAG AAG GAG GAG ATG GTG AAG ACG CTG CAG AAC   48
 N   R   E   K   R   K   K   E   E   M   V   K   T   L   Q   N    16

CGG CCC GAG CCC ACG GGT ACC GAA TGG GAG CTG ATC CGC ATC GTG ACC   95
 R   P   E   P   T   G   T   E   W   E   L   I   R   I   V   T    32

GAG GCT CAC CGC CAC ACT AAC GCC CAG GGC TCA CAC TGG AAA CAG AAA  143
 E   A   H   R   H   T   N   A   Q   G   S   H   W   K   Q   K    48

CGC AAG TTC CTG CCT GAA GAC ATT GGT CAG TCG CCT GTA GCC CCC ACG  191
 R   K   F   L   P   E   D   I   G   Q   S   P   V   A   P   T    64

TCA GAC GGG GAT AAA GTC GAC CTG GAA GCC TTC AGC GAG TTT ACC AAG  239
 S   D   G   D   K   V   D   L   E   A   F   S   E   F   T   K    80

ATC ATC ACG CCA GCC ATC ACC CGC GTC GTC GAC TTT GCC AAA AAA CTG  287
 I   I   T   P   A   I   T   R   V   V   D   F   A   K   K   L    96

CCC ATG TTT TCA GAG CTG CCT TGT GAG GAT CAG ATC ATC CTG CTG AAG  335
 P   M   F   S   E   L   P   C   E   D   Q   I   I   L   L   K   112

GGC TGC TGT ATG GAA ATA ATG TCC CTG CGG                          367
 G   C   C   M   E   I   M   S   L   R                           122
```

Figure 3.14 Partial nucleotide sequence and its deduced amino acids of green catfish thyroid hormone receptor alpha isoform (TRα). 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 number at right, beginning with first nucleotide of the fragment obtained from RT-PCR. Ligand-binding domain (LBD) found in all TRs is underlined.

```
Human                       NRERRRKEEMIRSLQQRPEP TPEEWDLIHIATEAHRSTNA
Rat                         ******************** *********V**********
Xenopus laevis              **Q********KT******* SS***E**R*V*********
Salmo salar                 **VK*K ***VKT**N**** *GS**E**RML*****H***
Oncorhynchus mykiss         ***K*K****VKT**N**** *GS**E**RML*****H***
Danio rerio                 ***K*K***IVKT*HN**** *VS**E**RMV*****H***
Paralichthys olivaceus      *****K***IVKT**N**** *GA**E**RMV*****H***
Anguilla anguilla           *****K****VKT**N**** CSS**E**RLV*****H***
Takifugu rubripes           **QK*K****V*M**A**** DTA**E**RMV*****H***
Mystus nemurus              ***K*K****VKT**N**** *GT**E**R*V*****H***
Conserved                   **  *  **    *  ****    ** **   ***** ***
                                               160                  180

Human                       QGSHWKQRRKFLPDDIGQSP IVSMPDGDKVDLEAFSEFTK
Rat                         ******************** ********************
Xenopus laevis              *************E****** MA******************
Salmo salar                 *******K*****E****** V*PTS***************
Oncorhynchus mykiss         *******K*****E****** VAPTS***************
Danio rerio                 **P****K*****E******  APTS*N*************
Paralichthys olivaceus      **AQ***K******K***** VAPTS***************
Anguilla anguilla           *C*****K*****E****F* VAPNS**********R****
Takifugu rubripes           E**T***K****S*****G* VVPSS***************
Mystus nemurus              *******K*****E****** VAPTS***************
Conserved                       *** ****   *** *      * ******** ****
                                               200                  220

Human                       IITPAITRVVDFAKKLPMFS E LPCEDQIILLKGCCMEIM SLR
Rat                         ******************** * ****************** ***
Xenopus laevis              *************E****** * *T**************** ***
Salmo salar                 ******************** G ****************** ***
Oncorhynchus mykiss         ******************** * ****************** ***
Danio rerio                 ******************** * ****************** ***
Paralichthys olivaceus      ******************** *Q****************** ***
Anguilla anguilla           *******G************ * ****************** ***
Takifugu rubripes           *M****************** * ****************** ***
Mystus nemurus              ******************** * ****************** ***
Conserved                   * ***** ************   * **************** ***
                                               240                  260 263
```

Figure 3.15 Comparison of amino acid sequence of the green catfish thyroid hormone receptor fragment to those from other fish and vertebrate species. The alignment was constructed using Cluster W algolithm. The numbering of amino acid is based on that of human TRα. The sequence for human TRα is written using a single-lettet amino acid abbreviation. Gap was introduced to aid alignment. The residues those in other animal species with identical amino acids to those of human are indicated by astyerisks. Amino acid sequence of green catfish was bold. Conserved residues are indicated by the blue asterisks.

Table 4.1 Comparison of identity (%) of nucleotide sequence and deduced amino acid sequence of green catfish thyroid hormone receptor alpha to those from other vertebrates

Vertebrate species	Nucleotide sequence (% identity)	Amino acid sequence (% identity)
Homo sapiens (Human)	79	84
Rattus norvegicus (Rat)	80	83
Xenopus laevis	75	87
Oncorhynchus mykiss (rainbow trout)	89	97
Salmo salar (Atlantic salmon)	86	90
Paralichthys olivaceus (Japanese flounder)	84	93
Danio rerio (zebrafish)	83	93
Takifugu rubripes (Taki puffer)	80	86

as well as during the thyroid hormone actions of the green catfish without a need of new pair of primers. The expression level of the TRα gene, then, was determined by relatively comparing or normalizing the amount of the TRα gene to the amount of the beta-actin gene, a known T_3-nonresponsive gene.

4.2 The gene expression of thyroid hormone receptor TRα during the growth of the green catfish larvae

To determine the TRα expression during development of the green catfish, whole body of the larvae at 3, 7, 15, 30, and 45 days of age were 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 expression level then was followed by semiquantitative RT-PCR determination. The amount of the TRα was relatively compared with that of beta-actin, a house-keeping gene.

The changes in the expression level of the TRα during the growth of the green catfish were shown in Figure 3.16. It clearly showed that the level of the receptor gradually decreased after the age of 3 days and reached the minimum level at 15 days after hatching. Then, the expression level rapidly increased, and was kept steadily from 30 to 45 days of age. At 45 days after hatching, the expression of the TRα was at the similar level as found in the larvae at 3 days of age. Thus, from the results, it clearly indicated an accumulation of the TRα during the growth of the catfish. Since the higher in TR level suggests the more capability of responding to thyroid hormone of the animals. It, thus, could be implied that thyroid hormone exerts more important role in the green catfish with an older of age. Moreover, as numerous investigations have implicated the involvement of the thyroid hormone in developmental and differentiation processes occur during the growth of fishes (for examples see Jones *et al.*, 2002; Yamano and Miwa, 1998; Donaldson *et al.*, 1979). It could be conceivable that thyroid hormones also exert their actions on development and organogenesis of the green catfish via TR.

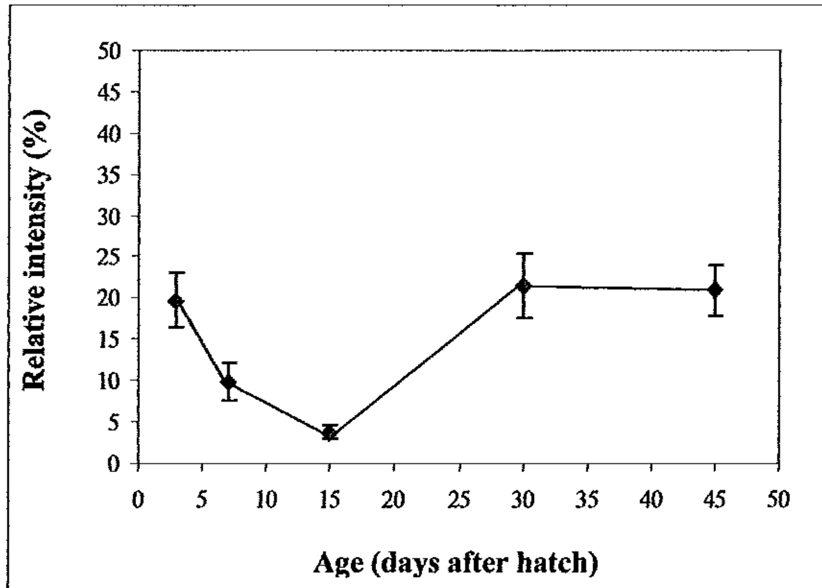


Figure 3.16 Change in the level of TRα gene during development of green catfish. Total RNA was purified from the fish body tissue, and RT-PCR was performed to amplify the TR gene. The PCR products were analyzed on an agarose gel electrophoresis in the presence of ethidium bromide. Intensity of the single band of the gene was measured by Document Gel. The percentage of relative intensity of the TR gene band to that of the house keeping (beta-actin) gene was calculated. At least 3 of independent PCR were performed for each point. Vertical bar indicates standard deviation (SD).

4.3 Changes in expression level of TRα during the actions of thyroid hormones

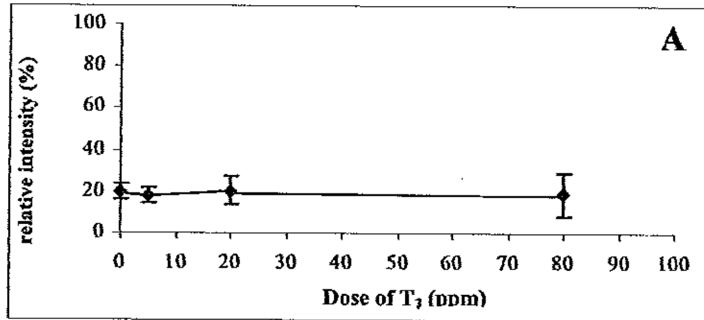
4.3.1 After the short-term treatment with T_3

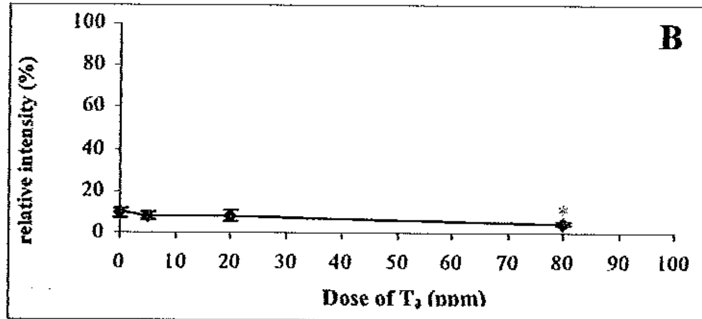
To examine the changes of TRα expression after the fish was treated with thyroid hormone for a short period, the larvae at 3, 7, and 15 days of age were exposed to T_3 for 24 h before the receptor expression level was analyzed. The changes of TRα level were shown in Figure 3.17. After 1 day of the exposure to T_3, no significant increase ($p > 0.05$) of the TR level in the treatments at 3 and 7 days old was found at every concentration of the hormone treatment, compared to the control (Figure 3.17A, B). In contrast, at the 80 ppm of T_3, a significant decline was detected in the larvae at 7 days of age (Figure 3.17B). In the fish larvae of 15 days after hatching, the expression level of TRα had a tendency to be increased at higher dosage of the hormone (Figure 3.17C). A significantly higher ($p \leq 0.05$) receptor level than that of the control was observed after the animals obtained the thyroid hormone at 80 ppm. The differences of the TRα expression level, i.e. unchanged or lower level, detected in 3 or 7 days old larvae and the higher of the level observed in 15 days old larvae well agreed with the effect of the thyroid hormone observed on their survival. That is, the receptor level was lower in the fish those T_3-treatment increased their mortality rate. Whereas, the larvae those T_3 decreased their mortality rate showed an increase of the TRα gene.

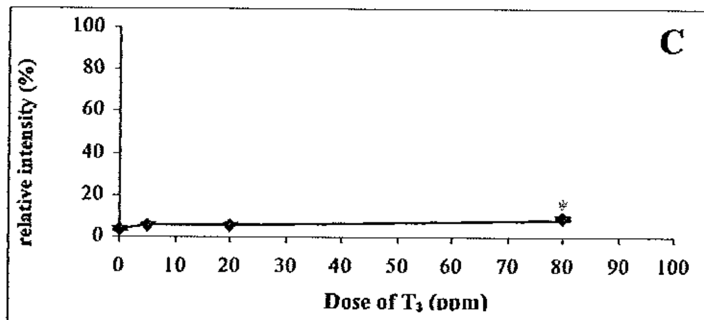
4.3.2 After the long-term treatment of T_3

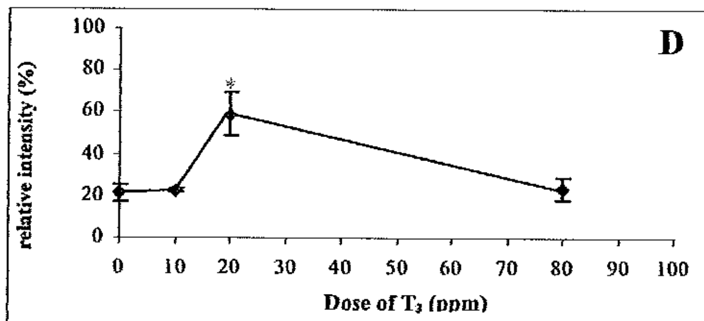
To investigate the expression of the TRα after the green catfish were exposed to the hormone for a long period, the larvae of 30 and 45 days of age were reared in the water containing T_3 for 15 days. The animals were fed, aerated, and concentrations of the hormone were maintained as described in Methods. The result clearly showed that T_3 at 20 ppm drastically increased the TR expression in the larvae at 30 days old of age, however, insignificant changes of the expression level was observed at other examined concentrations of the hormone (Figure 3.17**D**). The increase of the TR was significantly higher than that in the control at $p \leq 0.05$. At the same concentration of T_3, an increase, though at lower degree, of the TR level also

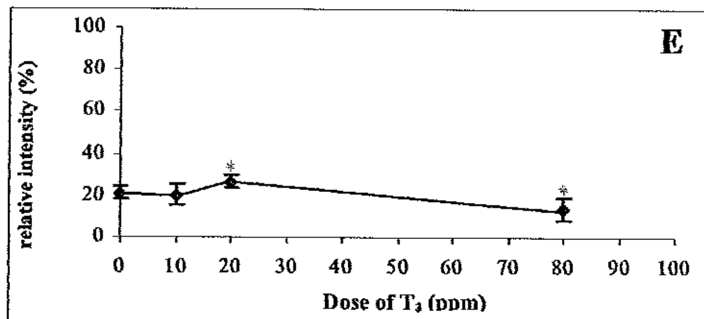
was observed in the fish larvae 45 days of age (Figure 3.17**E**). Interestingly, in this age, the expression of the receptor decreased after high dosage, i.e. 80 ppm, of the thyroid hormone was used.


A
relative intensity (%)
Dose of T_3 (ppm)


B
relative intensity (%)
Dose of T_3 (ppm)


C
relative intensity (%)
Dose of T_3 (ppm)


D
relative intensity (%)
Dose of T_3 (ppm)


E
relative intensity (%)
Dose of T_3 (ppm)

Chapter 4

DISCUSSION

The main target of this thesis is to reveal the effects of thyroid hormones in the green catfish (*M. nemurus*). In the investigation of the hormonal effects, experiments were conducted on hatchability, survival at selected ages, and the expression levels of two key enzymes in the carbohydrate metabolism, i.e. G6PDH and LDH, of the fish larvae. In addition, the TG was studied to determine the effect of thyroid hormone on the enzyme level, and the possibility of the cross-communication between thyroid hormone and retinoids actions in this catfish.

The observation on the effect of thyroid hormones on hatching of the green catfish demonstrated that the hormones in both T_3 and T_4 forms at concentrations in range of 6.25 to 300 ppm produced no effect on the fish hatchability. Exposure period to the hormones, i.e. 1 h or until hatch, did not alter the hatching percentage of the treatments comparing with that of the control. This indicated that the usage of exogenous thyroid hormones neither is essential nor improves the quality of eggs in this fish. It was demonstrated that while thyroid hormones significantly improved the egg viability of many fishes e.g. carp (Lam and Sharma, 1985), grouper and sea bass (Lam 1995), they could not improve the hatchability of other fish species such as rabbitfish and medaka (Ayson and Lam, 1993; Tagawa and Hirano, 1991). The administration of thyroid peroxidase inhibitor (0.003% thiourea) to medaka showed any influence on hatch rate and time of the fish eggs (Tagawa and Hirano, 1991). In addition, the embryonic thyroid hormone was first detected at the hatching time and developed during the yolk absorption of some fish species (Takawa and Hirano, 1987, 1989). Taken all the evidences have been reported and the results obtained in this thesis could convince that thyroid hormones (T_3 and T_4) are not essential for the embryonic development and hatching of the green catfish. However, as it has been well known that thyroid hormones are important for embryonic development of most of vertebrates and the transferring of thyroid hormones from the maternal circulation to eggs were demonstrated in several fish species (Brown *et al.*, 1987; Kobuke

et al., 1987; Ayson and Lam, 1993; Chang and Kang, 1998). It could not totally obliterate the possible role of thyroid hormones on physiological metabolism during the development of the green catfish embryos. As, thyroid hormones were possibly transferred from the maternal circulation in sufficient amount to the fish eggs that results in none of enhancement of the hatch rate could be detected when the exogenous hormones was supplied. In addition, as it was shown in some fish species that direct injection of thyroid hormone into female fish can address the hormone to the fish eggs and improved their hatching (Chang and Kang, 1998). Introduction of thyroid hormone into eggs via the immersion used in this thesis might not be appropriate. Considering the hydrophobic characteristic of thyroid hormone molecules, the hormones might be obstructed at the multi-layered fish chorion and could not enter the eggs. In order to obtain a precise conclusion of the possible role of thyroid hormones on eggs and the usage of the exogenous hormones to increase the hatchability of the green catfish, additional observations such as determination of thyroid hormones level in eggs and membrane component of the catfish eggs are required.

The effect of thyroid hormone on survival of the green catfish was emphasized in this thesis on both short and long-term of hormone treatments. In the short-term treatment, the fish larvae were maintained without feeding. The results obtained indicated that thyroid hormone did not enhance survival of the larvae at 3 and 7 days of age at all investigated concentrations of T_3. However, remarkable elevation in survival was detected in the larvae at 15, 30 and 45 days of age when they were exposed to 5 to 80 ppm of T_3. Though some dosages of T_3 showed a tendency to reduce the survival, the overall results demonstrated that the hormone in a range of 5 to 80 ppm is effective to be used to improve the survival of the green catfish larvae.

Thyroid hormone is not only unable to enhance the survival of 3 and 7 days old larvae but also accelerated the mortality rate of the fish at high dose (Figure 3.5**II A, B**). This could imply to the inappropriate concentration, possible too high, of T_3 used for these ages of fish. Selective dose at low concentration of the hormone, e.g. lower than 5 ppm, should be trial in order to definitely reveal the effect of thyroid hormones on survival of these newly hatched green catfish larvae. The short-term supplementation of thyroid hormones with the combination of other hormones,

successfully increased survival rate of threadfin (*Polydactylus sexfilis*) (Brown and Kim, 1995). Thus, using thyroid hormone as a combination, e.g. with other hormones, is suggested to be another choice of trial for better benefit of using thyroid hormone to increase survival of the green catfish.

In long-term treatment, catfish larvae at 15, 30, and 45 days of age were exposed to thyroid hormone for 15 days with feeding and aeration. At all examined concentrations, thyroid hormone did not elevate survival of the 15 days old catfish larvae. Moreover, higher mortality rate was observed at higher concentrations of thyroid hormone. The abnormal development and growth inhibition by high dosage and long-term treatment with thyroid hormone was reported in tilapia (Nacario, 1983). This could bring to the assumption that an inappropriate concentration, i.e. too high, of thyroid hormone was a factor that increased the mortality rate of 15 days green catfish larvae. On the contrary, an extraordinary increase of survival was detected in larvae at 30 and 45 days after hatch, at the particular concentrations of the hormone. Lam *et al.* (1985) demonstrated in milkfish (*Chanos chanos*) that the effective concentration of thyroid hormone for accelerating the growth of newly-hatch carp was 5 to 50 times lower than that for the larvae with an older of age. This is reverse to the correlation of the effective hormone concentration and age of larvae observed in the green catfish, i.e. 80 ppm of T_3 was the most effective concentration for the 30 days of age while 5 ppm was the most effective dose for the 45 days of age. However, it should confirm the idea that the stage of larvae is one of the factors involving in different sensitivity of the fish responses to thyroid hormone. In addition, based on the observation of the correlation between hormone dosage and catfish age, the possibility that too low concentration decreased survival of 15 days old catfish larvae could not be ruled out. Long-term treatment of 15 days old larvae at higher dose of T_3, thus, should be put to trial for survival enhancement.

During the development of flounder, the thyroid hormone level in plasma of the fish gradually increased to maximum during metamorphic climax and post-metamorphic climax stages, between 35 to 45 days after hatch (Tagawa *et al.*, 1990). This implied an important role of this hormone on differentiation and development of the fish during these stages. As mentioned above, the most effective dose of T_3 in elevating the survival of the fish at 30 days of age found in this thesis was higher than

that for the 45 days old larvae. A possible explanation of this observation is that the catfish larvae at these two ages require similar level, or a little bit higher in the older fish, of thyroid hormone for life maintenance and development. But, the thyroid hormones level in plasma of the 45 days old fish probably is much higher than in the younger fish. This, thus, resulted in a lower requirement for exogenous thyroid hormone supplementation in the 45 days larvae.

To determine the effect of thyroid hormone on carbohydrate metabolism, the expression levels of G6PDH and LDH were analyzed. The short-term treatment of the T_3 on the expression of enzymes was conducted with the purpose to determine the immediate responses to the hormone action in selective ages of the catfish larvae. For the expression level of G6PDH, it demonstrated that thyroid hormone at the concentration of 20 ppm was the most effective dosage to induce immediate response of the larvae with 3, 7, and 15 days of age to the hormone action on the enzyme level. Whereas, the concentration at 5 ppm of T_3 showed marked effect on the expression of G6PDH in the 30 and 45 days old larvae. This should be another evidence confirming the effect of larval stages on responsiveness to thyroid hormone action, and, in addition to the reverse correlation between concentrations of the hormone and ages of the fish.

In the observation on the effect of long-term treatment with thyroid hormone on expression level of G6PDH, the fish larvae were maintained in the hormone solution without feeding. So, the fish lacked energy resource, particularly in the form of glucose, from outside during the observation period. As it is well known that glucose is an important raw material for the energy production by several metabolic pathways including glycolysis which is the main pathway generating important metabolites for the energy production via oxidative pathway. In addition, glucose can be utilized to produce reducing equivalent, i.e. NADPH, which is important for reductive biosynthesis such as fatty acids through the pentose phosphate pathway, in which G6PDH is the rate limiting key enzyme. (Kletzien *et al.*, 1994). Thus, the results obtained here demonstrated that thyroid hormone increased capability of the treated larvae in lowering the enzyme expression level. The effect of thyroid hormone on decreasing the level of G6PDH might redirect the breakdown of glucose from the biosynthetic pathway of macromolecules, e.g. fat, to the effective energy-producing

pathway. The decrease of the enzyme level also occurred in the control, but with less immediate response than the treatments. Moreover, increase of the enzyme level in the control during the first two days was detected. The consumption rate of the blood glucose for the biosynthesis pathway in the control, should be higher compared with the treated larvae. Together with the observation of the higher survival in the treated larvae than the control, therefore it is conceivable that the decrease of enzyme level by thyroid hormone lengthened the blood glucose consumption and directed it to the more effective pathway for energy production during starving period. This resulted in prolonging the viability of the catfish larvae.

The effect of short-term T_3 treatment on the level of LDH revealed an immediate response to thyroid hormone action in the catfish larvae at 7, 30, and 45, but not at 3 and 15, days of age. The inconsistency of thyroid hormone responsiveness on LDH expression level was reported in some species of fish (Tripathi and Shukla, 1989; Tripathi and Verma, 2003). This unsteady response was suggested predominantly depending on stages of larvae. Seemingly, the immediate responsiveness to T_3 on the LDH expression of the green catfish was also affected by the same factor category, i.e. stages of larvae. The effect of long-term treatment showed reduction of LDH level profile in the treatments in comparison with the control, similarly to that observed on G6PDH. Since LDH is an enzyme catalyzing the interconversion of lactate and pyruvate, and the activity of LDH was determined via an alteration of the NADH level in this thesis. In addition, all of LDH isozymes were analyzed simultaneously without an individual separation. The decrease of LDH level, thus, implied to the reduction of NADH, not NAD^+, produced by the enzyme. In teleosts, the LDH-A_4 isozyme preferably catalyzes pyruvate to lactate and NADH to NAD^+, whereas the LDH-B_4 prefers to convert lactate to pyruvate and regenerate NADH. Thus, the decrease of LDH level by thyroid hormone action detected in the long-term treated green catfish larvae could indicate the reduction of LDH-B_4 expression, rather than LDH-A_4, and resulted in directing the role of LDH to restore NAD^+ for continued anaerobic glycolysis. Taken all into account, the results obtained in this thesis demonstrated that thyroid hormone affected the LDH of the green catfish larvae by decreasing the enzyme level so that more NAD^+ was regenerated for some continuous anaerobic activities, e.g. swimming, which is important for viability of the

fish. Moreover, since NAD^+ serves as the important cofactor for metabolism of the visual cycles of most teleosts (for review see Coppes, 1992) and catfish is one of the predator. Restoration of NAD^+, effected by thyroid hormone, might be important for an adaptation mechanism of the fish larvae for better survival in an environment with stresses, e.g. food depletion.

The observation on the effect of hormone on TG level with both short and long-term treatments indicated that thyroid hormone altered the level of the enzyme. In short-term treatment, decrease of the enzyme level was observed in most of the treated larvae. Effective doses of the hormone were varied and influenced by stages of the larvae, similarly to that experienced in G6PDH and LDH. In the long-term treatment, no specific pattern was found in the alteration of the enzyme profile, particularly in 45 days old catfish. Although, the important role of the TG could not be clarified as the definite physiological function of TG in fish has not been established (Chen and Metha, 1999) and the TG is a multifunctional enzyme. The observations generated from this thesis clearly showed that thyroid hormone has an effect on the expression of TG.

Since TG synthesis is known directly regulated by retinoic acid via retinoic acid receptor (RAR) and retinoid X receptor (RXR) (Chen and Metha, 1999). The observation in the green catfish, first demonstrated the influence of thyroid hormone on the retinoic acid pathway in fish via cross-communication among TR, RAR, and RXR. However, as non-genomic, independent of TR action of thyroid hormone has been evinced (for review see Davis and Davis, 1996). Several incidents including alterations in solute transport (Ca^{2+}, Na^+, glucose), changes in activities of several kinases, modulation of mitochondrial respiration, and regulation of actin polymerization, were consequent. Moreover, the suppression of TG was recently observed mediated by other signaling pathways (Dahler *et al.*, 2001). The action of thyroid hormone on expression of TG through non-genomic action mediated without TR involvement could not be ruled out.

The TRα cDNA of the green catfish was first isolated, and its sequence was analyzed. In comparison with TR sequences reported and available in databases of other fish species, green catfish TRα showed the highest identity to the TRα of rainbow trout. During growth of the catfish larvae, tremendous changes of the

receptor level were detected in the larvae from newly hatched to 30 days after hatch. The minimum level of TR was at 15 days of age. This indicated different degrees of importance or requirement of thyroid hormone actions during development of the catfish larvae. The differences of the expression level of TR and thyroid hormone in plasma during growth would reflect the different responsiveness to thyroid hormone among ages of fish.

Since the thyroid hormone action mediated via TR was determined through the expression level of TRα. The observation on short-term treatment effect of thyroid hormone should imply that the younger fish were less responsive to the hormone than the older ones. In addition, the expression level of the TRα was intensely increased in the older larvae with long-term treatment. This should confirm an idea that different level of importance of thyroid hormone actions and responsiveness of the catfish larvae to the hormone occur during development. The higher level of TR during growth should indicate higher requirement of thyroid hormone, leading to higher responsiveness to exogenous thyroid hormone detectable in the older fish larvae.

Chapter 5

CONCLUSION

All investigations of thyroid hormone effects on the green catfish in this thesis generated evidences that could be categorized as follows.

1. Effect on hatchability

1.1 Neither T_3 nor T_4 at concentrations of 6.25 to 300 ppm showed effect on hatching rate of the fish.

1.2 Neither short nor long-term exposed to T_3 at concentrations of 6.25 to 300 ppm altered hatchability of the green catfish eggs.

2. Effect on survival

2.1 Short-term treatment with T_3 at concentrations of 5 to 80 ppm could enhance survival of the catfish larvae at 15, 30, and 45, but not 3 and 7, days of age.

2.2 Long-term treatment with T_3 at concentrations of 10 to 80 ppm could intensely enhance survival of the catfish larvae at 30 and 45, but not 15, days of age.

2.3 The responsiveness on survival of fish to thyroid hormone was dependent on dosage and developmental stage.

3. Effect on metabolic enzymes

3.1 The G6PDH expression during the development of the green catfish was non-linearly correlated to the age of larvae. Whereas, that of LDH showed age dependent manner, i.e. the enzyme expression increased more in the older larvae. In addition, the expression profile of TG was reverse to that of LDH.

3.2 Immediate responses to thyroid hormone action resulted in the alterations of G6PDH and LDH expression were detected in the T_3-treated larvae.

3.3 Decrease of G6PDH and LDH levels was mediated by thyroid hormone action and was suggested to redirect the carbohydrate metabolic pathways for better survival and adaptation to environment of the catfish larvae.

3.4 Effect of thyroid hormone on the retinoic pathway was demonstrated from the detectable of TG level alteration in fish larvae those were short and long-term treated with T_3.

4. Thyroid hormone action mediated via TR

4.1 The α-isoform of TR of the green catfish (*M. nemurus*) was successfully isolated for the construction of cDNA partial fragment and its sequence was analyzed. In comparison with other vertebrates, the green catfish TRα has the highest identity to that of the rainbow trout (*O. mykiss*).

4.2 TRα expression level during growth of the green catfish extremely changed from 3 to 30 days after hatch, and maintained at the high level from 30 days after hatch.

4.3 An alteration of TRα level during action of thyroid hormone in the green catfish indicated different levels of responsiveness to thyroid hormone of the larvae, reflecting the different role and requirement of the hormone during the fish growth.

In summary, thyroid hormone generated both short and long-term effects on survival, level of some metabolic enzymes of the green catfish (*M. nemurus*) and mediated through TR. Influence on the retinoic acid pathways of thyroid hormone was also evidenced. Most of these effects of thyroid hormone occurred in different manners relating to the concentration of the hormone and developmental stages of the larvae. To be successful in enhancing the mass production of the green catfish by thyroid hormone supplement, more insights are the must. Further studies on thyroid hormone level in plasma, as well as molecular level study of TR and TREs during fish development are recommended.

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APPENDIX A

1. Enzymatic activity assay

(All enzymatic activity was determined using UV/Visible Spectrophotometer of Hewlet Packard model 8452)

1.1 G6PDH activity assay (Lupiáñez *et al.*, 1987; Corpas *et al.*, 1995)

Assay condition

The mixture is prepared and the reaction is allowed to initiate in 1 ml-quartz cuvette. Each reaction mixture is composed of 50 µl of 0.5 M Tris-HCl (pH7.4), 50 µl of 0.1 M EDTA (pH 7.4), 100 µl of 0.02 M G6P, 700 µl of enzyme diluted in 0.05 M Tris-HCl (pH 7.4). Then, mix the solution thoroughly by inversion and place the cuvette in the spectrophotometer. Set background at 340 nm to zero, follow by adding 50 µl of 0.012 M NADP and start the program which is set to record changes of absorbance in every 15 s for 5 min, at 25 °C

Calculation

According to the molar extinction coefficient of NADP which is 6.22 litre/cm/moles, the activity of enzyme is calculated by using the following formula.

$$\mu\text{mole/min/mg protein(U/mg protein)} = \frac{(\Delta A/\text{min}) \times \text{vol} \times 10^{6}}{\varepsilon \times \text{mg protein}}$$

where,

(ΔA/min)	is	the change of absorbance in 1 min
vol	is	volume of reaction mixture
ε	is	molar extinction coefficient

1.2 LDH activity assay (Adolf and Koho, 1977; Wahlefeld, 1983)

Assay condition

The assay mixture is prepared and the reaction is allowed to initiate in 1 ml-quartz cuvette. Each reaction mixture is composed of 400 μl of 0.025 M Tris-HCl (pH 8.8), 400 μl of 0.25 M lactate solution, and 100 μl of enzyme solution diluted in 0.01 M Tris-HCl (pH 8.8). The mixture is thoroughly mixed by inversion. Set background of the instrument to zero, follow by adding 100 μl of 0.01 M NAD^+ to initiate the reaction. Start the program to read changes of the absorbance in every 15 s for 5 min at 25 °C

NADH standard curve preparation

Prepare tubes contained the following proportion of NADH and buffer

reagent	tube					
	1	2	3	4	5	6
0.001 M NADH (ml)	0.00	0.0025	0.005	0.010	0.020	0.040
0.01 M Tris-HCl, pH8.8 (ml)	1.500	1.498	1.495	1.490	1.480	1.460

Mix the solution in each tube thoroughly, then measure the absorbance at 340 nm. The amount in μmoles of NADH is plotted against the absorbance. This standard curve is used to determine the amount of NADH produced from an unknown enzymatic reaction.

2. Protein concentration determination by Micro-Biuret assay (Itzhaki and Gill, 1964)

Prepare tubes contained the following proportion of bovine serum albumin (BSA) and distilled water.

reagent	tube					
	1	2	3	4	5	6
1 mg/ml BSA (ml)	0.000	0.005	0.010	0.020	0.040	0.080
water (ml)	1.00	0.495	0.490	0.480	0.460	0.420

Each tube is then added with 0.5 ml of 0.21%$CuSO_4.H_2O$ in 30% NAOH, and mix vigorously. To allow maximum forming of the copper-protein complex, the solution is left at room temperature for 10 min, after that the absorbance at 310 nm of the mixture is read in 1 ml-quartz cuvette. Standard curve prepared by plotting the amount of BSA (in mg) against the absorbance at 310 nm is used to determine the concentration of an unknown protein sample.

3. Agarose gel electrophoresis

Prepare the gel mold by sealing the open end of the plastic tray and place the comb on at the position of 1 mm above the tray for the well forming, then dissolve 0.50 g of agarose to 50 ml of 1 x TAE buffer and heat until the agarose is melted. After that, cool it down to about 50°C and add ethidium bromide to a final concentration of 0.4 µl/ ml, mix thoroughly and pour to the gel mold. When the gel is set (5-10 min) place the gel mold to the electrophoresis chamber contained 1 x TAE buffer with the volume that the gel is covered with the buffer to a dept of 0.5-1 cm. Then remove the comb. Prepare the sample by mixing 15 µl of PCR sample with 3 µl 10 x loading buffer, then slowly load into the wells. Running the gel with constant voltage of 80 volts. Turn off the power supply when

the dye runs to ¾ of the gel, then photograph and analyze the intensity of bands by Gel Document.

4. Formaldehyde agarose gel electrophoresis for RNA (Sambrook *et al.*, 1989)

(This method is for checking the integrity of total RNA used for RT-PCR step)

Prepare the gel by melting 0.60 g of agarose (Analytical grade) with 24.85 ml water and cool it down to about 50 °C. Then add 8 ml of 5 x formaldehyde gel running buffer and 7.15 ml of formaldehyde (Sigma), mix thoroughly and pour into the gel mold. After the gel is set (10-15 min), place the gel mold to the electrophoresis chamber contained 1 x formaldehyde gel running buffer with the volume that the gel is covered with the buffer to a dept of 0.5-1 cm, and remove the comb. Thereafter, prepare the sample by mixing 4.5 μl of total RNA, 2 μl of 5 x formaldehyde gel running buffer, 3.5 μl of formaldehyde, and 10 μl of formamide (Sigma) in 1.5 ml-sterile tube. Incubate at 65°C for 15 min then chill on ice, then add 2 μl of formaldehyde gel loading buffer and 1 μl of 1 mg/ml ethidium bromide, mix gently and slowly load into the well. Running the gel at constant voltage of 100 volts. Turn off the power supply when the dye runs to the end of the gel, then photograph the gel by Gel Document. An example of the figure of total RNA is shown in Figure. 6.1.

5. Preparation of some chemical reagents

5.1 5 x MOPs solution

Dissolve 20.6 g of 3(N-Morpholino) propanesulfonic acid (M.W. 209.3) in 800 ml of 5 mM sodium acetate, and adjust pH to 7 with 2 N NaOH. Then add 10 ml of 0.5 M EDTA, pH 8, and adjust volume to 1000 ml with distilled water.

5.2 50 x TAE buffer

Dissolve 121 g of Tris base (121.14) in 28.55 ml glacial acetic acid and 50 ml of 0.5 M EDTA, pH 8, then adjust volume to 500 ml with distilled water.

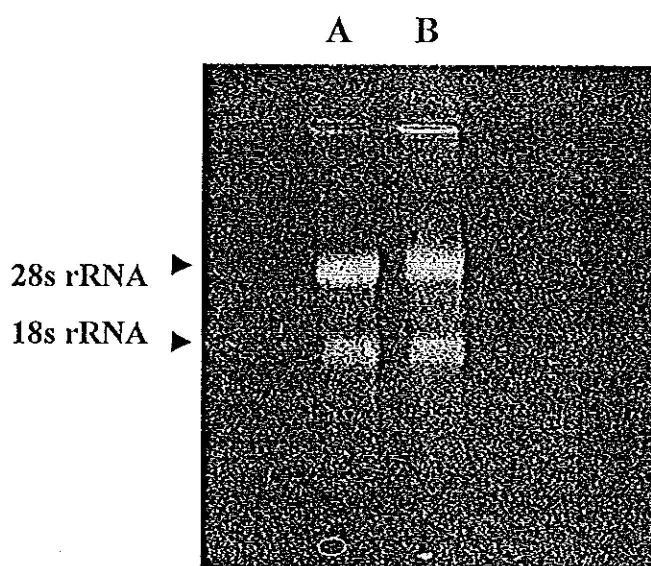

Figure 6.1 Formaldehyde agarose gel of total RNA isolated from whole body of green catfish larvae age of 45 days, using RNeasy Mini Kit (QIAGEN). (A) the first elution of total RNA eluted from spin column (5.2 μg RNA), (B) the second elution of total RNA eluted from the column (4.8 μg RNA).

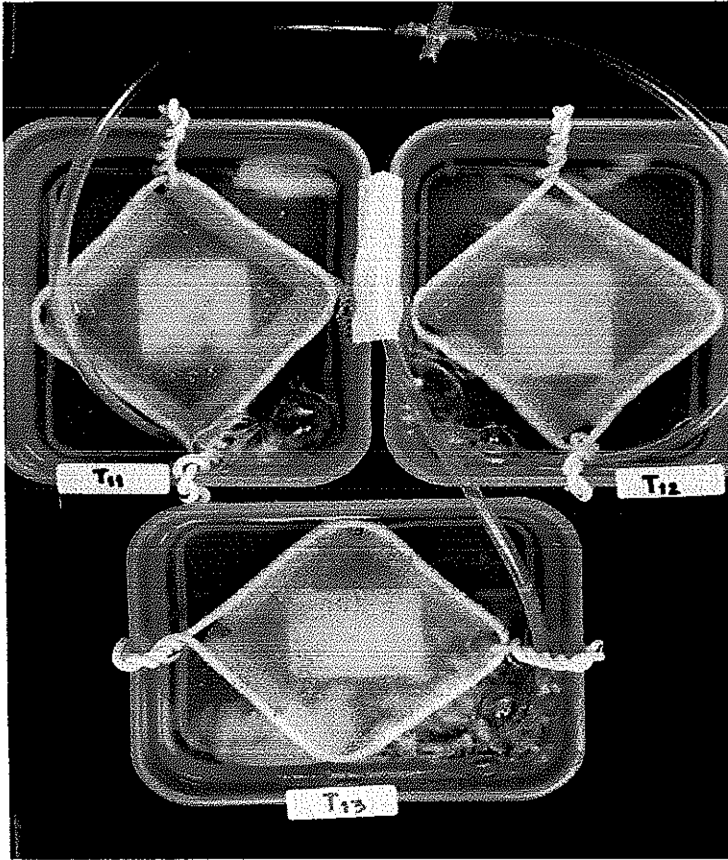

Figure 6.2 Small hatchery set for the hatching study.

APPENDIX B

Table 6.1 The alteration of G6PDH, LDH and TG activity during growth of the green catfish

Age	Specific Activity (μmole/min/mg protein) [2]		
(days after hatch)	G6PDH[1]	LDH[1]	TG[1]
3	19.364 ± 2.389	0.191 ± 0.028	0.221 ± 0.002
7	28.006 ± 4.214	0.358 ± 0.044	0.107 ± 0.009
15	29.30.1± 6.807	0.263 ± 0.030	0.111 ± 0.009
30	17.858 ± 1.174	0.658 ± 0.052	0.041 ± 0.008
45	31.835 ± 1.327	0.994 ± 0.094	0.052 ± 0.004

[1] G6PDH- glucose-6-phosphate dehydrogenase, LDH- lactate dehydrogenase and TG-transglutaminase

[2] Specific activity are present as mean ± SD. Crude protein was extracted (from 100-50, 25-40, 3-5 of the larvae at 3 and 7, 15, and 30 and 45 days of age, respectively), and analyzed for enzyme activity at least 3 times.

Table 6.2 Effect of short-term treatment with different doses of T_3 on G6PDH activity in fish of each age

Age (days after hatch)	Specific Activity (µmole NADPH/min/mg protein) [1]			
	$T_3 = 0$ ppm	$T_3 = 5$ ppm	$T_3 = 20$ ppm	$T_3 = 80$ ppm
3	12.140 ± 0.809	13.734 ± 1.551	18.667 ± 0.425	14.443 ± 1.351
7	16.047 ± 2.316	18.784 ± 0.916	22.435 ± 0.793	19.571 ± 0.408
15	16.231 ± 1.527	16.854 ± 1.564	20.570 ± 0.490	19.101 ± 0.490
30	29.530 ± 7.009	22.246 ± 3.425	33.124 ± 3.209	33.714 ± 5.026
45	28.337 ± 3.329	33.432 ± 3.404	28.987 ± 3.147	25.649 ± 2.343

[1] Results are present as mean ± SD of the enzyme specific activity.

Table 6.3 Changes of G6PDH specific activity of green catfish at 15, 30 and 45 days of age exposed to T_3 at different concentrations for 4 days

Day of exposure	Specific Activity (μmole NADPH/min/mg protein) [1]			
	$T_3 = 0$ ppm	$T_3 = 5$ ppm	$T_3 = 20$ ppm	$T_3 = 80$ ppm
15 days of age				
Day1	16.231 ± 1.527	16.854 ± 1.564	20.570 ± 0.490	19.101 ± 0.490
Day2	22.940 ± 0.982	23.001 ± 2.637	18.061 ± 1.226	17.700 ± 1.276
Day3	18.969 ± 1.528	11.484 ± 0.796	19.068 ± 1.122	18.751 ± 0.996
Day4	11.508 ± 1.354	12.224 ± 0.638	11.884 ± 1.379	13.691 ± 1.760
30 days of age				
Day1	29.530 ± 7.009	22.246 ± 3.426	33.124 ± 3.209	33.714 ± 5.0269
Day2	45.076 ± 9.584	30.619 ± 4.452	28.745 ± 4.987	30.680 ± 4.509
Day3	23.958 ± 3.766	24.869 ± 3.908	25.609 ± 4.150	30.317 ± 2.766
Day4	29.035 ± 4.311	20.906 ± 1.980	18.919 ± 7.435	28.380 ± 5.250
45 days of age				
Day1	28.337 ± 3.329	33.432 ± 3.404	28.990 ± 3.147	25.649 ± 2.343
Day2	31.052 ± 6.671	26.014 ± 4.314	24.806 ± 2.385	22.246 ± 1.700
Day3	26.322 ± 2.462	23.929 ± 1.282	21.791 ± 1.674	20.563 ± 2.718
Day4	18.126 ± 1.528	21.509 ± 3.862	22.703 ± 0.729	18.207 ± 2.328

[1] Results are present as mean ± SD of the enzyme specific activity.

Table 6.4 Effect of short-term treatment with different doses of T_3 on LDH activity in fish of each age

Age (days after hatch)	Specific Activity (μmole NADH/min/mg protein) [1]			
	$T_3 = 0$ ppm	$T_3 = 5$ ppm	$T_3 = 20$ ppm	$T_3 = 80$ ppm
3	0.292 ± 0.035	0.240 ± 0.024	0.254 ± 0.038	0.307 ± 0.033
7	0.383 ± 0.042	0.462 ± 0.027	0.413 ± 0.018	0.576 ± 0.042
15	0.362 ± 0.048	0.315 ± 0.030	0.369 ± 0.013	0.344 ± 0.027
30	0.669 ± 0.040	0.824 ± 0.0270	0.696 ± 0.090	0.594 ± 0.040
45	0.758 ± 0.129	0.931 ± 0.071	0.876 ± 0.074	0.837 ± 0.081

[1] Results are present as mean ± SD of the enzyme specific activity.

Table 6.5 Changes of LDH activity of the green catfish at 15, 30 and 45 days of age exposed to T_3 at different concentrations for 4 days

Day of exposure	Specific Activity (μmole NADH/min/mg protein) [1]			
	T_3 = 0 ppm	T_3 = 5 ppm	T_3 = 20 ppm	T_3 = 80 ppm
15 days of age				
Day1	0.362 ± 0.048	0.315 ± 0.030	0.369 ± 0.013	0.344 ± 0.027
Day2	0.406 ± 0.041	0.308 ± 0.033	0.364 ± 0.040	0.347 ± 0.016
Day3	0.461 ± 0.019	0.380 ± 0.039	0.373 ± 0.016	0.397 ± 0.000
Day4	0.504 ± 0.052	0.380 ± 0.034	0.352 ± 0.018	0.359 ± 0.080
30 days of age				
Day1	0.669 ± 0.040	0.824 ± 0.270	0.696 ± 0.090	0.594 ± 0.040
Day2	0.795 ± 0.040	0.652 ± 0.100	0.633 ± 0.030	0.873 ± 0.090
Day3	0.774 ± 0.050	0.750 ± 0.017	0.903 ± 0.320	0.645 ± 0.110
Day4	0.898 ± 0.020	0.744 ± 0.140	0.709 ± 0.030	0.724 ± 0.130
45 days of age				
Day1	0.758 ± 0.129	0.931 ± 0.071	0.876 ± 0.039	0.837 ± 0.008
Day2	0.822 ± 0.065	0.679 ± 0.029	0.876 ± 0.074	0.835 ± 0.049
Day3	0.757 ± 0.057	0.695 ± 0.057	0.594 ± 0.064	0.629 ± 0.023
Day4	0.881 ± 0.160	0.924 ± 0.111	0.968 ± 0.067	0.940 ± 0.061

[1] Results are present as mean ± SD of the enzyme specific activity.

Table 6.6 Effect of short-term treatment with different doses of T_3 on TG activity in fish of each age

Age	Specific Activity (µmole hydroxamate/min/mg protein) [1]			
(days after hatch)	$T_3 = 0$ ppm	$T_3 = 5$ ppm	$T_3 = 20$ ppm	$T_3 = 80$ ppm
3	0.124 ± 0.007	0.103 ± 0.011	0.086 ± 0.005	0.109 ± 0.010
7	0.104 ± 0.007	0.087 ± 0.007	0.099 ± 0.010	0.092 ± 0.007
15	0.092 ± 0.006	0.089 ± 0.006	0.063 ± 0.007	0.072 ± 0.006
30	0.081 ± 0.007	0.071 ± 0.011	0.051 ± 0.011	0.051 ± 0.040
45	0.049 ± 0.012	0.064 ± 0.008	0.053 ± 0.003	0.068 ± 0.010

[1] Results are present as mean ± SD of the enzyme specific activity.

Table 6.7 Changes of TG activity of the green catfish at 15, 30 and 45 days of age exposed to T_3 at different concentrations for 4 days

Day of exposure	Specific Activity (μmole hydroxamate/min/mg protein) [1]			
	T_3 = 0 ppm	T_3 = 5 ppm	T_3 = 20 ppm	T_3 = 80 ppm
15 days of age				
Day1	0.092 ± 0.006	0.089 ± 0.006	0.063 ± 0.007	0.072 ± 0.006
Day2	0.093 ± 0.008	0.080 ± 0.007	0.072 ± 0.012	0.081 ± 0.005
Day3	0.097 ± 0.008	0.086 ± 0.004	0.076 ± 0.005	0.085 ± 0.009
Day4	0.096 ± 0.005	0.087 ± 0.004	0.087 ± 0.004	0.076 ± 0.013
30 days of age				
Day1	0.081 ± 0.007	0.071 ± 0.011	0.051 ± 0.011	0.051 ± 0.004
Day2	0.072 ± 0.011	0.052 ± 0.005	0.053 ± 0.008	0.047 ± 0.008
Day3	0.064 ± 0.004	0.042 ± 0.011	0.042 ± 0.012	0.046 ± 0.005
Day4	0.068 ± 0.009	0.050 ± 0.010	0.048 ± 0.006	0.048 ± 0.007
45 days of age				
Day1	0.049 ± 0.012	0.064 ± 0.008	0.053 ± 0.003	0.068 ± 0.010
Day2	0.023 ± 0.017	0.039 ± 0.004	0.040 ± 0.012	0.060 ± 0.019
Day3	0.027 ± 0.007	0.027 ± 0.010	0.034 ± 0.008	0.042 ± 0.023
Day4	0.039 ± 0.012	0.052 ± 0.007	0.093 ± 0.006	0.034 ± 0.012

[1] Results are present as mean ± SD of the enzyme specific activity.

Table 6.8 Changes in TRα gene expression level during development of the green catfish

Age of larvae (days after hatch)	Relative intensity (%)[1]
3	19.70 ± 3.29
7	9.85 ± 2.21
15	3.74 ± 0.74
30	21.49 ± 3.97
45	20.97 ± 3.05

[1] Relative intensity (%) is the percentage intensity ratio of TRα and beta-actin bands. The data are shown as mean ± SD.

Table 6.9 Effect of short-term treatment with T_3 on the expression of TRα of green catfish larvae at 3, 7 and 15 days of age

Age (days after hatch)	Relative intensity (%)[1]			
	T_3 = 0 ppm	T_3 = 5 ppm	T_3 = 20 ppm	T_3 = 80 ppm
3	19.70 ± 3.29	18.10 ± 3.35	20.10 ± 6.43	18.70 ± 10.14
7	9.85 ± 2.21	8.00 ± 1.97	8.31 ± 2.49	4.33 ± 0.70
15	3.74 ± 0.74	5.66 ± 0.24	5.83 ± 0.80	9.03 ± 0.14

[1] Relative intensity (%) is shown as mean ± SD.

Table 6.10 Effect of long-term treatment with T_3 on the expression of TRα of green catfish larvae at 30 and 45 days of age

Age (days after hatch)	Relative intensity (%)[1]			
	$T_3 = 0$ ppm	$T_3 = 5$ ppm	$T_3 = 20$ ppm	$T_3 = 80$ ppm
30	21.49 ± 3.97	22.44 ± 0.75	58.94 ± 10.63	23.30 ± 5.35
45	20.97 ± 3.05	19.92 ± 4.90	26.50 ± 3.25	13.28 ± 5.52

[1] Relative intensity (%) are shown as mean ± SD.

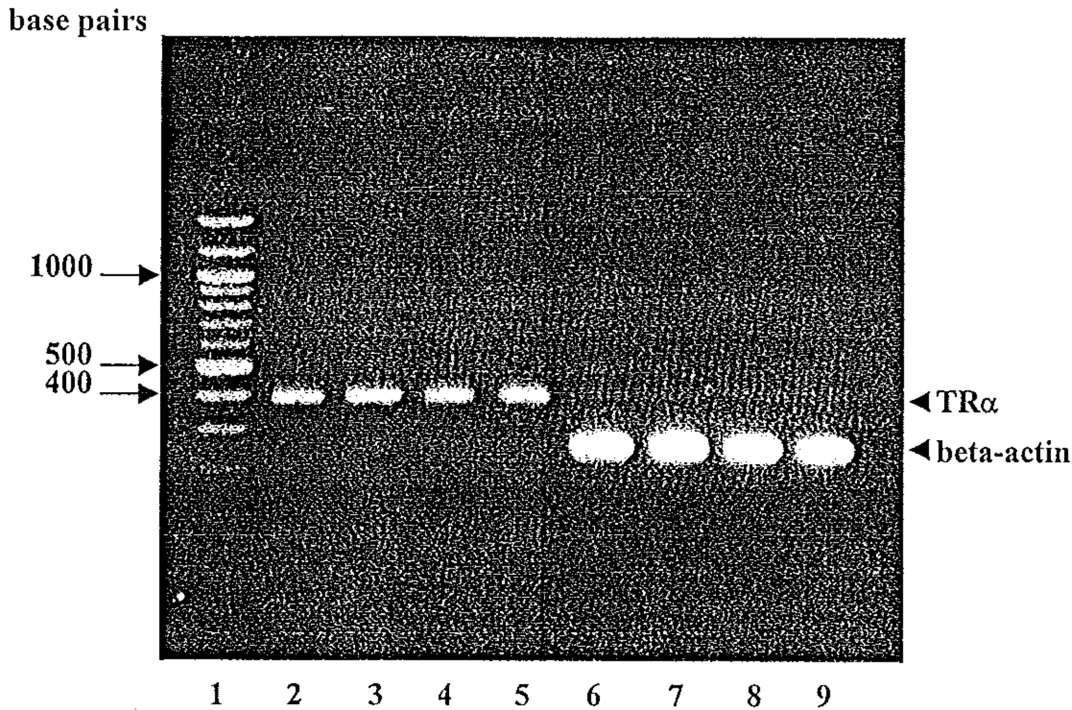

Figure 6.3 RT-PCR analysis of TRα mRNA. Total RNA of the green catfish was determined by RT-PCR using specific primers and the PCR product was analyzed on 1% agarose gel. The expected size of the TR and beta-actin cDNA fragments are ~ 400 and 200 bp, respectively. Beta-actin was used to normalize the amount of RNA of each unknown sample in semi-quantitative RT-PCR step. Lane 1 is standard DNA marker. Lane 2, 3, 4, and 5 represent the PCR products of TRα from total RNA of the catfish larvae at 30 days of age. Lane 6, 7, 8, 9 are beta-actin band obtained by RT-PCR of the same RNA used in lane 2, 3, 4, and 5, respectively.

VITAE

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ชื่อวิทยานิพนธ์ ผลของฮอร์โมนต่อมไธรอยด์ต่อพัฒนาการในระยะวัยอ่อนของปลากดเหลือง (*Mystus nemurus*)

ผู้เขียน นางสาวลัดดา ลีละวัฒน์วัฒนา

สาขาวิชา ชีวเคมี

ปีการศึกษา 2546

บทคัดย่อ

เป็นที่ทราบกันดีกว่าฮอร์โมนต่อมไธรอยด์มีบทบาทสำคัญต่อการเจริญเติบโตและพัฒนาการของสัตว์มีกระดูกสันหลังชนิดต่างๆ วิทยานิพนธ์นี้ได้ทำการศึกษาถึงบทบาทที่สำคัญของฮอร์โมนดังกล่าวในปลากดเหลือง (*Mystus nemurus*) โดยศึกษาผลที่มีต่ออัตราการฟัก, อัตราการรอดตายของลูกปลา ระดับของเอนไซม์หลักของวิถีคาร์โบไฮเดรตที่สำคัญ 2 ชนิด คือ glucose-6-phosphate dehydrogenase (G6PDH) และ lactate dehydrogenase (LDH) นอกจากนี้ยังได้ทำการศึกษาความสัมพันธ์ระหว่างฮอร์โมนต่อมไธรอยด์กับวิถีของวิตามินเอ โดยพิจารณาจากการเปลี่ยนแปลงของระดับเอนไซม์ transglutaminase (TG) รวมทั้งศึกษาการออกฤทธิ์ในนิวเคลียสของฮอร์โมนโดยอาศัย thyroid hormone receptor (TR)

ผลการศึกษาพบว่าฮอร์โมนต่อมไธรอยด์ทั้งในรูป T_4 และ T_3 ที่ระดับความเข้มข้น 6.25 ppm ถึง 300 ppm และระยะเวลาที่ไข่ปลาได้รับฮอร์โมนไม่มีผลเปลี่ยนแปลงอัตราการฟักเป็นตัวอ่อนของไข่ปลากดเหลือง และพบว่าลูกปลากดเหลืองที่อายุต่างๆที่ได้รับฮอร์โมน T_3 เป็นช่วงระยะสั้น มีอัตราการรอดที่แตกต่างกัน ขึ้นอยู่กับอายุของลูกปลา โดยฮอร์โมนสามารถเพิ่มอัตราการรอดอย่างมีนัยสำคัญทางสถิติในลูกปลาอายุ 15, 30 และ 45 วัน ส่วนลูกปลาที่ได้รับฮอร์โมนเป็นช่วงระยะเวลานาน มีอัตราการรอดที่เพิ่มขึ้นโดยมีความสัมพันธ์ระหว่างความเข้มข้นของฮอร์โมนที่ออกฤทธิ์กับอายุของลูกปลาเป็นแบบผกผัน

ฮอร์โมนมีผลต่อระดับการสร้างเอนไซม์ G6PDH และ LDH โดยลูกปลาทุกช่วงอายุที่ทำการศึกษามีการเปลี่ยนแปลงระดับเอนไซม์ทั้งสองชนิดแบบทันที หลังจากที่ได้รับฮอร์โมนเป็นช่วงระยะเวลาสั้น ส่วนลูกปลาที่ได้รับฮอร์โมนเป็นช่วงระยะเวลานานนั้น ระดับของเอนไซม์ทั้งสองชนิดลดลงอย่างต่อเนื่อง โดยอาจจะทำให้กลูโคสในเลือดถูกนำไปใช้ในการสร้างพลังงานด้วยวิถีที่มีประสิทธิภาพมากขึ้น ส่งผลให้ลูกปลาสามารถดำรงชีวิตอยู่ได้นานขึ้น การเปลี่ยนแปลงระดับของเอนไซม์ TG ที่เกิดขึ้นหลังจากที่ลูกปลาได้รับฮอร์โมน ยังสามารถบอกความเป็นไปได้ของบทบาท