CHAPTER II

HORMONE ASSAY

INTRODUCTION

Thyroid hormones are produced upon activation of the neuroendocrine hypothalamo-pituitary-thyroid (HPT) axis (Eales, 2006; Zoeller et al., 2007). Thyrotropin or thyroid stimulating hormone (TSH) is a member of the vertebrate glycoprotein hormone family, which also comprises the pituitary and chorionic gonadotropins. All are composed of two different, non-covalently bound subunits, and encoded by separate genes. In a given species, the subunit is common to all glycoprotein hormones whereas the subunit is specific to each hormone (Pierce and Parsons, 1981; Pierce, 1988; Quérat, 1994). In vertebrates, TSH stimulates the thyroid gland to produce the thyroid hormones (THs) thyroxine (T4) and triiodothyronine (T3). The thyroid gland is the only tissue in the body that is capable of accumulating iodine in large quantities, with its endocrine function relying on the effects of two hormones, T4 and the more potent T3. T4 generally represents >95% of the thyroid hormone output and it is typically present in higher quantities than T3 in the blood circulation, with the higher T4 concentrations serving as a pool of prohormone that can be converted into T3 by 5’-iodothyronine deiodinases in target tissues (Eales, 2006; Zoeller et al., 2007).

The synthesis of THs occurs in the thyroid follicle, a single layer of epithelial cells enclosing a colloid-filled space and T4 is the predominant hormone secreted. T4 has few direct actions and is considered to act principally as a precursor for T3, the biologically active form of the hormone (Hadley, 1992). The conversion of T4 to T3 occurs in the peripheral tissue by the enzymatic removal (5’- monodeiodination) of one of the iodide units of the outer ring of T4 (Eales and Brown, 1993). THs circulate in plasma bound to thyroid hormone-binding proteins that include albumin, transthyretin TTR, and thyroxine-binding globulin in vertebrates (Power et al., 2000).

The THs T3 and T4 have a wide range of biological effects in physiological processes of vertebrates (Power et al., 2001; Schnitzler et al., 2008). THs have been
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reported to play a major role in the migration (Matty, 1985), regulation of metabolism (Gupta and Thapliyal, 1991), breeding cycle (Volkoff et al., 1999), sexual maturation (Monteverdi and Di Giulio, 2000), electrolyte, water and oxidative metabolism (Peter et al., 2000), growth and development (Power et al., 2001). Their reduction or absence of production is known to decrease metabolic rate and cause the muscle to become sluggish (Thangavel et al., 2004). Moreover, they have profound effects on lipid, carbohydrate and protein metabolism (Plisetskaya et al., 1983).

In fish, THs have been implicated as being important regulators in differentiation, growth, metabolism, and salinity adaptation (Griffin, 2000; Power et al., 2001; Liu and Chan, 2002; Crane et al., 2004; Eales, 2006). In fish, thyroid follicles secrete primarily T4, a prohormone which is converted to biologically active T3 almost entirely in peripheral tissues, a process catalyzed by two microsomal iodothyronine deiodinases, type I (Dio1) and type II (Dio2) (Orozco and Valverde, 2005). Plasma T4 is a more reliable reflection of THs production than T3, since most of circulating T3 (around 80%) is produced extra-thyroidally from T4 deiodination (Sapin and Schlienger, 2003).

A number of recent reviews have pointed out the advantages of fish models for laboratory-based testing (Powers, 1989; Winn, 2001) because of thyroid system operates in basically the same way in all vertebrates including humans (Zoeller et al., 2007). Environmental contaminants can affect the thyroid system at different sites and can directly interfere with TH synthesis; TH metabolism and TH-blood transport (Boas et al., 2006). Endocrine responses through their integrative and early warning capacity may offer as potential indicators that may be useful in the detection and assessment of toxic stress in fish exposed to polluted environments (Hontela et al., 1993) and measurement of circulating levels of hormones can provide additional information on the lethal effects of many chemicals (Folmar, 1993). Recent attention has focused on the ability of these synthetic chemicals to interfere with the normal function of the endocrine system. Currently, over 116 environmental contaminants are suspected of exerting toxic effects on thyroid (Howdeshell, 2002) affecting several steps, namely synthesis, regulation, metabolism and action of thyroid hormones (Leatherland, 2000).
Scientifically stress is a common phenomenon; stressors disturb an animal’s homeostasis, which in turn can elicit compensatory as adaptive responses. These responses occur in many target organs, especially those under multiple endocrine controls (McDonald and Wood, 1993; Wendelaar Bonga, 1997). For example, handling of fish during blood sampling has been shown to affect the plasma TH levels (Todd and Eales, 2002). Restriction of feed declined plasma T_4 and T_3 in rainbow trout (Farbridge et al., 1992) and catfish (Gaylord et al., 2001). These regulatory mechanisms by which TH establishes its homeostasis deliver these hormones to target cells. The transport of THs to the intracellular sites and the activation of thyroid hormone receptors are the other regulatory events which regulate TH action at the cellular level.

Variations in the circulating levels of T_4 and T_3 of fish are reportedly controlled by fluctuations in ambient temperature, physical activity, photoperiod, feeding, etc. (Matty, 1985; Leiner et al., 2000). And also the gender, age, nutritional status, reproductive phase and health condition determine the effects of THs on fishes (Grau, 1988). Natural variation in thyroid status of fish has been demonstrated in response to developmental state and/or age (McLeese and Eales, 1998), water temperature (Eales and Fletcher, 1982; Johnston and Eales, 1995) and nutritional status (Leatherland and Farbridge, 1992; Eales and Brown, 1993) and, in many cases, these situations call for an increase in thyroid activity. In Japanese flounder, exogenous administration of TH or elevation of endogenous T_4 levels by TSH induces precocious metamorphosis by increasing the rate of transformation (Inui et al., 1989). The relevance of THs to metamorphosis has also been demonstrated in fishes exhibiting a relatively less dramatic transformation, such as conger eel (Conger myriaster), telescopic-eye black goldfish (Carassius auratus), zebrafish (Danio rerio) grouper (Epinephelus coioides) and tarpon (Megalops cyprinodes) (Yamano et al., 1991; Reddy and Lam, 1992; Brown, 1997; de Jesus et al., 1998; Shiao and Hwang, 2006).

A number of naturally occurring and man-made chemicals are able to interact with the endocrine system of humans and wildlife, which can lead to a disturbance of hormone metabolism or hormone-regulated cellular and physiological processes (Damstra et al., 2002). Awareness regarding environmental contaminants and their effects on endocrine function in fish is on the rise (Kime, 1998; Harvey and Johnson, 2002).
Chemical pollutants present in the aquatic environment can disrupt vital physiological processes in fish, such as growth, reproduction, osmoregulation, and thyroid and immune functions (Folmar et al., 1982; Thomas and Khan, 2005). Waterborne toxicants generally are known to cause endocrine disturbances in fish (Zhou et al., 2000; van der Oost et al., 2003; Thangavel et al., 2005), and hormone regulation may be impaired due to exposure to environmental pollutants (Folmar, 1993; Ernst et al., 2007).

Chemical pollutants have been reported to detrimentally affect THs in a number of fish species (Xu et al., 2002; Scott and Sloman, 2004; Vander Ven et al., 2006). Thus, a great variety of natural or synthetic chemicals of different classes and sources are thought to exert an acute and chronic effect at different levels of the thyroid cascade (Ibrahim et al., 2000). The effects of chemical compounds exposure may be multiple targets with the complex regulatory network of thyroid hormone metabolism and action. For example, the lethal effect of low amounts of copper and sublethal level results increased hormone levels in fish (Khangarot and Rathore, 1989). Aluminium, copper and cadmium exposures showed an increased activity in hormone level of trout (Marc et al., 1995; Le Guevel et al., 2000).

Determination of TSH (thyrotropin) in plasma is recognized as a sensitive method in the diagnosis of primary and secondary hypothyroidism. Increased TSH level in blood and decreased level of TSH in pituitary was observed in response to the prostaglandins in Heteropneustes fossilis (Singh and Singh, 1977). Significant lower and higher levels of the TSH in blood were noticed in pituitary and plasma of O. kisutch during smoltification (Larsen et al., 2011). Chronic fasting reduces the response of TSH in rainbow trout, Oncorhynchus mykiss (Leatherland and Farbridge, 1992). Significant lower and higher levels of the TSH in blood were noticed in pituitary and plasma of O. kisutch during smoltification (Larsen et al., 2011). Singh and Singh (1980) reported that a reduced pituitary and TSH content in the freshwater catfish, H. fossilis (Bloch) treated with cythion and hexadrin.

In addition to these, Braverman et al. (2006) found an inhibition of thyroid iodide uptake and the serum levels of TSH fish. TSH levels were significantly higher in fish Liza aurata collected from contaminated sites of Ria de Aveiro (Portugal) (Oliveira et al., 2011). Similar observation was also noted in Zebra fish exposed to
triadimefon (Liu, et al., 2011). Likewise, an increase in TSH activity was noted in *Fundulus heteroclitus* (Grau et al., 1985) and in *Channa punctatus* (Ghosh et al., 1989). In contrast, a significant decrease in TSH activity was noted in *Labeo rohita* exposed to endosulfan (Saravanan et al., 2010). The combination of T₃ with bisphenol A had an additive effect in suppressing TSH release in bullfrog (Kaneko et al., 2008).

*C. batrachus* exposed to the pesticide, carbaryl showed a higher level of T₄/T₃ ratio in serum during 96 hr treatment (Sinha et al., 1991). Similarly, selenium also increases plasma T₄ and T₃ levels in juvenile rainbow trout (Miller et al., 2007) and T₄ of Coho salmon, *Onchorhyncus kisutch* during smoltification (Larsen et al., 2011). On the other hand, a decreased level of T₄ and T₃ were also observed many fishes in previous reports. For example, a decreased T₄ and T₃ levels was found in rainbow trout (Hansen et al., 1999), in common carp, *C. carpio* to acidic water (Nagae et al., 2001), to phenobarbital, 3-methylcholanthrene, and polychlorinated biphenyls (Vansell and Klassen, 2002; Hood et al., 2003).

A similar findings were observed in T₄ and T₃ levels in plasma of rainbow trout *O. mykiss* to sublethal cyanide exposure (Ruby et al., 1993), in Sea bream *S. aurata* (Ortuno et al., 2001), in air-breathing fish *C. gariepinus* (Gupta and Premabati, 2002), in rainbow trout (*O. mykiss*) in response to nutritional state (Valentea et al., 2003), in the sea bream, *S. aurata* exposed to diethylstilbestrol and ioxynil (Morgado et al., 2009), to prochloraz (Liu et al., 2011). In some cases, T₃ alone were significantly decreased in an Anadromous Arctic charr fed A1254 (polychlorinated biphenyls) exhibit reduced plasma T₃ levels (Jorgensen et al., 2004) and in Cu exposed fish (Oliveira et al., 2008).

Li et al. (2009) reported that plasma T₄ and T₃ levels of larval and adult rare minnow (*Gobiocypris rarus*) at various concentrations of acetochlor was showed no significant changes among treatments. A similar response was also found in Atlantic salmon *Salmo salar* exposed to sub-lethal concentrations of zinc (Ibrahim et al., 2000); also in *C. gariepinus* (Gupta and Premabati, 2002); in *A. anguilla* after Cu exposure (Oliveira et al., 2008) and in zebrafish larvae exposed to Perfluorooctane sulfonates (PFOS) (Shi et al., 2009). The foregoing review of literature reveals that various environmental chemicals induced stress which causes disturbances in the homeostasis
of fish. From the previous reports, these three endpoints, TSH, T₄, and T₃ are routinely used for evaluating thyroid hormone homeostasis in fish (O’Connor et al., 2000; Yamasaki et al., 2002). The TSH, T₄ and T₃ determination are important factors in thyroid disease diagnosis.

Since THs play important roles in all physiological functions a toxicity test is needed to provide a compromise fitness and survival of aquatic organism through toxicity assessment of pharmaceuticals. On the other hand, the information on environmental contaminants effects on thyroid function is mostly provided by many investigations in mammals and few only on fish (Laflamme et al., 2000; Monteiro et al., 2005; Gagnon et al., 2006). However, studies on the effects of various chemicals on thyroid system in fish have been limited. The potential risk assessment of pharmaceuticals CA and DCF on thyroid hormones has not been fully elaborated in fish.

Therefore, the present study is aimed to examine the ecotoxicological effects of most commonly used pharmaceuticals CA and DCF on thyroid hormones (TSH, T₄ and T₃) of a freshwater fish C. mrigala with various concentrations (environmentally relevant concentrations). Further, this study will help to establish the mechanisms of CA and DCF on thyroid status in fish.
MATERIALS AND METHODS

Specimen

The fish *C. mrigala* is the most important among Indian major carps and it is exotic to India but enjoy global distribution in the tropical as well as temperature region. It is an intensively cultivated species, non predatory, voraciously omnivorous. *C. mrigala* was selected as an experimental animal based on the following reasons.

- It is widely distributed through the India.
- Mainly herbivorous feeding on diatoms, blue green algae, insect etc.
- Mainly a fresh water fish mostly used in aquaculture.
- It is easily adaptable to laboratory conditions.
- It multiples rapidly without much effort.
- It is an ideal animal for toxicity studies in aquatic biology.
- It is easily adaptable to laboratory conditions.
- It is widely distributed through the India.

Acclimatization and maintenance of fish

The test organism *C. mrigala* were procured from Tamil Nadu Fisheries Development Corporation Limited Aliyar Fish Farm, Aliyar, Tamil Nadu. Fish were transported to the laboratory in plastic baskets packed in ploythene bags containing aerated water. They were acclimatized to laboratory conditions for about 20 days in a large cement tank (containing 1000 L of water) disinfected with potassium permanganate and washed thoroughly prior to introduction of fish (to prevent fungal infection). During acclimatization, fish were fed with *ad libitum* with rice bran and ground nut oil cakes. Water was changed daily to ensure sufficient oxygen supply and to avoid accumulation of metabolic water of fish. Prior to the commencement of experiments, a suitable number of fish with an average length of 7.0 cm and an average weight of 8.0 g were selected and kept in separate clean rectangular glass aquarium tanks (120 x 80 x 40 cm) and fish belonging to both sex were used. Feeding was slopped two days prior to the commencement of experiments, to keep the experimental animals in a more (or) less state of metabolic requirements.
Analytical technique for water quality

Tap water free from chlorine was used for the present study. The hydrobiological feature such as temperature pH, salinity, dissolved oxygen, total hardness, calcium, magnesium and total alkalinity were determined for each set of experiment as these factors have significant influence on the biodegradability and toxicity of pollutants. Temperature of the water was monitored by a thermometer. The pH was determined by pen type pH meter (pH scan, Eutechcybermetics, Singapore). The salinity of the water was estimated by Mohr’s method using 0.5195N silver nitrate and potassium chromate as an indicator. Dissolved oxygen was determined by Winkler’s method. Total hardness was determined by using ammonia buffer solution and Erichrome black ‘T’ as an indicator and total alkalinity was determined by using methyl orange as an indicator. Calcium level was determined using murexide indicator and magnesium content was calculated by subtracting calcium.

The above physico-chemical analysis of water used in the present experiments was carried out as per APHA et al. (1998). The analytical data for a single set of experiments for the above parameters are given in Table 1 as the value varied negligibly for the waters used for the other sets of experiments.

Table 1. Physico-chemical parameters of water used for the present investigation

<table>
<thead>
<tr>
<th>Physico-chemical parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>26.0 ± 1.5°C</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 ± 0.08</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>6.2 ± 0.04 mg/L</td>
</tr>
<tr>
<td>Total alkalinity</td>
<td>18.1 ± 7.0 mg/L</td>
</tr>
<tr>
<td>Total hardness</td>
<td>18.3 ± 0.5 mg/L</td>
</tr>
<tr>
<td>Salinity</td>
<td>0.3 ± 0.02 ppt</td>
</tr>
<tr>
<td>Calcium</td>
<td>4.1 ± 0.3 mg/L</td>
</tr>
<tr>
<td>Magnesium</td>
<td>2.3 ± 0.6 mg/L</td>
</tr>
</tbody>
</table>

Values are mean ± S.E. of five individual observations
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Toxicant

Analytical grade Clofibric acid α-(p-Chlorophenoxy) isobutyric acid (CA, 97% pure); CAS No. 882-09-7 and Diclofenac 2-[(2, 6-Dichlorophenyl) amino] benzene acetic acid sodium salt (DCF, 99.9% pure); CAS No. 15307-79-6 were purchased from Sigma-Aldrich Chemie GmbH, Germany. Dimethyl sulfoxide (DMSO) (CAS No. 67-68-5) was purchased from Fischer Scientific India Pvt. Ltd, India and 0.2 mL/L used to prepare the stock solution at different concentrations (1, 10, and 100 µg/L) due to their low water solubility.

Chemical properties

CA:

IUPAC Name : α-(p-Chlorophenoxy) isobutyric acid
Formula : C_{10}H_{11}ClO_{3}
Molecular Weight : 214.65
Structural formula :

DCF:

IUPAC Name : 2-[(2,6-Dichlorophenyl) amino] benzeneacetic acid sodium salt
Formula : C_{14}H_{10}Cl_{2}NNaO_{2}
Molecular Weight : 318.13
Structural formula :
Preparation of test solution

The pharmaceutical drugs CA and DCF were used to evaluate their toxicity to a freshwater fish *Cirrhinus mrigala* at short and long-term exposures. Stock solution of CA and DCF (1000 ppm) was prepared by dissolving 1 gm of CA and DCF in 0.2 ml of DMSO and appropriate quantity of tap water.

Experimental protocol

Two experimental series were performed *viz.*, short and long-term toxicity studies. In each study one control and four replicates of treatment groups were maintained.

Short-term toxicity study

For short-term toxicity studies, healthy fish were taken from the stock and were maintained in the glass tank. Two days prior to experiments and during the experimental period feeding was discontinued. The pH of the water was maintained at 7.2 ± 0.08. The nominal concentrations of CA and DCF including 1, 10 and 100 µg/L were added in each glass aquaria (120 cm × 80 cm × 40 cm) containing 60 L of water. Four replicates were maintained for each concentration groups and 30 fish of equal size and weight were introduced. The test water was renewed at the end of 24 h and freshly prepared solution was added to maintain the concentration of CA and DCF at a constant level. A concurrent control of 30 fish in four different glass aquaria was maintained under identical conditions. No mortality was observed during the above study period. Feeding was withheld during the bioassay experiment. At the end of 96 h period fish from the control and drug treated groups were taken for further analysis.

Sampling

At the end of 96 h, live fish from control and CA and DCF treated groups were taken and sacrificed for blood chemistry studies. A minimum of 20 fish per treatment, four replicates per treatment and 20 fish per replicate were used for short-term studies. Care was taken to avoid stress during sampling.
Long-term toxicity studies

For long-term toxicity studies, the nominal concentrations of CA and DCF (1, 10 and 100 µg/L) were added in each glass aquaria (100 liter capacity) containing 90 L of water. The pH of the control and experimental glass aquaria was maintained at 7.2 ± 0.08. Four replicates were maintained for each concentration groups and 45 fish of equal size and weight were introduced. Water was changed daily in order to avoid accumulation of faecal matter and excess feed and renewed with the toxicant. A separate control was maintained by stocking 45 fish in a glass tank without adding toxicant. Four replicates were also maintained. Fish were fed *ad libitum* every day.

Sampling frequency

Experiments were conducted for a period of 35 days with 7 days sampling frequency. No mortality was observed during the experimental period. Upon completion of the stipulated exposure period of 7, 14, 21, 28 and 35 days, 20 fish were randomly selected from control and drug treated glass aquarium and sacrificed without anesthetizing for further analysis. After removal of fish at various intervals of time, the volume of the control and drug treated glass aquarium were adjusted to maintain a constant density of fish per unit volume of water.

Sample preparation

**Plasma**

Blood samples were collected by heart puncture using plastic disposable syringes fitted with 26 gauge needles. The syringe and needle were prechilled and coated with heparin (Beparine R heparin sodium, IP 1000 IU/ml derived from beef intestinal mucosa containing 0.15% w/v chlorocresol IP preservative), an anticoagulant manufactured by Biological E Limited, Hyderabad, India. It was transferred into small vials, which is previously rinsed with heparin. Whole blood sample was centrifuged at 10,000 rpm for 20 minutes to separate the plasma, which was used for the estimation of hormonal assays (TSH, T₄ and T₃), electrolytes (Na⁺, K⁺ and Cl⁻), biochemical parameters (glucose and protein), and enzymes (GOT and GPT). Pooled blood sample was used for determination of all the parameters.
Gills

After drawing the blood, fish were washed with double distilled water and blotted dry with absorbent paper. Then the gills were separated from the control and drug treated (CA and DCF exposed) fish and 100 mg gill tissue from each was weighed. They were homogenized with ice-cold 1.0 ml of 0.1M Tris-HCl buffer (pH 7.4) using a Teflon homogenizer, and then centrifuged at 1000 rpm at 4°C for 15 min. The supernatant was used for the estimation of Na⁺/K⁺-ATPase activity following the method of Shiosaka et al. (1971).

Hormonal studies

TSH, T₄ and T₃ levels were estimated in blood plasma of an Indian major carp, C. mrigala exposed to various concentrations (1, 10 and 100 µg/L) of CA and DCF using the following methods.

**Thyroid-stimulating hormone (TSH or thyrotropin) assay**

TSH assay was performed with TSH ELISA Kit (ANOGEN, Canada).

**Principle**

This TSH enzyme linked immunosorbent assay (ELISA) applies a technique called a quantitative sandwich immunoassay. The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific for TSH. Standards or samples are then added to the microtiter plate wells and TSH if present will bind to the antibody pre-coated wells. In order to quantitatively determine the amount of TSH present in the sample, a standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody, specific for TSH are added to each well to “sandwich” the TSH immobilized on the plate. The microtiter plate undergoes incubation, and then the wells are thoroughly washed to remove all unbound components. Next, a TMB (3,3',5,5' tetramethyl-benzidine) substrate solution is added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only those wells that contain TSH and enzyme-conjugated
antibody will exhibit a change in colour. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450 nm.

In order to measure the concentration of TSH in the sample, TSH ELISA Kit includes a set of calibration standards (6 standards). The calibration standards are assayed at the same time as the samples and allow the operator to produce a standard curve of Optical Density (O.D.) versus TSH concentration (μIU/mL). The concentration of TSH in the samples is then determined by comparing the O.D. of the samples to the standard curve.

**Reagents**

All reagents provided are stored at 2-8°C.

1. **Microtiter plate** (Part EL12-1) 96 wells - Pre-coated with anti-human TSH monoclonal antibody.

2. **Conjugate** (Part EL12-2) 12 ml - Anti-human TSH polyclonal antibody conjugated to horseradish peroxidase (HRP) with preservative.

3. **Standard - 40 μIU/ml** (Part EL12-3) 1 vial - Lyophilized human TSH in a buffered protein base with preservative that will contain 40 μIU/ml after reconstitution.

4. **Standard - 20 μIU/ml** (Part EL12-4) 1 vial - Lyophilized human TSH in a buffered protein base with preservative that will contain 20 μIU/ml after reconstitution.

5. **Standard - 10 μIU/ml** (Part EL12-5) 1 vial - Lyophilized human TSH in a buffered protein base with preservative that will contain 10 μIU/ml after reconstitution.

6. **Standard - 5 μIU/ml** (Part EL12-6) 1 vial - Lyophilized human TSH in a buffered protein base with preservative that will contain 5 μIU/ml after reconstitution.
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7. Standard - 1 μIU/ml (Part EL12-7) 1 vial - Lyophilized human TSH in a buffered protein base with preservative that will contain 1 μIU/ml after reconstitution.

8. Standard - 0 μIU/ml (Part EL12-8) 1 vial - Lyophilized buffered protein base with preservative that will contain 0 μIU/ml of human TSH after reconstitution.

9. Substrate A (Part EL12-9) 10 ml - Buffered solution with H₂O₂.

10. Substrate B (Part 30007) 10 ml - Buffered solution with TMB.

11. Stop solution (Part 30008) 7 ml - 2N Sulphuric Acid (H₂SO₄).

Procedure

All TSH standards were prepared before starting assay procedure. All standards and samples (plasma from CA treated fish) was added in duplicate to the Microtiter Plate. First, the desired numbers of coated wells in the holder was secured and then 50 μl of standards or samples was added to the appropriate well of the antibody pre-coated Microtiter Plate. Then, 2 drops or 100 μl of conjugate was added to each well. The contents were mixed well and covered and incubate for 2 hours at 37°C. Then, the substrate solution was prepared no more than 15 minutes before end of the incubation. The microtiter plate was washed by manually. Then, to each well 100 μl substrate solution was added and covered and incubated for 15 minutes at 37°C. Finally, 100 μl of stop solution was added to each well and mixed well. Then, the optical density (O.D.) was read at 450 nm using a microtiter plate reader within 30 minutes. The same procedure was followed for the determination of DCF toxicity also.

Calculation

The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the six standard concentrations on the vertical (Y) axis versus the corresponding TSH concentration (μIU/ml) on the horizontal (X) axis. This standard curve is used to determine the amount of TSH in an unknown sample. The mean O.D. value for each standard and sample was calculated. Then, all O.D. values were subtracted...
by the mean value of the zero standards (0 μIU/ml) before result interpretation. The standard curve was constructed using statistical software. The amount of TSH in each sample was determined by locating the O.D. value on the Y-axis and extended a horizontal line to the standard curve. At the point of intersection, a vertical line to the X-axis was drawn and the corresponding TSH concentration was noted.

**Thyroxine (T₄) test**

T₄ was estimated by using the method of Skelley *et al.* (1973) and Walker, (1977).

**Principle**

To measure T₄ by competitive immunoassay techniques, a plasma sample is mixed with labeled T₄ and T₄ antibody. The labeled T₄ solution contains 8-anilino-1-napthalene sulfonic acid (ANS) to inhibit binding of T₄ to plasma proteins, which would otherwise interfere with the assay. During incubation, a fixed amount of labeled T₄ competes with the unlabeled T₄ in the sample, standard, or control for a fixed number of binding sites on the specific T₄ antibody. Separation of the unbound T₄ from antibody-bound T₄ and the subsequent measurement of the labeled fraction of the bound phase complete the test. By comparing results of the unknown sample with those obtained from a series of T₄ standards, an accurate measurement of the T₄ concentration in the sample can be obtained. In the T₄ (Thyroxine) (Total) ELISA, T₄ antibody is coated on a solid phase (microtiter well). A measured amount of plasma and a constant amount of T₄ labeled with horseradish peroxidase are added. During incubation, T₄ in the sample and enzyme-labeled T₄ compete for the limited binding sites on the T₄ antibody. After 60 minute incubation at room temperature, the solid phase is washed with water to remove unbound labeled T₄. A solution of tetramethylbenzidine (TMB) is added and incubated for 20 minutes, resulting in the development of a blue color. The color development is stopped with the addition of 1N HCl, and the resulting yellow color is measured spectrophotometrically at 450 nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of T₄ in the plasma. By reference to a series of standards processed in the same way, the concentration of T₄ in the unknown sample is determined.
Reagents

1. Antibody-Coated Wells - Microtiter wells coated with sheep anti-T₄.

2. Enzyme Conjugate Concentrate - Contains T₄-HRP Conjugate.


4. Standard Set - Contains 0, 2.0, 5.0, 10.0, 15.0, and 25.0 μg/dL in T₃/T₄-free plasma, 1 set, liquid, ready to use.

5. TMB Reagent - Contains 3, 3', 5, 5' tetramethylbenzidine (TMB) stabilized in buffer solution.

6. Stop Solution (1N HCl) - Contains diluted hydrochloric acid.

Procedure

The desired number of coated wells in the holder was secured. Then, 25 μl of standards, samples (plasma from CA treated fish), and controls were added into the appropriate wells. Again 100 μl of working conjugate reagent was added into each well and thoroughly mixed for 30 seconds. The contents were incubated at room temperature (18-25°C) for 60 minutes. After that, incubation mixture was removed by flicking plate contents into a waste container. The microtiter wells were rinsed and flicked for 5 times with distilled or deionized water. Then, the wells were struck sharply onto absorbent paper to remove all residual water droplets. Then, 100 μl of TMB reagent was added into each well and gently mixed for 5-10 seconds. Finally, the contents were incubated at room temperature, in the dark, for 20 minutes. The reaction was stopped by adding 100 μl of stop solution to each well and gently mixed for 30 seconds. The blue color changes completely to yellow. The absorbance was read at 450 nm with a microtiter plate reader within 15 minutes. The same procedure was followed for the determination of DCF treated fish also.
Calculation

The mean absorbance value (O.D 450) for each set of standards, controls, and samples were calculated using standard curves. A standard curve was constructed by plotting the mean absorbance obtained for each standard against its concentration in μg/dL on log-log paper, with absorbance on the vertical (Y) axis and concentration on the horizontal (X) axis. Using the mean absorbance value for each sample, the corresponding concentration of T$_4$ in μg/dL was determined from the standard curve.

**Triiodothyronine (T$_3$) hormone assay**

T$_3$ was estimated by using the method of Skelley *et al.* (1973) and Walker, (1977).

**Principle**

In the T$_3$ ELISA, a second antibody (goat anti-mouse IgG) is coated on microtiter wells. A measured amount of plasma, a certain amount of mouse monoclonal Anti-T$_3$ antibody, and a constant amount of T$_3$ conjugated with horseradish peroxidase are added to the microtiter wells. During incubation, the mouse anti-T$_3$ antibody is bound to the second antibody on the wells. T$_3$ and the enzyme conjugated-T$_3$ compete for the limited binding sites on the anti-T$_3$ antibody. After a 60 minute incubation at room temperature, the wells are washed 5 times with water to remove unbound T$_3$ conjugate. A solution of TMB is then added and incubated for 20 minutes at room temperature, resulting in the development of a blue color. The color development is stopped with the addition of 1 N HCl, and the absorbance is measured spectrophotometrically at 450 nm. The intensity of the color formed is proportional to the amount of enzyme present, and is inversely related to the amount of unlabeled T$_3$ standards assayed in the same manner. The concentration of T$_3$ in the unknown sample is then calculated.
Reagents

1. **Antibody Coated Wells** - Microtiter wells coated with goat anti-mouse IgG.

2. **Enzyme Conjugate Concentrate** - Contains $T_3$-HRP Conjugate, with TRIS buffer, pH=7.60 and ProClin-300.

3. **Enzyme Conjugate Diluent** - Contains ANS, TRIS buffer, pH=7.60 and ProClin 300.

4. **Standard Set** - Contains 0, 0.5, 1.0, 2.5, 5.0, and 10.0 ng/ml triiodothyronine in $T_3/T_4$ free (stripped) plasma and ProClin-300.

5. **Antibody Reagent** - Contains mouse monoclonal anti-$T_3$ in phosphate buffer, pH=7.60 and ProClin-300.

6. **TMB Reagent** - Contains 3,3',5,5' tetramethylbenzidine (TMB) stabilized in buffer solution.

7. **Stop Solution** (1 N HCl) - Contains diluted hydrochloric acid.

Procedure

The desired number of coated wells in the holder was secured. Then, plate map with appropriate identification was prepared. 50 μl of standards, samples (plasma from CA treated fish), and controls were added into appropriate wells. Again, 50 μl of antibody reagent was added into each well and mixed thoroughly for 30 seconds. Further, 100 μl of working enzyme conjugate reagent was added to each well and again mixed thoroughly for 30 seconds, incubated at room temperature (18-25°C) for 60 minutes. Then, the incubation mixture was removed by flicking plate contents into a waste container. Microtiter wells were rinsed and flicked 5 times with distilled or deionized water. The wells were struck sharply onto absorbent paper or paper towels to remove all residual water droplets. Then, 100 μl of TMB reagent was added into each well and gently mixed for 5-10 seconds, incubated at room temperature in the
dark for 20 minutes. Then, the reaction was stopped by adding 100 μl of stop solution to each well and gently mixed for 30 seconds. The blue color completely changes to yellow. Finally, the absorbance was read at 450 nm with a microtiter well reader within 15 minutes. The same procedure was followed for the determination of DCF toxicity also.

**Calculation**

The mean absorbance value (O.D 450) from the duplicate set of standards, controls, and samples were calculated. A standard curve was constructed by plotting the mean absorbance obtained for each standard against its concentration in ng/ml on linear graph paper, with absorbance on the vertical (Y) axis and concentration on the horizontal (X) axis.

**Statistical analysis**

The statistical analysis was made individually on each sample and the mean value of five individual observations was taken for each parameter. All values were expressed as means and analyzed by ANOVA, followed by a DMRT test to determine the significant differences ($P < 0.01$ and $P < 0.05$) among the concentrations, between the drugs, and the difference between the concentrations and drugs on each parameter. The analytical data together with Tables and Graph/Bar - diagrams are presented on appropriate places in the text for all the chapters.
RESULTS

Plasma TSH, T₄, and T₃: Short-term assay

The hormones viz., TSH, T₄ and T₃ in C. mrigala exposed to various concentrations of CA and DCF for 96 h exposure showed alterations when compared to control groups. TSH exhibited lower level in fish exposed to all concentrations of CA and an elevation in this hormone level was observed in all concentrations of DCF exposure (Table 2 and Fig. 1). T₄ was found to be increased in all concentrations of CA treatment fish (except in 10 μg/L) (Table 2 and Fig. 2), whereas in DCF exposure, a significant decrease in T₄ was noted in all concentrations. Similarly, T₃ level was also increased in 10 and 100 μg/L of CA treatments. However, the T₃ level was decreased in all concentrations of DCF exposure (Table 2 and Fig. 3). A significant \( P < 0.01 \) difference was observed in TSH, T₄ and T₃ among the concentrations of both CA and DCF, between drugs and also between the concentrations and drugs.
Fig 1. Changes in the plasma TSH level in a freshwater fish *C. mrigala* treated with nominal concentrations of CA and DCF (1, 10 and 100 μg/L; 96 h). Means in the bars followed by common letters for the drug are not significantly different ($P < 0.05$) according to DMRT.

Fig 2. Changes in the plasma $T_4$ level in a freshwater fish *C. mrigala* treated with nominal concentrations of CA and DCF (1, 10 and 100 μg/L; 96 h). Means in the bars followed by common letters for the drug are not significantly different ($P < 0.05$) according to DMRT.
Fig 3. Changes in the plasma T\textsubscript{3} level in a freshwater fish *C. mrigala* treated with nominal concentrations of CA and DCF (1, 10 and 100 μg/L; 96 h). Means in the bars followed by common letters for the drug are not significantly different (*P* < 0.05) according to DMRT.
Plasma TSH, T4 and T3: Long-term assay

Plasma TSH level of fish *C. mirgala* was decreased in CA treatments throughout the study period (except 1 and 10 µg/L at the end of 14th day and 1 µg/L at 21st day) when compared to control groups (Table 3 and Fig. 4). However, in DCF treated fish, a significant increase was noted throughout the study period. In this study, a significant (*P* < 0.01) difference was observed in TSH among the concentrations of both CA and DCF, between drugs and also between the concentrations and drugs.

T4 activity was lowered at all concentrations of CA exposed fish upto 28th day (except on day 7th in 10 µg/L and 28th day in 10 and 100 µg/L). At the end of the exposure period i.e. 35th day, the hormone level was found to be increased in all concentrations when compared with control groups (Table 4 and Fig. 5). All concentrations of DCF induced a considerable decrease throughout the study period except 100 µg L⁻¹ at the end of 35th day (Table 4 and Fig. 5). A significant (*P* < 0.01) difference was observed in T4 level among the concentrations of both CA and DCF, between drugs and also between the concentrations and drugs (except at the end of 28th day). On day 28 there was no significant (*P* < 0.01) difference in T4 level among the concentrations of both CA and DCF, between drugs and also between the concentrations and drugs.

T3 level at 1 and 10 µg/L of CA exposed fish were decreased throughout the study period (except 10 µg/L on 35th day). In contrast, T3 level was found to be higher in all concentrations of CA exposed fish. In DCF treatment, a decrease in T3 level was noticed with different concentrations throughout the study period (35 days) (Table 5 and Fig. 6). A significant (*P* < 0.01) difference was observed in plasma T3 level among the concentrations of both CA and DCF, between drugs and also between the concentrations and drugs.
Fig 4. Changes in the plasma TSH level in a freshwater fish C. mrigala treated with nominal concentrations of CA and DCF (1, 10 and 100 μg/L; 35 days). Means in the bars followed by common letters for the drug are not significantly different ($P < 0.05$) according to DMRT.

Fig 5. Changes in the plasma $T_4$ level in a freshwater fish C. mrigala treated with nominal concentrations of CA and DCF (1, 10 and 100 μg/L; 35 days). Means in the bars followed by common letters for the drug are not significantly different ($P < 0.05$) according to DMRT.
Fig 6. Changes in the plasma T₃ level in a freshwater fish *C. mrigala* treated with nominal concentrations of CA and DCF (1, 10 and 100 μg/L; 35 days). Means in the bars followed by common letters for the drug are not significantly different (*P* < 0.05) according to DMRT.
DISCUSSION

Thyroid hormones influence the activity of a wider variety of tissues and biological functions when compared to any other hormones (Janz, 2000). In fishes thyroid hormones contribute to the control of growth and development, metabolism and osmoregulation, often in association with growth hormone and cortisol. Thyroid hormones also appear to be involved in triggering the migratory behavior and part of the adaptive osmoregulatory changes (Prunet et al., 1989). In teleosts, unlike mammals, the thyroid gland produces quite exclusively T4, whereas circulating T3 results from T4 peripheral deiodination (Eales and Brown, 1993). They also pointed out that thyroid activity is regulated, at least in part, via a “peripheral control model” whereby thyroid status is dictated by a tissue specific T3 set-point or the availability of T3 to receptor sites in specific tissues. The T3 set point is dependent primarily on T4•T3 conversion rate, although change in tissue sensitivity and hormone degradation rate may play roles as well (Oba et al., 2000).

It is generally believed that thyroid activity is regulated through the hypothalamic-pituitary-thyroid axis. This “central control model” involves hypothalamic secretion of thyrotropin releasing hormone (TRH) that stimulates release of TSH from the pituitary. TSH, in turn, stimulates secretion of T4 which is subsequently converted to the more biologically active T3 (Larsen et al., 2011). Although T4 is the major circulatory thyroid hormone in fish, there is strong evidence that T3 is the physiologically relevant hormone, monodeiodination of T4 probably occurs in target tissues (Hazon and Balment, 1997). One of the most spectacular actions of T4 is the stimulation of metamorphosis (Das and Bhattacharya, 2008).

Xenobiotics induced alterations in plasma thyroid hormones may be a result of a variety of mechanisms, corresponding to changes on thyroid status and/or alterations upstream or downstream of the thyroid hormones (THs) release (Oliveira et al., 2008). These mechanisms include alterations on the: (i) hypothalamus and/or pituitary status (Alkindi et al., 1996), (ii) biosynthesis and secretion steps of T4 and T3 (Capen, 1997), (iii) uptake by peripheral tissues, (iv) hepatic 5’-monodeiodinase activity (Waring et al., 1996), or (v) hormone catabolism and clearance rates (Saito et al., 1991;
Hormone Assay

Hontela et al., 1995). In this case, the need for development and validation of an *in vivo* assay for effects of thyroid system disrupting chemicals arises from concern that a considerable number of compounds have the potential to interact with different suspects of thyroid function and TH action (Kloas, 2002; Zoeller, 2003). Further, alteration in thyroid function is frequently determined by measuring TH-blood levels which is assumed to give a fairly good indication of central thyroid axis activity (De Vito et al., 1999).

Thyroid hypo function due to environmental toxicants may be mediated through inhibition of iodide peroxidase as this enzyme from fish kidney source is readily inactivated by toxicants or inhibition of lysosomal protease. Mensi et al. (1982) reported that nitrate intoxication in rainbow trout causes lysosomal damage. The head kidney peroxidase from *C. punctatus* is found to form I$_3$ suggesting that this peroxidase is of physiological importance (De and Bhattacharya, 1976). Chavin and Bouwman (1965) demonstrated that thyroid hormone synthesis does take place in the kidney of some fish and this is catalysed by iodide peroxidase (Kumar et al., 1973). Evidences are presented that these complex interactions at the hypothalamic-pituitary levels as well as at the levels of hormonal actions support a role for thyroid hormones in stress response of fish (Larsen et al., 2011). Their reduction or absence of production is known to decrease metabolic rate and cause the muscle to become sluggish (Thangavel et al., 2004). And also handling of fish during blood sampling has been shown to affect the plasma TH levels (Todd and Eales, 2002). For example, the lethal effect of low amounts of copper and sublethal level results increased hormone levels in fish (Anderson, 1996).

TSH is responsible for regulating synthesis and release of T$_4$ and T$_3$ in vertebrates (Zoeller et al., 2007). TSH assay could be employed to determine whether xenobiotic chemicals can affect thyroid gland sensitivity to TSH (Zoeller and Tan, 2007a). Pituitary and plasma TSH levels were used as indices of translation/storage and secretion of TSH, respectively (Larsen et al., 2011). TH exerts a negative feedback on the expression of TRH and TSH (Manchado et al., 2008). TSH levels were significantly higher in fish *Liza aurata* collected from contaminated sites of Ria de Aveiro (Portugal) (Oliveira et al., 2011). Similar observation was also
noted in Zebra fish exposed to triadimefon (Liu, et al., 2011). Likewise, an increase in TSH activity was noted in *Fundulus heteroclitus* (Grau et al., 1985) and in *Channa punctatus* (Ghosh et al., 1989).

Perchlorate induced lower level of TH production in zebrafish which causes an increased secretion of TSH by the pituitary (Patino et al., 2003). A slight decrease in plasma TSH activity was observed in European eel *A. anguilla* after Cu (Oliveira et al., 2008) and β-naphthoflavone exposures (Teles et al., 2005). In the present study also DCF may induce lowered level of TH production in fish which results an increased secretion of TSH by the pituitary. Therefore, the lesser concentration of TSH would result in lesser thyroid hormone concentrations (Shengliu et al., 2011). In response to reduced plasma T₄, an increase in plasma TSH would be expected (Teles et al., 2005). Previous studies concerning mammals exposed to microsomal enzyme inducers revealed divergent responses, i.e., increased (Hood et al., 2003) or unaltered (Liu et al., 1995; Hood and Klaassen, 2000) plasma TSH concomitantly with decreased plasma T₄. According to Hood and Klaassen (2000), these mechanisms are still poorly understood. Oliveira et al. (2008) reported that plasma TSH was not influenced by Cu exposure in *A. anguilla* whereas plasma T₃ was decreased despite the unchanged T₄ levels. TSH increase would be expected upon a plasma T₄ decrease (Oliveira et al., 2011). In the present study, the significant increase in plasma TSH level during drug treatment reflects a response to reduced plasma T₄ level.

In fish, corticotrophin-releasing hormone (CRH) is a more potent factor than TRH for stimulating TSH release (De Groef et al., 2006). For example, down-regulation of CRH could result in down-regulation of TSH. Therefore, the lesser concentration of TSH would result in lesser thyroid hormone concentrations. In this study, CA and DCF resulted in significant lesser amounts of T₄ and T₃ in *C. mrigala* which respond to be decrease in TSH level in this study. Previously, it has also been demonstrated in rats exposed to PCZ (Laier et al., 2006). These results suggest that CA and DCF down-regulates CRH and TSH and results in lesser concentrations of T₃ and T₄. Further, drugs interfere with the metabolism of THs by modifying deiodinase enzyme activity or abundance this might modify hormone concentrations in blood and
tissue (De Vito et al., 1999). In the present study, CA induced a significant decrease in TSH activity of *C. mrigala* during short and long-term treatments. In this case, the increased T<sub>4</sub> and T<sub>3</sub> might be resulted in decrease of plasma TSH in *C. mrigala*.

Alterations on plasma TSH levels observed in our study could suggest that pituitary was affected by these drugs. The plasma TSH levels shows different responses to different concentrations of CA (decreased at maximum) and DCF (increased at maximum) of during short and long-term treatments. Further, some marginal changes in TSH may suggest some physiological responses to weak thyroid modulators. In conclusion, the present study looked at a range of elements of the fish thyroid system and assessed how they were modified by the exposure to CA and DCF.

Plasma T<sub>4</sub> was decreased as a result of RM1 goitrogenic effects, whereas circulating T<sub>3</sub> remained steady, reflecting the regulation of T<sub>4</sub> deiodination. PCBs can also alter plasma TH levels by directly affecting TH synthesis and/or inhibiting the proteolysis of thyroglobulin, a T<sub>4</sub> precursor (Van Birgelen et al., 1995). Plasma T<sub>4</sub> decreased significantly in *A. anguilla* (L) owing to exposure to chromium and copper (Teles et al., 2005). PCZ down-regulates CRH and TSH and results in lesser concentrations of T<sub>4</sub> by reducing concentrations of E2 (Shengliu et al., 2011). Restriction of feed declined plasma T<sub>4</sub> and T<sub>3</sub> in rainbow trout (Farbridge et al., 1992) and catfish (Gaylord et al., 2001). Typical effects such as lowering of endogenous T<sub>4</sub> and/or T<sub>3</sub> levels and thyroidal hypertrophy are also observed in fish (Patino et al., 2003). Mercury and methyl-mercury, in addition to some other metals (e.g., cadmium), have been reported to decrease plasma T<sub>4</sub> and T<sub>3</sub>, possibly via direct thyroid cell effects, as well as by interfering with deiodinase activity (Eales et al., 1999; Brown et al., 2004a; Soldin et al., 2008).

Decrease in T<sub>4</sub> levels were observed in CA and DCF exposures in this study which can be explained by the drugs ability to increase the activity of hepatic drug-metabolizing enzymes, in particular UDP-transferase, responsible for glucuronidation and clearance of T<sub>4</sub> (Zhou et al., 2001; Hallgren and Darnerud, 2002) or their capacity to bind to TH-transporter transthyretin and thereby decrease plasma T<sub>4</sub> levels by replacing T<sub>4</sub> (Hallgren and Darnerud, 2002). Dependent upon the various concentrations, plasma T<sub>4</sub> concentrations exhibited significant differences in both CA
and DCF. However, CA and DCF exhibited significantly reduced T\textsubscript{4} activity in fish. The decreased plasma TH concentrations in fasted juvenile trout may be due to a combination of reduced T\textsubscript{4} release from the thyroid and reduced peripheral monodeiodination of T\textsubscript{4} (Rainea \textit{et al.}, 2005). In the present study also the observed decrease in T\textsubscript{4} during CA and DCF treatments may be due to a combination of reduced T\textsubscript{4} release from the thyroid and reduced peripheral monodeiodination of T\textsubscript{4}.

An increase in T\textsubscript{4} levels following melatonin treatment might be either due to increase in TSH secretion or sensitivity of thyroid follicles to TSH (Grau \textit{et al.}, 1985) and/or due to a direct thyrotropic action of melatonin. Alternatively, the increase in T\textsubscript{4} concentrations in the fish blood may result due to melatonin-induced inhibition of 5'-deiodinase - the enzyme responsible for accelerating peripheral deiodination of T\textsubscript{4} to T\textsubscript{3} in plasma of \textit{C. gariepinus} (Gupta and Premabati, 2002). In other study, plasma T\textsubscript{4} level was increased in an exposure-dependent pattern in acute and sub-chronically selenite exposed juvenile rainbow trout fish (Miller \textit{et al.}, 2007). Bleau \textit{et al.} (1996) reported that \textit{O. mykiss} exposure to mercury lead to increased T\textsubscript{4} levels despite their return to levels similar to control after 72 and 168 h. The increase of T\textsubscript{4} in PTU treated fish suggests that the increase in T\textsubscript{4} could be a consequence of the decreased GH levels. The PTU induced lowering in GH levels may contribute to this increase in F\textsubscript{T4}, levels, since GH is known to increase thyroxine 50-monodeiodinase activity (MacLatchy and Eales, 1990). In this study, the observed increase in T\textsubscript{4} levels following CA treatment might be either due to increase in TSH secretion or sensitivity of thyroid follicles to TSH and/or due to a direct thyrotropic action of CA. It is also possible that less T\textsubscript{3} binds to receptor sites of fish, which may be competitively blocked by CA. If the effectiveness of T\textsubscript{3} binding were affected, the higher plasma T\textsubscript{4} levels might result from elevated TSH due to an altered feedback mechanism in the hypothalamus or pituitary.

According to Eales (1995), the production of T\textsubscript{4} is most likely controlled by the brain, via the hypothalamus pituitary-thyroid axis. The subsequent regulation of T\textsubscript{4} availability to tissues, the conversion of the less biologically active T\textsubscript{4} to the more active T\textsubscript{3} by monodeiodinase activity, and specific tissue regulation of thyroid hormone receptors are controlled by a complex endocrine feed-back mechanism involving thyroid hormones and other factors (Ebbesson \textit{et al.}, 1998). Plasma T\textsubscript{4}
frequently follows a response pattern similar to that one of plasma cortisol, and T_4 may also activate the interrenal function (Hontela et al., 1995). The profile of blood T_4 and T_3 concomitantly traced during the exposure of fish to the toxicants, in most cases demonstrated a significant decline as noted in *Anabas testudineus* exposed to phenol, ammonia, mercury and cadmium (Chatterjee and Bhattacharya, 1985).

Leatherland (1992) suggested that low level of plasma T_3 may be a result of goiter condition and the inability of the organism to produce plasma T_3 optimum level. The lowered T_3 levels may have been the result of chemical interaction along the hypothalamic-pituitary-ovarian axis (Ruby et al., 1993). Ruby et al. (1993) examined plasma T_4 and T_3 in rainbow trout following exposure to cyanide in which T_3 levels were lower in cyanide-treated fish. It has previously been reported that PTU decreased concentrations of T_4 and T_3 in the plasma of zebrafish (van der Ven et al., 2006). Plasma T_3 decrease may be either due to its increased uptake by peripheral tissues, decreased extra-thyroidal tissues 5'-monodeiodinase activity or increased T_3 catabolism and plasma clearance rates (Oliveira et al., 2011). A similar mechanism may be operated in the present study on T_3 activities of CA and DCF treated fish.

Liver disorder also one of the reason for decreased level of T_3 in *C. mrigala* exposed to CA and DCF. Because the liver is also a major site of T_3 production, an enlarged liver may adversely affect thyroid hormone metabolism or T_4 to T_3 conversion (Zhou et al., 1999) and also a high level of cortisol may secret due to stress induced by the drugs causes a decrease in T_4 to T_3 conversion (Eales and MacLatchy, 1989). In the present study also the decreased plasma T_3 concentration in the fish, *C. mrigala* during CA and DCF exposures may be due to inhibition of extrathyroidal conversion of T_4 to T_3. The observed T_3 decrease may have a serious impact in fish metabolism since T_3 is the biologically active thyroid hormone (Brown et al., 1998).

On the other hand, various chemicals such as PCBs and pesticides can lead to increase of T_3 in blood by altering 5'-deiodinase activity in fish (Adams et al., 2000; Brown et al., 2004a; Coimbra et al., 2005; Picard-Aitken et al., 2007). In all vertebrates including fish, the bulk of plasma T_3 concentrations are derived from peripheral conversions of T_4 by 5'-deiodinases (Eales et al., 1999; Plohman et al., 2002;
Eales, 2006; Zoeller et al., 2007). In this study, a significant increase in plasma T₃ level in C. mrigala during short and long-term exposures was noticed which might be a response to maintenance of the basal metabolic rate of the fish to ensure its survival. And the marginal changes in T₃ may suggest some physiological responses to weak thyroid modulators. Elevated serum T₃ in nimbecidine exposed fish suggesting a role for TH in stress acclimation (Peter et al., 2009). In addition, exposure of perch to neem extract, rotenone (Peter and Oommen, 1991; Peter, 1996) and nimbecidine (Peter and Peter, 1997) demand compensatory metabolic changes in the liver.

The environmental contaminants has been altered the thyroid system in fishes by causing decreases in the circulating levels of THs and hepatic 5’-monodeiodinase activity, although the sites and mechanisms of chemical interference with thyroid function remain poorly understood (Movahedinia et al., 2011). The relationship between environmental contaminants and thyroid function in fishes are less known, despite many studies (Brown, 1993; Wendelaar Bonga, 1997; Peter and Peter, 2007). Liu et al. (2011) hypothesize the lesser concentrations of T₄ and T₃ in zebrafish is a compensatory mechanism by up regulation of expression of some HPT axis genes induced by toxicant. Li et al. (2009) found a decrease in THs levels at environmentally relevant concentrations of acetochlor treated fish as a result of tissue-specific alternations and THs related genes expressions. Hence, environmental toxicants are known to depress thyroid functions in fish (Grant and Schoettger, 1972). Convincing evidences at the molecular and evolutionary perspectives have yet to be established on the interaction of thyroid and endocrine stress axes that may operate at the extra-thyroid deiodination pathways and functional hormonal levels (Larsen et al., 2011).

The present investigation concludes that different concentrations (1, 10, and 100 µg/L) of CA and DCF have a profound influence on the THs (TSH, T₄ and T₃) levels in plasma of a freshwater fish C. mrigala during short and long-term exposures. These parameters could be effectively used as potential biomarkers of pharmaceutical toxicity to freshwater fish in the field of environmental biomonitoring.