Chapter I

Hormonal Profile of O.mossambicus exposed to agrochemical toxicity

Introduction

Agrochemicals (Insecticide, Fungicide, and Herbicide etc) have brought tremendous benefits to mankind by increasing production and controlling the vector of man and animal diseases. At the same time use of these agrochemicals has affected the health of aquatic organisms as it has posed potential health hazards to life of fishes (Osman *et al*., 2011). Agrochemicals are major cause of concern for aquatic environment because of their toxicity, persistency, and tendency to accumulate in the organisms (Joseph and Raj, 2010). The impacts of these on aquatic organisms is due to their movement from various diffuse or point sources and are posing a great threat to aquatic fauna especially to fishes, which constitute one of the major sources of protein rich food for mankind (Ray *et al*., 2015).Agrochemicals in the form of synthetic compounds are commonly referred as EDCs (Kojima *et al*., 2004) have hormone-like activity. Of the agrochemicals proved to have endocrine disrupting property, 46% are insecticides, 21% herbicides and 31% fungicides (Mnif *et al*., 2011). The endocrine and reproductive effects of these agrochemicals are believed to be due to their ability to: mimic the effect of endogenous hormones, antagonize the effect of endogenous hormones, disrupt the synthesis and metabolism of endogenous hormones and disrupt the synthesis and metabolism of hormone receptors (Raun *et al*., 2002; Blair *et al*., 2002; Tabb, and Blumberg, 2006). They may also bind to these receptors without activating them, these antagonistic accomplishment blocks the receptors and inhibits their action. Finally, they may also interfere with the synthesis, transport, metabolism and elimination of hormones, thereby decreasing the concentration of natural hormones (Cocco, 2002).

The intricate relationship between brain and endocrine action allows adaptability of an organism to the environment while maintaining homeostasis. In vertebrates, the hypothalamus represents a master regulator of homeostasis and is the critical nexus between the nervous and endocrine systems. The hypothalamus mediates responses to homeostatic imbalance mainly through regulation of the pituitary gland, which, in turn, produces hormones that are able to affect systemic change, for example within the gonad. The central role of the hypothalamic–pituitary–gonadal (HPG) axis makes it particularly susceptible and sensitive to perturbation by a variety of

environmental contaminants. Chemical disruption of the HPG axis will often result in modifications of circulating hormones, leading to an inability to mitigate environmental stress, as well as, direct impacts on reproduction and development, which has been demonstrated to produce population-level impact on fish (Kidd *et al*., 2007 and Miller *et al*., 2007).

In the vertebrate HPG-axis, a small group of neurons in the hypothalamus control the release of neuropeptides that subsequently cause release and/or synthesis of hormones into the blood stream, thereby influencing target cells. The neuroendocrine system of the hypothalamus–pituitary–gonad (HPG) axis regulates reproduction in vertebrates and can be influenced by chemicals, therefore affecting the reproductive system. Neurotoxic environmental contaminants recognized as endocrine-disrupting chemicals (EDCs) have aroused considerable interest in the field of neuroendocrinology (Gore 2000; Pillai *et al*. 2003; Panzica *et al*. 2005; Gore 2008a, b). Among these pollutants, some of the selected pesticides are considered to be hazardous because they are very persistent, are nonbiodegradable, and are ubiquitously found in the environment.

Agrochemicals have been known to interrupt the production and secretion of circulating hormones such as cortisol and thyroid hormones (Billiard *et al*., 2002).These hormones play important roles in the responses of most animals such as fish to stress (Teles *et al*., 2005; Brar *et al*., 2010). Thyroid hormones (T_3 and T_4) and cortisol secretion are under the control of the hypothalamo-pituitary-thyroid and hypothalamo-pituitary-interrenal systems (Bernier and Peter, 2001; Power *et al*., 2001). Thyroid hormones have crucial role in regulating the growth and balance of the hydro-mineral conditions of body fluids (Van Anholt *et al*., 2003), while cortisol has a major role in regulating metabolic energy, stress responses, and immune system function (Belanger *et al*., 2001). Thyroid hormones and cortisol influence the metabolism of hydrocarbons (Yarahmadi *et al*., 2016). Besides, they are used in teleost as biomarker. Plasma levels of T_3, and T_4, and T_3/T_4 ratio can be used as suitable indices for estimating the metabolism state with respect to growth, rate of protein synthesis, and oxygen consumed by tissues (Brar *et al*., 2010; Fontainhas-Fernandes *et al*., 2000; Gad and Saad, 2008). There are at least three independent, but possibly interacting, mechanisms that may explain the ability of agrochemical to reduce circulating and tissue levels of thyroid hormones (Schnitzler *et al*., 2011). First, they have been shown to change thyroid gland structure, possibly by interfering with thyroid gland function and directly disrupting hormone synthesis in the thyroid gland (Boas *et al*., 2006;

Brown *et al*., 2004; Ishihara *et al*., 2003). Second, they can target thyroid hormone metabolism. They may affect extrathyroidal iodothyronine deiodinases enzymes that control the conversion of thyroid hormones and are thus essential in the regulation of levels of biologically active T_3 locally and systemically (Ishihara *et al*., 2003; Zoeller and Tyl, 2007). One more possible mechanism has been been reported where agrochemical exposure increases bile flow rate as well as the biliary excretion of T_4 (EPA 2015).

One of the recently explored mechanism is T_4 clearance by hepatic mechanism where agrochemical exposure induces the expression and activity of the phase-II enzymes glucuronosyltransferase and sulfotransferase as conjugate group acceptors and increase T_4 conjugation reducing the biological half-life of T_4 (Klaassen and Hood, 2001). Agrochemicals may also competitively bind to thyroid hormone binding proteins like transthyretin in blood (Boas *et al*., 2006; Ishihara *et al*., 2003; Wade *et al*., 2002) and can potentially displace thyroid hormones from their carrier molecules. Moreover, these may interact to produce summative effects. Besides these direct effects, indirect effects via disruption of thyroid hormone receptors and accessory proteins that directly control the gene expression through thyroid hormone responsive elements can also interfere with the thyroid system (Blanton and Specker, 2007; Ishihara *et al*., 2003).

Animals are known to exhibit primary response by way of showing changes in the hormone profile in the blood upon exposure to changed environment/toxic conditions. Fish are reported to respond to the exposure of various pesticides by way of showing changes in the profile of various hormones. The studies on the steroidogenesis of male and female sex hormones in fishes towards the improvement of fisheries and aquaculture are carried out by many researchers. On the other hand, the primary endocrine responses of fish upon exposure to toxic conditions were investigated only in few fish species. A survey of literature on fish toxicology with special reference to endocrine response indicates that more information are available on the changes in hormone profile of fishes exposed to heavy metals compared to lesser information on the effects of pesticides on the hormone profile of fish (Swarnalatha 2015).

Some agrochemicals may exert their action by interfering with the brain's release of hormones, which in turn regulate the production of other hormones that control the growth and the activity of many other endocrine glands. Indeed, the pituitary has been termed the conductor of the endocrine orchestra, and pollutants that cause the pituitary region in the brain malfunction may therefore have multiple effects. In aquatic systems, agrochemicals easily join food webs, and their concentrations increase with each trophic level (Sun *et al*., 2006). In the case of fish, accumulation of these compounds in gonads may result in reduced reproduction potential, as well as in a decrease in fry number and developmental disorders. Agrochemicals-induced endocrine disruption often mimics sex steroidal action resulting in physiological functional disarray of hypothalamo–pituitary–gonadal (HPG) system at multiple levels (Senthilkumaran, 2015). Among various group of agrochemicals, he opines that organochlorine and organophosphate family of agrochemicals impart sex steroidal mimicking activity with slightly higher resemblance to estrogens when compared to androgenic action and suggests that the effects of sex-steroid analogues where in sex reversal to reproductive dysfunction is evident, which may imply the extent of sexual plasticity in teleosts compared to other vertebrates.

There is increasing evidence that a wide range of chemicals can interfere with thyroid and adrenal functions (Maranghi *et al*., 2003; De Angelis *et al*., 2007; Khatun and Mahanta, 2014; Veeraiah *et al*., 2014) and that the developmental life stages are critically vulnerable to agrochemicals (Mantovani, 2006). Furthermore, the residues of agrochemicals in edible parts of fish are another problem (Darko *et al*. 2008, Li *et al*., 2008).Thyroid hormones regulate a number of biological processes essential for growth, metabolism as well as brain maturation (Bernal *et al*., 2003). Thyroid hormone disruption can result in negative impacts on foetal brain development (Ghisari and J.Bonefeld, 2005). Specifically, a few studies have suggested that chlorpyrifos may target thyroid and adrenal homeostasis both in human and animal models. (Jacobsen *et al*., 2004; Jeong *et al*., 2006). Hence pollutants such as insecticides may significantly damage certain physiological and biochemical processes when they enter into the organs of fishes (John, 2007; Banaee *et al*., 2011). Since fishes are important sources of protein and lipids for humans and domestic animals, so health of fishes is very important for human beings. Although the focus of much recent research has been towards impacts on reproduction and associated sex steroids (Tyler *et al*. 1998), contaminant interference in other endocrine

systems such as those of thyroid hormones (Eales and Brown 2005) and glucocorticoid stress hormones (Hontela 2005; Vijayan *et al*. 2005) are also of growing interest.

The response to stress in fish is characterized by the stimulation of the hypothalamus, which results in the activation of the neuroendocrine system and a subsequent cascade of metabolic and physiological changes (Lowe & Davison 2005; Martínez-Porchas *et al*. 2009). These changes enhance the tolerance of an organism to face an environmental variation or an adverse situation while maintaining a homeostasis status (Martínez-Porchas *et al*. 2009). Under conditions of stress, the body of the fish emits immediate responses recognized as primary and secondary responses. The primary response is the perception of an altered state by the central nervous system (CNS) and the release of the stress hormones, cortisol and catecholamines (adrenaline and epinephrine) into the bloodstream by the endocrine system (Martínez-Porchas *et al*. 2009). Secondary responses occur as a consequence of the released stress hormones (Martínez-Porchas *et al*. 2009), causing changes in the blood and tissue chemistry, e.g. an increase of plasma glucose (Begg & Pankhurst 2004; Martínez-Porchas *et al*. 2009). Cortisol is a hormone secreted from the hypothalamo pituitary gland-internal axis (HPI) and used in many studies as the stress indicator (Flik *et al*., 2006; Gagnon *et al*., 2006; Sepici-Dinçel *et al*., 2009). The effects of prolonged exposures to agrochemicals on cortisol production have been examined in both the laboratory, and in wild populations. Many studies have indicated HPI axis exhaustion as a common phenomenon - prolonged cortisol elevation eventually creates negative feedback on the HPI axis, down-regulating receptors and causing atrophy of cells (e.g., pituitary corticotrophs; Basu *et al*. 2002; Laflamme *et al*. 2000; Levesque *et al*. 2003; Gravel *et al*. 2005; Marentette *et al*., 2012).

From the foregoing review of literature, it is evident that investigations on the effect of agrochemicals at hormonal level leads to atypical sexual development, irregular sex ratios, males feminization and disturbed mating behaviour are well documented in different fishes. It is apparent from the literature cited above that there are apparent inconsistencies in these experiments which may potentially reflect the interaction of multiple hormones with each other. Furthermore, a comparative and comprehensive investigation on the effect of different agrochemicals on hormonal patterns of a single fish species is not available excepting that of Sumathiral (2006) and Maheswari (2010). ***Hence, the present study is focused on the***

alterations in the hormonal profile as an adaptive change of the freshwater teleost, Oreochromis mossambicus exposed to sub-lethal concentration of agrochemicals: IMI, CZ, MN and PE.

Materials and Method

Animal Maintainence:

Tilapia O.mossambicus were obtained from the pure brooders of Vadodara district and transferred to the laboratory. The acclimation period was for 15 days at 27 ± 4^0C, pH 7.4 ± 0.5, dissolved oxygen 8 ± 0.3 mg/L, total hardness 188 mg/L $CaCO_3$ with a 12:12 light:dark photoperiod. Fish were supplied daily with commercial fish food during acclimation.Animal maintenance and experimental procedures were in accordance with the guideline of A.P.H.A., A.W.W.A. and W.P.C.F. (1998).

Experimental protocol:

On basis of LC_{50} value of each agrochemical sub lethal study dose LC_{50}/10 was chosen for hormonal assay studies. The experimental regime was maintained in the laboratory for 14 days with a control group having three replicates in each group. The experiment was performed semi statically with a group of 10 fishes in experimental aquaria. Hormonal assays of the experimental as well as the control fish were carried out at 15^{th} day of exposure. All the groups were kept under continuous observation during the experimental period. After the completion of the exposure fish were caught very gently using a small dip net, one at a time with least disturbance. They were slowly released in the tough containing 1% clove oil to make it immobile, blotted dry and blood was collected by tail ablation.

Hormonal assays:

The blood was collected and plasma was separated and was further used for hormonal assays. Hormonal titers like cortisol *(Cyaman Cat # 500360),* TSH (*Biodetect kit # 1003),* T_3 (*Biodetect kit # 1001),* T_4 (*Biodetect kit # 1002),* 17-βestradiol *(Cyaman Cat #582251)* and 11-keto testosterone *(Cyaman Cat # 582751)* were performed. The detailed procedure of the hormonal assays is discussed in materials and method.

Statistical Analysis:

The difference between the control and the exposed fishes was determined by One-Way ANOVA. If there was significant difference, Dunnett's multiple comparison tests were utilized to recognize specific differences in the alterations found between the control and the exposed groups. The significant level of the tests was set at α=5% ($p<0.05$).

Results

The calculated values for hormones and standard deviation, along with per cent change over the control in different hormones of fish *O.mossambicus*, with different agrochemical exposure are given in Fig. 1.1-1.6.

Sublethal exposure of agrochemicals resulted in a decrease in cortisol concentration ranging from 2.2 ± 0.15 ng/ml to 5.9 ± 0.6 ng/ml in response to agrochemical exposure (Table 1.1, 1.2). At individual level however, the significance was found only with exposure of CZ (-61%), a fungicide and IMI (-58%) an insecticide in comparison to the normal control group. In contrast Mn and PE resulted in an insignificant decrease. Impairment of thyroid function was demonstrated by the significant decrease ($p < 0.01$) in plasma T_3, T_4 and TSH levels by CZ (-65%, -74% and -57% for T_3, T_4 and TSH respectively) and IMI (-74%, -54% and -47% for T_3, T_4 and TSH respectively) exposure (Table-1.3). Thyroid titer in PE exposed group resulted into a significant increase ($p<0.05$) in T_3, T_4 and TSH level (67%, 103% and 57% for T_3, T_4 and TSH respectively). An insignificant decrease in T_3, T_4 and TSH level (-31%, -15% and -26% for T_3, T_4 and TSH respectively) in MN exposed group compared to the normal control group.

In Tilapia, *O. mossambicus*, agrochemicals exposure resulted into a significant increase plasma estrogen (20%) and testosterone (49%) levels in CZ exposed group of fish and Significant increase (119%) in estrogen with a decrease testosterone (-18%) in PE exposed group of fish in comparison to the normal control group. On exposure of IMI and MN the estrogen titer was found to decrease (-13%,-20%) whereas, there was an increase (76%, 10%) in the titer of Testosterone in comparison to the normal control group (Table 1.1, 1.2 and Fig 1.5, 1.6).

Hormones	Control	IMI	CZ	MN	PE
Cortisol	5.93 ± 0.66	2.26 ± 0.66	2.43 ± 0.152	5.1 ± 1.21	5.06 ± 1.52
T_3	5.57 ± 0.15	1.93±0.404	1.40 ± 0.10	3.83 ± 0.57	9.33 ± 1.36
T_4	4.60 ± 0.12	1.20 ± 0.43	2.13 ± 0.15	3.93 ± 0.37	9.47 ± 0.60
TSH	7.36 ± 0.32	3.1 ± 0.55	3.83 ± 0.58	5.43 ± 0.15	11.60 ± 0.62
17-β estradiol	3.4 ± 0.2	2.7 ± 0.1	8.53 ± 0.2	3.87 ± 0.2	7.46 ± 0.05
11-ketotestosterone	4.8 ± 0.1	7.17 ± 0.5	8.47 ± 0.58	4.3 ± 0.8	5.67 ± 0.987

Table 1.1: Represents the mean ± SE values of cortisol, T_3,T_4, TSH, Estradiol and 11 ketotestosterone of female tilapia *O.mossambicus*, exposed to agrochemicals.

Hormones	Control	IMI	CZ	MN	PE
Cortisol	4.6±0.15	1.26±0.15	1.43±0.15	4.1±0.23	4.06±0.78
T_3	3.9±0.11	1.17±0.04	0.90±0.03	2.73±0.31	8.17±0.24
T_4	3.30±0.26	1.00±0.26	2.17±0.30	2.60±0.56	8.13±0.83
TSH	6.03±0.55	2.2± 0.51	3.51± 0.1	4.5±0.45	10.27±0.15
17-β estradiol	2.4±0.24	2.14±0.1	7.53±0.23	2.87±0.25	6.46±0.05
11-keto testosterone	3.8±0.14	6.17±0.56	7.80±0.18	2.96±0.85	4.67±0.97

Table 1.2: Represents the mean ± SE values of cortisol, T_3,T_4, TSH, 17-β Estradiol and 11 ketotestosterone of male tilapia *O.mossambicus* exposed to agrochemicals.

Hormones	CZ	IMI	MN	PE
Cortisol	-61%	-58%	-14%	-14%
TSH	-57%	-47%	-26%	57%
T_3	-65%	-74%	-31%	67%
T_4	-74%	-54%	-15%	103%
Estradiol(E_2)	-20%	150%	13%	119%
11-Testosterone	49%	76%	-10%	18%

Table 1.3: Depicts the mean percentage difference of cortisol, T_3, T_4, TSH, Estradiol and 11-ketotestosterone of tilapia *O.mossambicus* exposed to agrochemicals.

Ratio	Control	CZ	IMI	MN	PE
T_3/T_4	1.210145	1.611111	0.65625	0.974576	0.985915

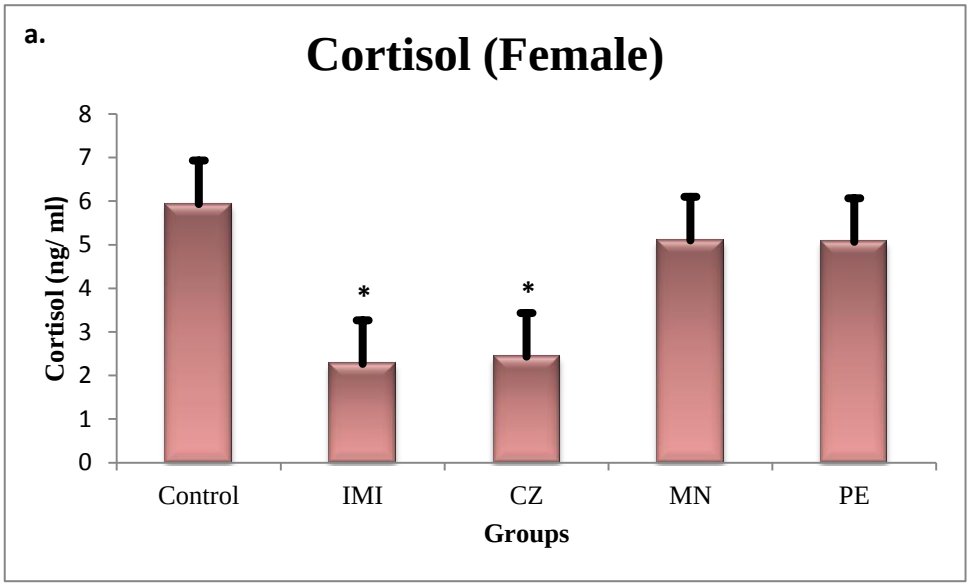
Table 1.4: Depicts the ratio of T_3 and T_4 of *O.mossambicus* exposed to agrochemicals.

Hormones	IMI	CZ	MN	PE
Cortisol	0.02*	0.03*	0.3	0.3
T_3	0.02*	0.03*	0.04*	0.03*
T_4	0.034*	0.037*	0.046*	0.038
TSH	0.021*	0.039*	0.048*	0.035*
17-β estradiol	0.49	0.010*	0.09	0.01*
11-keto testosterone	0.04*	0.032*	0.65	0.976

Table 1.5: Shows the p value of Dunnettes multiple comparison tests of various hormones of male *O.mossambicus* exposed to agrochemicals. (*) denotes the level of significance at *p<0.05,**p<0.01,***p<0.001

Hormones	IMI	CZ	MN	PE
Cortisol	0.024*	0.035*	0.4	0.4
T_3	0.025*	0.037*	0.041*	0.032*
T_4	0.036*	0.033*	0.041*	0.042
TSH	0.022*	0.031*	0.042*	0.033*
17-β estradiol	0.42	0.011*	0.07	0.014*
11-keto testosterone	0.03*	0.022*	0.85	0.981

Table 1.5: Shows the p value of Dunnettes multiple comparison tests of various hormones of female *O.mossambicus* exposed to agrochemicals. (*) denotes the level of significance at *p<0.05,**p<0.01,***p<0.001



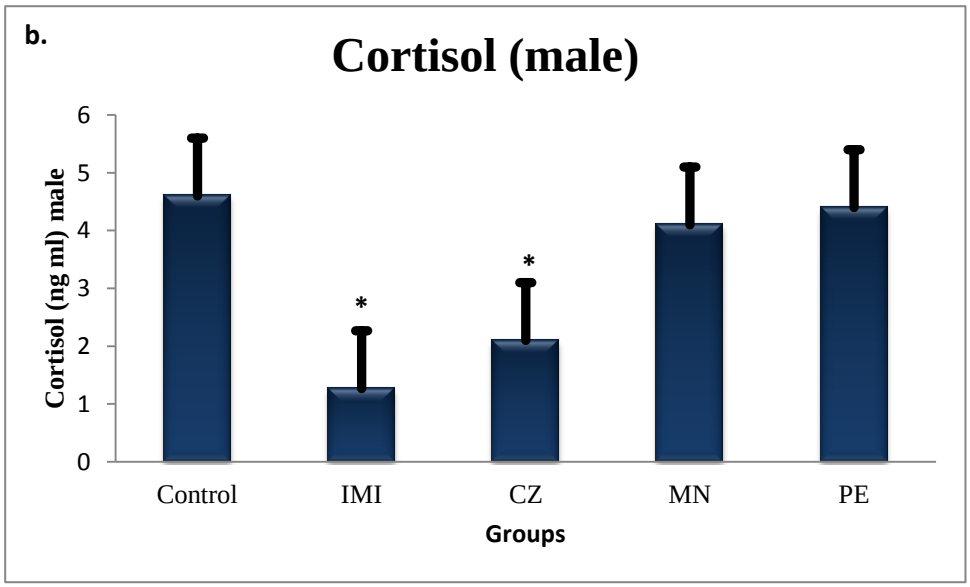
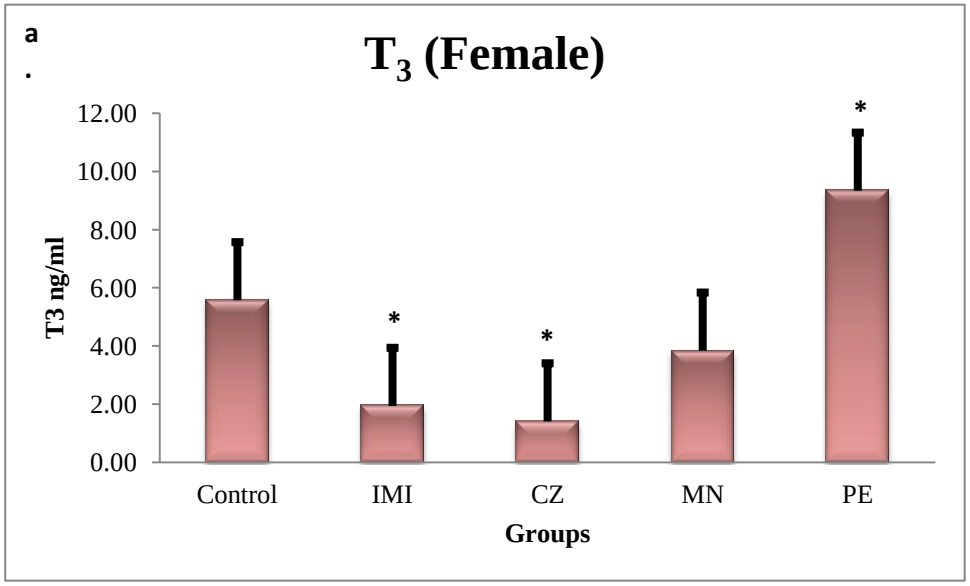


Figure 1.1 (a, b): Represents the Graphs of plasma cortisol in female (a) and male (b) *O.mossambicus* exposed to agrochemicals. (*) denotes the *p<0.005,**p<0.01,***p<0.001



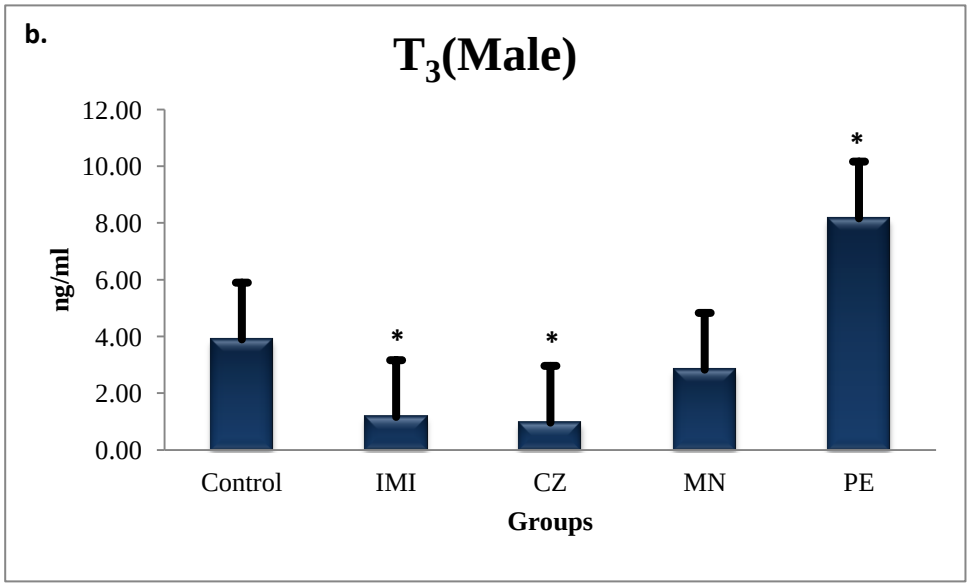
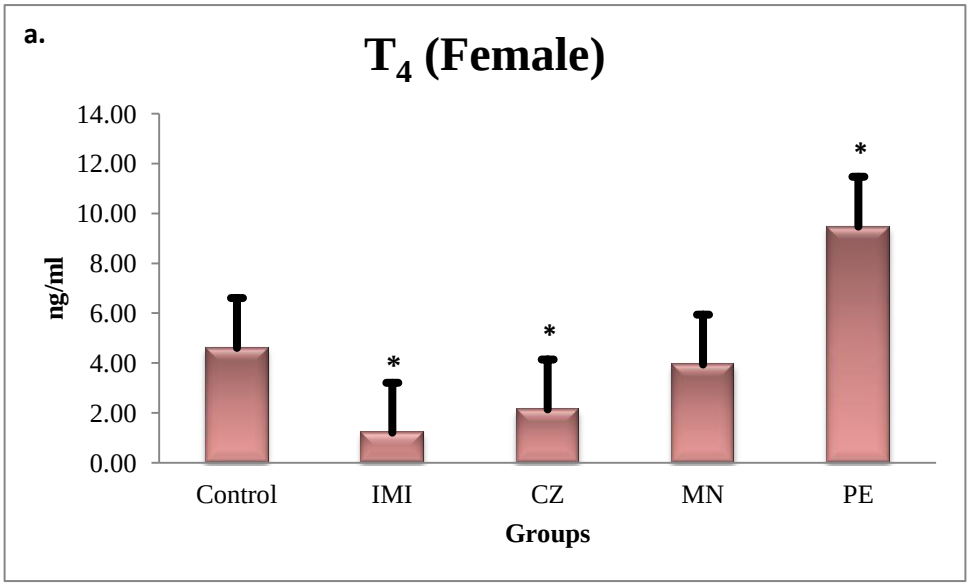


Figure 1.2 (a, b): Represents the Graphs of plasma T_3 in female (a) and male(b) *O.mossambicus* exposed to agrochemicals. (*) denotes the *p<0.005,**p<0.01,***p<0.001



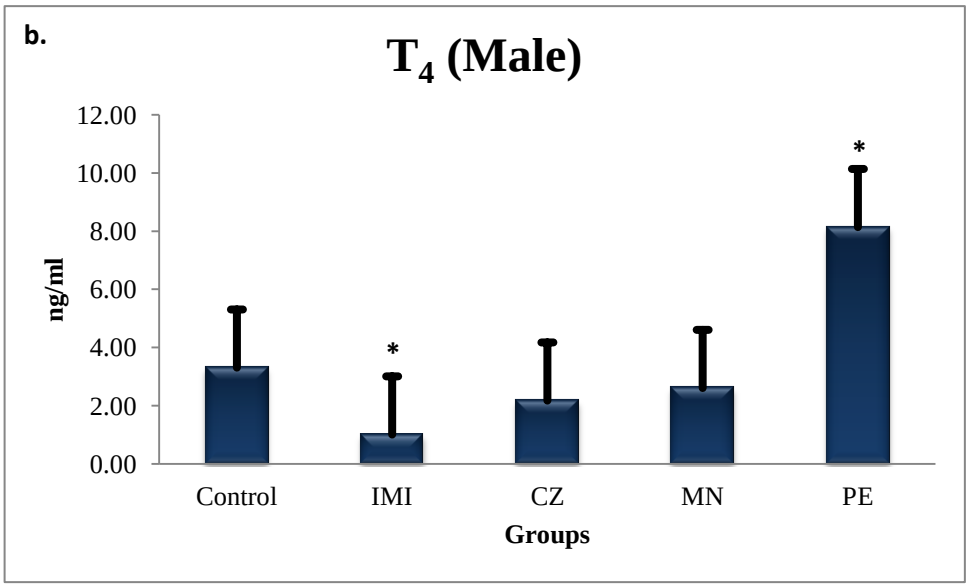
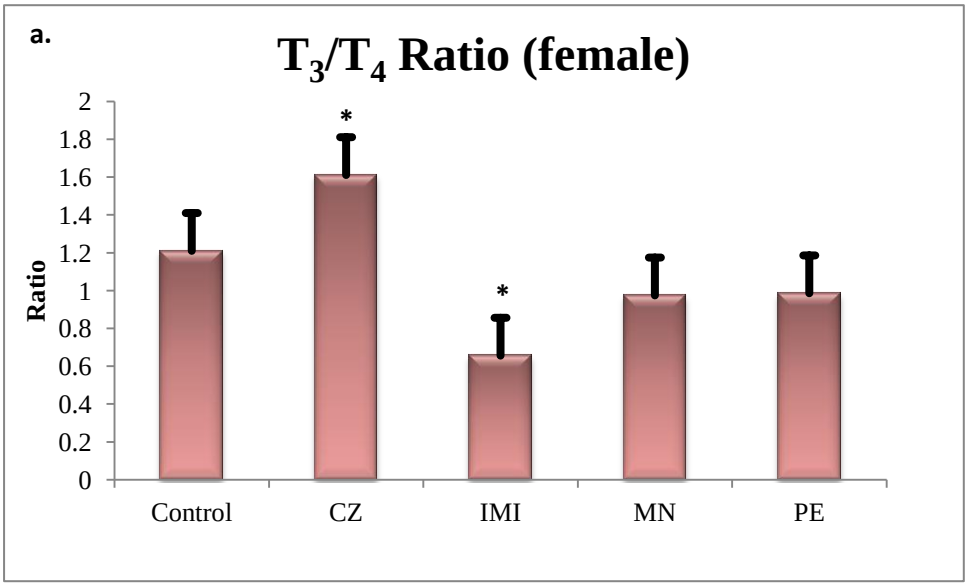


Figure 1.3 (a, b): Represents the Graphs of plasma T_4 in female (a) and male(b) *O.mossambicus* exposed to agrochemicals. (*) denotes the *p<0.005,**p<0.01,***p<0.001



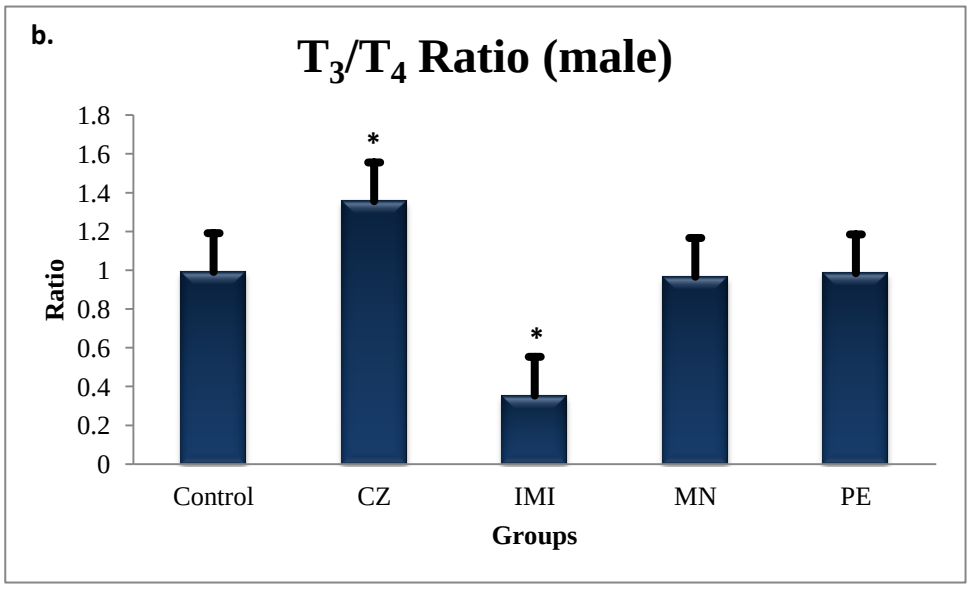
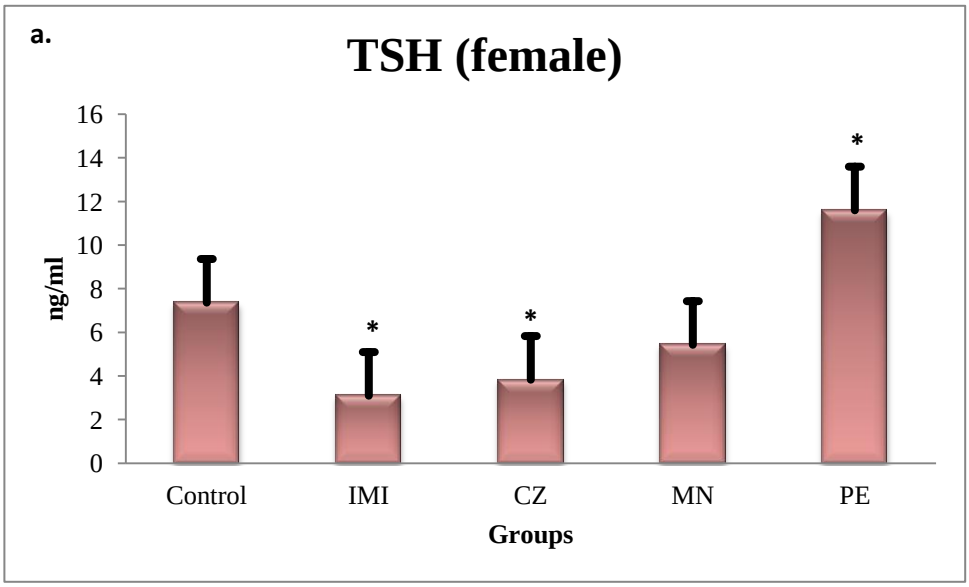


Figure 1.4 (a, b): Represents the Graphs of plasma ratio of T_3/T_4 in female (a) and male(b) of *O.mossambicus* exposed to agrochemicals. (*) denotes the *p<0.005,**p<0.01,***p<0.001



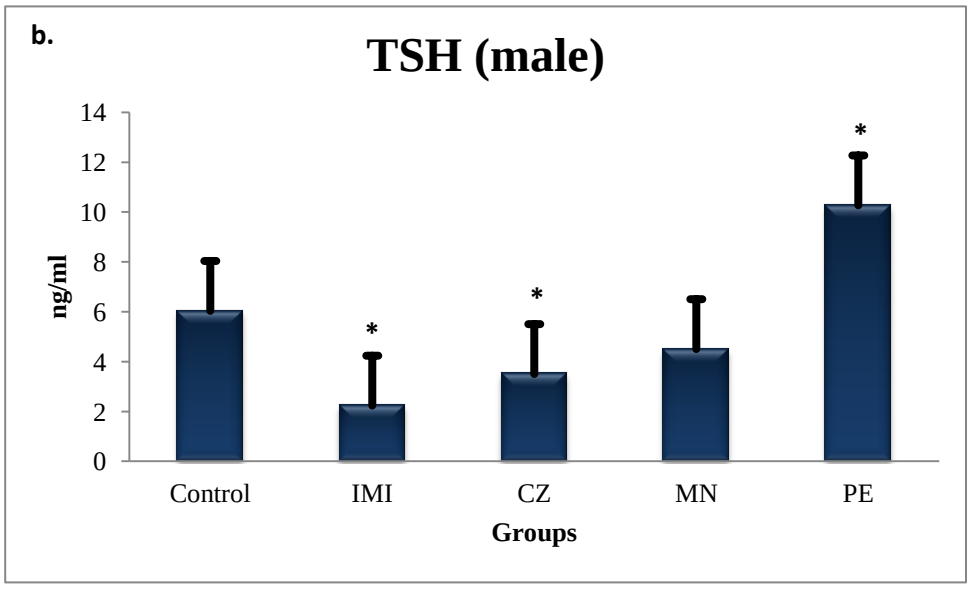
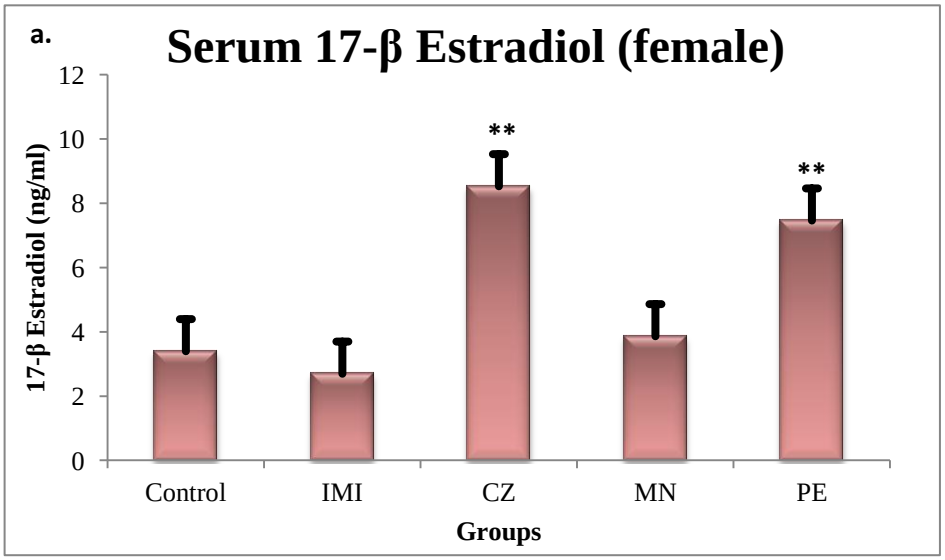


Figure 1.5 (a, b): Represents the Graphs of plasma TSH in female (a) and male(b) of *O.mossambicus* exposed to agrochemicals. (*) denotes the *p<0.005,**p<0.01,***p<0.001



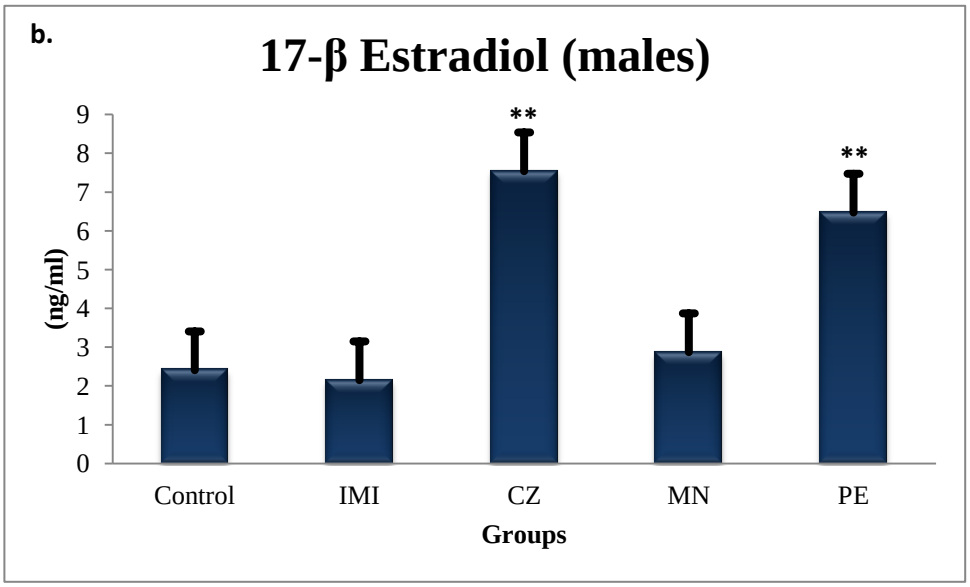
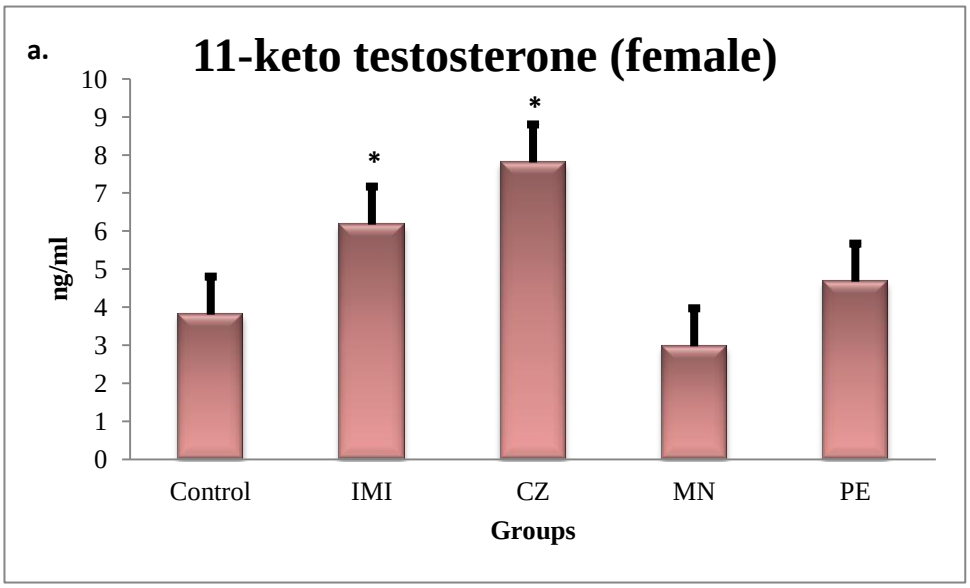


Figure 1.6 (a, b): Represents the Graphs of plasma 17-β Estradiol in female (a) and male (b) of *O.mossambicus* exposed to agrochemicals. (*) denotes the *p<0.005,**p<0.01,***p<0.001.



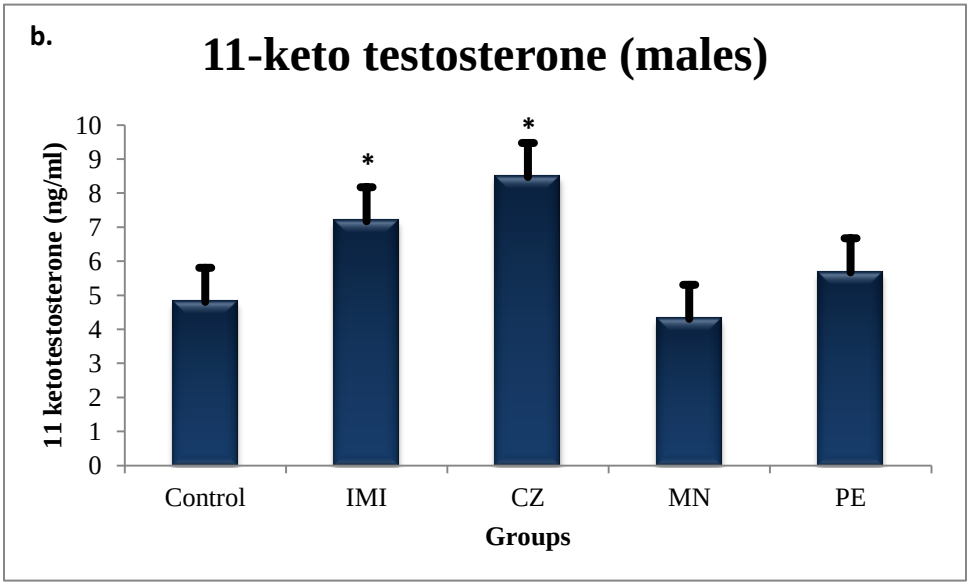


Figure 1.7 (a, b): Represents the Graphs of plasma 11-ketotestosterone in female (a) and male (b) of *O.mossambicus* exposed to agrochemicals. (*) denotes the *p<0.005,**p<0.01,***p<0.001.

Discussion

The chemical coordination by endocrine system in organisms is known to regulate a number of hormone dependent physiological functions which are essential for the survival of the organism both in normal as well as in an altered environmental condition. This endocrine system is a potential target of xenobiotics and its vulnerability resides in part in the finely tuned mechanisms through which the endocrine control system operates in animals (Hontela,1997). The xenobiotics that enter into the body can have either direct adverse effects on the endocrine gland and tissues, or their effects can be indirect through alterations of homeostasis and damaging activities of non-endocrine organs (Atterwill and Flack,1992).

The hypothalamic–pituitary interrenal (HPI) axis is responsible for releasing corticosteroids and catecholamines in response to a stressor. Cortisol is the major corticosteroid in teleost fish and most mammals (Miller , 2006). When a fish perceives a stimulus as a stressor, the hypothalamus releases corticotropin releasing hormone (CRH) that stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH enters the bloodstream and stimulates the steroidogenic interrenal cells in the anterior head kidney to synthesize cortisol (Hontela 2005). ACTH binds to its receptor in the cell membrane, and cyclic adenosine monophosphate (cAMP) activates protein kinase A. Protein kinase A activates cholesterol ester hydrolase releasing free cholesterol from cholesterol esters within the cellular matrix (Stocco 2000). At the outer mitochondrial membrane, steroidogenic acute regulatory (StAR) protein regulates the passage of cholesterol to the inner membrane where cytochrome P450 side chain cleavage (cP450scc) mediates the transformation of cholesterol to pregnenolone (Stocco 2000). This is a rate limiting step in cortisol synthesis and can be disrupted by contaminants (Walsh *et al*. 2000). Pregnenolone moves to the cytoplasm where cytochromes P45017α and P450C21 create 11-deoxycortisol, that is shunted back to the mitochondria to be transformed into cortisol by cytochrome P45011ß (Hontela 2005). Cortisol regulates its own production through a negative feedback loop by altering ATCH secretion at the pituitary and hypothalamus (Hontela, 2006). Cortisol affects a variety of systems that regulate homeostasis (Dorval, 2003). High plasma cortisol levels indicate the animal is under acute or sub-chronic stress, while low levels indicate no stress, interrenal exhaustion or impairment of the HPI axis (Martinez-porchas *et al*., 2009; Miller and Hontela, 2011). Corticosteroids can also be influenced by factors other than stressors.

Genetic differences between species may result in very different cortisol levels and developmental stage may also be important. Some fish are more sensitive to stress during smolting and may decrease their cortisol production as they age (Barton 2002; Jasmin Christal *et al*., 2003).

Several studies have corroborated the impairment in cortisol synthesis and secretion due to the action of agrochemicals (Aluru *et al*., 2004-06; Gravel and Vijayan, 2006; Hontela, 2006). The impairment is either at the level of CRH, ACTH or at the level of StAR protein , and N6, 2'-o-dibutryl adenosine 3': 5'-cyclic monophosphate (dbc-AMP) (Leblond *et al*., 2001) which is the common target of many environmental pollutants ranging from pesticides to pharmaceuticals (Hontela, 2006; Martinez-porchas *et al.,* 2009). Therefore many pollutants halt cortisol secretion and even if the fish is under stress this will probably not be reflected in the cortisol response. In the present study cortisol level in *O. mossambicus* was found to be lower than that of control level on exposure to IMI and CZ, suggesting an impairment of the interrenal exhaustion or impairment of the HPI axis (Miller, 2003; Martinez-porchas *et al*., 2009).However, gene expression and receptor studies will help in better understanding of the exact mechanism behind it. On the other hand, a decreased level of serum cortisol under toxic conditions has been reported in *Fundulus heteroclittus* on exposure to naphthalene as a result of severe necrosis of interrenal tissues (Swarnalatha 2015).

Hontela and her co-workers (2006), while investigating the impact of toxicants on fish interrenal tissues, based on the *in vitro* studies, also reported inhibiting effects of pollutants on interrenal function and cortisol secretion. *Oreochromis mossambicus*, under sub-lethal thiodon exposure registered significant reductions in serum cortisol levels following 1, 6 and 12 hours of exposures (Parvatham *et al*., 2004). Similar reductions in serum cortisol level were also reported in *Oreochromis mossambicus* exposed to sub-lethal concentration of dimecron (Karthikeyan *et al*., 2004), in *Channa striatus* exposed to lethal and sub-lethal concentrations of sevin (Sumathiral, 2006), in *Labeo rohita* fingerlings exposed to endosulfan and dietary pyridoxine (Akhtar *et al*., 2010), in *Clarias gariepinus* exposed to varying concentrations of endosulfan (Ezemonye and Ikpesu, 2010) and in *Sarotherodon mossambicus* under endosulfan toxicity (Thangavel *et al*., 2010). Wedemeyer and Yasutake (1997) stated that too low levels of cortisol are indicative of interrenal exhaustion from severe stress, whereas, too high levels indicated the

fish to be under chronic or acute stress. Similar reductions in cortisol level as a function of interregnal exhaustion were also reported in fish exposed to various pesticides (Parvatham *et al*., 2004; Karthikeyan *et al*., 2004; Sumathiral, 2006 and Thangavel *et al*.,2010; Swarnalatha, 2015) and during *in vitro* exposure of head kidney tissues of fish to pesticides (Leblond *et al*., 2001) and to heavy metals (Brodeur *et al*., 1997). The reduced cortisol level in the present study, together with similar reports of interrenal exhaustion and reduction in cortisol level in other fish under different types of toxic conditions could be considered as an adaptive response by the fish by way of maintaining low metabolic rate under pesticide. However, at this juncture as ACTH , StAR and 17-β hydroxylase are not being studied and hence the actual mode of action cannot be illucidated.

Thyroid hormones are important in growth, metabolism, and homeostasis. Secretion of thyrotropin-releasing hormone from hypothalamus releases thyroid-stimulating hormone (TSH) from pituitary gland into the plasma, which stimulates the release of thyroid hormones (Bernier *et al*., 2001; Van Anholt *et al*., 2003; Yarahmadi *et al*., 2016). The main difference between T_3 and T_4 is their ability to bind to the receptors. T_3 binds to receptors ten times more than that T_4; therefore, it is considered as the main active biological form (Brar *et al*.,2010). Thyroid hormones regulate a number of biological processes essential for growth, metabolism as well as brain maturation (Bernal *et al*., 2003). The hypothalamo-pituitary-interrenal axis modulates the thyroid axis in fishes and other vertebrates. Corticosteroids hormones produced by adrenocortical cells in fish, synergize with TH at target tissues to promote morphogenesis (Denver, 2009). Further, as reported by Terrien and Prunet (2013) TH and CS can regulate each other, in a positive or negative way depending on molecular, cellular and physiologic context. This cross-regulation is important to amplify hormone signals, regulation of hormone activity, and coordinate hormone action. Plasma levels of T_3, and T_4, and T_3/T_4 ratio can be used as suitable indices for estimating the metabolism state with respect to growth, rate of protein synthesis, and oxygen consumed by tissues (Fontainhas-Fernandes, 2000; Gad 2008). Organic pollutants may decrease thyroid hormones production and change their circulating concentrations (Hood *et al*., 2003; Teles *et al*., 2004). However, the exact mechanism of effect of these pollutants is not clear. Decreased thyroid hormones and T_3/T_4 ratio variations in regions polluted with crude oil are reported earlier. Increased deiodination, biliary excretion of thyroid hormones (Gad *et al*., 2008), negative feedback in the hypothalamo-pituitary-thyroid axis, and disruption in rate limiting

function of brain and hepatic deiodination enzyme (5'- monodeiodinase) (Brown *et al.,* 2004; Yarahmadi 2016) would decrease T_3 and T_4 in plasma, and change T_3/T_4 ratios. The present studies on *O.mossambicus* showed that exposure of agrochemicals at sublethal concentration induce alternations in plasma concentration of T_3, T_4 and TSH in comparison to the normal control group. In particular, possible impairment of thyroid function was demonstrated by the significant decrease in plasma T_3, T_4 and TSH levels in CZ and IMI exposed fishes (Table-1.1,1.2).

A reduction in T_3, T_4 and TSH hormone levels indicates reduced thyroid function and reduced metabolic activity. Similar results have been reported in *Salmo salar* exposed to endrin toxicity (Freeman and Sangalong, 2000). Similar reductions in thyroid hormone levels were also reported in *Cyprinus carpio* exposed to sublethal endosulfan (Jenkins *et al*., 2003), in *Oreochromis mossambicus* (Karthikeyan *et al*., 2004), in *Sarotherodon mossambicus* (Thangavel *et al*., 2005a) exposed to dimecron and in *Channa striatus* exposed to lethal and sublethal concentrations of sevin (Sumathiral, 2006), in fresh water fish *Clarias batrachus* exposed to IMI (Padma priya *et al*., 2014). Furthermore, occurrence of T_3 in lesser amount may indicate the rapid turnover from T_4 to T_3 usage (Ray *et al*., 2015). The regulatory pathways involved in thyroid homeostasis are numerous and complex. As a consequence agrochemicals can act at many levels in the thyroid system (Ishihara *et al*., 2003). There are at least three independent, but possibly interacting, mechanisms that may explain the ability of agrochemicals to reduce circulating and tissue levels of thyroid hormones. First, agrochemicals have been shown to change thyroid gland structure, possibly directly interfering with thyroid gland function and disrupting directly the hormone synthesis in the thyroid gland (Ishihara *et al*., 2003; Brown *et al*., 2004; Boas *et al*., 2006; Schnitzler *et al*., 2011). Secondly, agrochemicals can target thyroid hormone metabolism. They may affect extrathyroidal iodothyronine deiodinases, enzymes that control the conversion of thyroid hormones and are thus essential in the regulation of levels of biologically active T_3 locally and systemically (Zoeller and Tyl, 2007). It has been shown that exposure of agrochemicals increases bile flow rate as well as the biliary excretion of T_4 (Collins and Capen, 1980a). Agrochemical exposure also induces the expression and activity of specific enzymes that utilize thyroid hormones as conjugate group acceptors and increase T_4 conjugation (Klaassen and Hood, 2001). These actions facilitate T_4 clearance by hepatic metabolism, reducing the biological half-life of T_4. Finally, agrochemicals can also competitively bind to thyroid hormone

binding proteins like transthyretin (TTR) in blood (Wade *et al*., 2002; Ishihara *et al*., 2003; Boas *et al*., 2006) and can potentially displace thyroid hormones from their carrier molecules. Moreover, these may interact to produce summative effects. Besides these direct effects, indirect effects via disruption of thyroid hormone receptors and accessory proteins that directly control the gene expression through thyroid hormone responsive elements can also interfere with the thyroid system (Ishihara *et al*., 2003; Blanton and Specker, 2007).

Results of the present work confirm the observations of the previous authors and the reduced thyroid titers on exposure of CZ and IMI is possibly mediated through either direct effects or through indirect effects through receptors. The increase in T_3, T_4 and TSH levels in the plasma of *O. mossambicus* following PE exposure probably suggests the onset of an increased metabolic activity triggered by the pesticide exposure. The main metabolic pathways for thyroid hormones are deiodination, glucuronidation and sulfation (Brown *et al.,* 2004). Iodothyronine deiodinase are enzymes involved likewise in the activation of thyroid hormone. In fish, apparently more than in other vertebrates, these important thyroid hormone transformations are controlled outside the thyroid and occur mainly in peripheral tissues (liver, brain, kidney, gill) as proposed by Schnitzler *et al*., (2011), an increased titer of the thyroid hormones on PE exposure is probably routed through the peripheral tissues, and hence, the tissue specific thyroid titer will confirm and shade more light.

Several pesticides have been identified as endocrine disruptors in other vertebrate fishes, including atrazine, 2,4-D, DDE, DDT, diazinon, diuron, endosulfan, fenthrothion, glyphosate, lindane, parathion and permethrine; for instance, it has been shown that DDT methoxychlor have xenoestrogenic activity, meaning that they are chemically similar to estrogens and trigger hormonal responses in exposed organisms, including the induction of vitellogenesis in young male fishes (Pait and Nelson, 2013).Agrochemicals at low concentrations may act as mimics or blockers of sex hormones, causing abnormal sexual development, feminization of males, abnormal sex ratios, and unusual mating behavior. The unique plasticity of sex differentiation in fish suggests that these animals may be very susceptible to disruption of sexual characteristics by pollutants (Murthy *et al*., 2013). Many studies show a direct relationship between concentrations of pesticides and related chemicals in fish gonads and alter the hormone concentrations. Agrochemicals are reported to cause degenerative changes in gonads and arrest gametogenic

processes either by acting directly on the gonads or by interfering with the secretary activity of the hypothalamohypophyseal-gonadal/thyroid axis that regulates various reproductive events. Secretion of hormones such as gonadotropin-releasing hormone (GnRH), gonadotropin, growth hormone, adrenocorticotropic hormone (ACTH), testosterone, estrogens, 17,20β-dihydroxyprogesterone and thyroid hormones are in general lowered, leading to cessation of gametogenesis, vitellogenesis, Oocyte maturation, ovulation, spermiation, etc.

Adverse effects of pesticides have also been demonstrated on fecundity, fertilization, hatching, and postembryonic development. The effects are highly variable and depend on the nature, dose, and mode of application of the pesticides (Lal, 2007). Various fish species have also been used to investigate the adverse effects of agrochemicals for reproductive fecundity (Sisneros *et al*., 2004; Strehler and Treiman, 2004; Hoeger *et al*., 2005; Diamanti-Kandarakis *et al*., 2009; Mortensen and Arukwe, 2007; Sadekarpawar and Parikh, 2013; Desai and Parikh 2010 and 2011). Majority of the studies have focused on morphological and biochemical alterations, studies of Wirbisky *et al*., (2015) has provided a genetic profile and has connected to physiological changes and responses as a result of the developmental atrazine exposure . Ecotoxicological manifestation of a commercial pyrethroid, devicyprin, (cypermethrin- 25%) on gonadal impairment in freshwater food fish, *Channa punctatus* has been studied by Srivastava *et al*., 2008. These substances have multiple modes of action since they can potentially act on the synthesis, secretion, transport, action and elimination of endogenous hormones (Segner *et al*., 2003). The reproductive system in vertebrates is regulated by the hypothalamic-pituitary-gonadal (HPG) axis, where the HPG axis begins in the hypothalamus and serves as the integration center through the release of gonadotropin releasing hormone (GnRH) into the hypophyseal portal system which stimulates the synthesis and secretion of the gonadotropins (luteinizing hormone-LH and follicle stimulating hormone- FSH) from the anterior pituitary gland (Plant, 2015). In females, LH exerts its function on the ovaries to promote ovulation; while FSH promotes follicular maturation and the synthesis of ovarian estrogens (Wang *et al*., 2015). In males, LH acts on Leydig cells to promote the synthesis and secretion of testosterone, while FSH binds to Sertoli cells in order to promote spermatogenesis (Jin and Yang, 2014).

Estradiol, progesterone, and testosterone produced by the ovaries and testes regulate the positive and negative feedback loops for proper reproductive function. Estrogens are steroid hormones

found in representatives of all classes of vertebrates, including fish, amphibians, reptiles, birds and mammals (Lange *et al*., 2003), 17b-estradiol (E_2) is the form responsible for most biological activities, such as its involvement in the control of sexual differentiation, maturation and reproduction. However, these hormones also exert numerous other effects on the development, differentiation and homeostasis of diverse target organs. Estrogens are also involved in the control of the cell cycle and proliferation. Estrogens act via two main mechanisms: genomic via nuclear receptors and non-genomic that takes place through membrane receptors (Acconcia *et al*., 2005; Kelly and Levin, 2001; Loomis and Thomas, 2000). Genomic estrogen actions require a relatively long time (from hours to days) to be accomplished. However, more rapid effects (from seconds to few minutes) also exist, referred to as ‘‘non-genomic signaling’’ (Falkenstein *et al*., 2000).

Several enzymes are involved in the process of estrogen biosynthesis. At the rate-limiting step, androgens are converted into estrogens via aromatization. This reaction is catalyzed by an enzymatic complex formed by the cytochrome P450 aromatase, a heme binding protein encoded by the cyp19 gene (aromatase CYP19), functioning in combination with the flavoprotein, NADPH-cytochrome P-450 reductase. A variety of agrochemicals, many of which are in use throughout the world, have been shown to affect (generally inhibit) in vitro aromatase activity in different vertebrate systems (Mason *et al*., 1987; Vinggaard *et al*., 2000; Sanderson *et al*., 2002; Zarn *et al*., 2003; Heneweer *et al*., 2004). Controlled experimentation has demonstrated that aromatase inhibitors can produce profound effects on the reproductive endocrine system and spawning success of fish (Ankley *et al*., 2002), and recent studies suggest that altered steroidogenesis is associated with adverse effects observed in fish from the field (Noaksson *et al*., 2003; Lavado *et al*., 2004). Studies conducted by Diamanti-Kandarakis *et al*., 2009 have opined the agrochemicals in the form of EDCs interact with the steroid hormones as analogs or antagonists and have suggested that these compounds are EDCs as well as more generalized toxicants.

In line of these, the present studies have obtained a significant increase in estradiol and testosterone on exposure of CZ which is agreement of the work of Anlkey *et al*., (2005) along with the other scientists who have reported that fungicides may act as both aromatase inhibitors and androgen agonists (Noriega *et al*., 2005; Vinggaard *et al*., 2000, 2002, 2005). In contrast, the

exposure of IMI and MN resulted in increase in testosterone titer and a decrease in estradiol indicating that the both the agrochemicals may be acting on aromatase (CYP19) and thus can be regarded as EDCs as it has resulted in reducing the estradiol levels (Villeneuve *et al*., 2006; Shalaby *et al*., 2007; Argemi *et al*., 2013; Reyes *et al*., 2014). Exposure of PE resulted in a decrease in testosterone titer with a concomitant increase in estradiol, probably by stimulating the CYP enzymes, in addition to aromatase, involved in steroidogenesis (Jensen *et al*., 2004; Wilson *et al*., 2004; Leino *et al*., 2005; Ankley *et al*., 2002; 2005 and 2009). Overall, the set of responses suggests that the PE is androgen antagonists and estradiol agonists, whereas, IMI and MN are androgen agonists and estradiol antagonists and the mode of action of CZ suggest that in agonists to estradiol as well as testosterone.

Conclusion:

The present study deals with alteration in the hormonal titer in a freshwater, cichlid Tilapia. O.mossambicus exposed to sub-lethal concentration of agrochemicals. The observed reductions in the plasma cortisol level is an indication of interrenal exhaustion and also an adaptive stress response of the fish by way of maintaining low basal metabolic rate (BMR) under agrochemical toxicity. Reduced thyroid titers on exposure of CZ and IMI is mediated through direct or indirect effects via specific nuclear receptors and increase in T_3, T_4 and TSH levels in the plasma of O. mossambicus following PE exposure probably suggests the onset of an increased metabolic activity triggered by the pesticide exposure. Overall, the set of responses suggests that the PE is androgen antagonists and estradiol agonists, whereas, IMI and MN are androgen agonists and estradiol antagonists and the mode of action of CZ suggest that in agonists to estradiol as well as testosterone.