DETERMINATION OF AROMATASE ACTIVITY, GONADAL HORMONE AND CELLULAR ACTIVITY IN *LABEO ROHITA* (HAM 1822) EXPERIMENTALLY EXPOSED TO SOME ENDOCRINE DISRUPTION CHEMICALS (EDCS).

Thesis

SUBMITTED TO THE
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
LUCKNOW

BABASAHEB
BHIMRAO
AMBEDKAR
UNIVERSITY
LUCKNOW
प्रज्ञा शील करुणा
ESTABLISHED 1996

FOR AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

APPLIED ANIMAL SCIENCES

Submitted By

RITA VERMA

UNDER THE SUPERVISION OF

Prof. KAMAL JAISWAL

DEPARTMENT OF APPLIED ANIMAL SCIENCES
SCHOOL FOR BIOSCIENCES AND BIOTECHNOLOGY
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
(A CENTRAL UNIVERSITY)
VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW-226 025 (U.P.), INDIA

&

Dr. A. K. SINGH

ICAR- DIRECTORATE OF COLD WATER FISHERIES RESEARCH,
BHIMTAL

Enrolment No.921/13 **Year 2016**

DECLARATION

I hereby declare that the thesis entitled **"Determination of aromatase activity, gonadal hormone and cellular activity in *Labeo rohita* (Ham, 1822) experimentally exposed to some Endocrine Disruption Chemicals (EDCs)."** embodies the results of the original research work carried out by me at the Department of Applied Animal Science in the School for Bioscience and Biotechnology Sciences, Babasaheb Bhimrao Ambedkar (Central) University, Lucknow. The work presented in this thesis has not been submitted for the award of degree or diploma to this or any other University.

(Rita Verma)

CERTIFICATE

This is certified that the thesis titled **"Determination of aromatase activity, gonadal hormone and cellular activity in *Labeo rohita* (Ham, 1822) experimentally exposed to some Endocrine Disruption Chemicals (EDCs)."** submitted by **Mrs. Rita Verma** is in original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or other university.

The thesis is submitted to Babasaheb Bhimrao Ambedkar University Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulation – 1999 as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Date:

(Prof. Kamal Jaiswal)
Supervisor
Department of Applied Animal Science
School for Bioscience and Biotechnology
BBAU, Lucknow (U.P.)

(Dr A K Singh)
Co-Supervisor
ICAR-Directorate of Cold Water Fisheries Research, Bhimtal

Head of Department

ACKNOWLEDGEMENT

After a long journey of three years of my research work, time has come to look back again and remember all those without whom this journey would have been impossible. Nothing comes worthwhile in life without struggle and these are the people who, in their own ways, have helped me to overcome the difficult situations. It is because of some special people that I have reached to a wonderful destination.

I am indebted to my mentor Prof. Kamal Jaiswal, Department of Applied Animal Sciences for introducing me to the exciting field of research in Animal sciences and allowing me to work under his supervision. I will ever remain grateful to him for his excellent guidance valuable suggestions, unending encouragement, constant cooperation and affection throughout the period of my research work.

I am indebted to my co-guide Dr. A.K. Singh, Director, Directorate of Cold Water Fisheries, Bhimtal, Nanital for his able guidance, valuable advice, constant encouragement and constructive criticism during the course of the work without which it would not have been possible for me to complete the thesis work and stand in my research career.

I take the opportunity to express my profound thanks to faculty members of the Department of Applied Animal Sciences, B. B. Ambedkar University, Dr. Suman Mishra, Dr. Abha Mishra, Dr V. Elengovan, and Dr Vankatesh Kumar. I am immensely thankful to staff members, Mr. Ramesh kumar (Technical Assistant), Miss Dipti Singh (Lab Attendant) and Mr. Sasank (Office Assistant) for their valuable cooperation regarding research work.

I also express my gratitude to the Council of Science and Technology, Uttar Pradesh, Lucknow for partly supporting my thesis work through the

awarded research project of which a small work particularly the methodology development and estimation of hormones are included.

I gratefully acknowledge the support and cooperation rendered throughout my research tenure directly or indirectly by my seniors and co workers Ms. Anita Singh, Miss Muktamayee Kumbhar, Ms. Jyoti Pandey, Miss Dipti Kashyap, Mr. Ravi Kumar Angare and Mr. Neeraj Kumar. I offer my humble thanks to Dr. Ruchi Singh, Dr Sharad Chandra Srivastava, Dr Dinesh Kumar, Dr Abubakar Anshari, and Mool Singh for their encouragement, love and for being a source of strength and motivation over the years in every walk of my Research Tenure.

Last but not least I have no words to say thanks to my family members mummy (Mangra Verma) papa (D P Verma) sister (Urvashi Verma) and brother (Karuneesh Verma), Law's family members, mother-in-Law (Vidhya devi) father-in-law (Ram Lal Verma) Sister-in-law (Kavita Verma) and brother-in law (Rahul Patel) and my husband (Dr.Sunil Kumar) because without the love, affection, guidance, extreme patience and constant support this would be not possible.

(Rita Verma)

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

µ	Micron
%	Percent
A	Alpha
B	Beta
<	Less than
>	Greater than
±	Add or subtract
L	Liter
mL	Milliliter
µL	Microliter
Kg	Kilogram
G	Gram
Mg	Milligram
Pg	Pico-gram
Ng	Nanogram
M	Meter
Cm	Centimeter
Ppm	Parts per million
EDCs	Endocrine disrupting chemicals
ARs	Aromatase receptor
ERs	Estrogen receptor
GSI	Gonadosomatic index
VTG	Vitellogenin
CYPs	Cytochrome P450
HSDs	Hydroxysteroid dehydrogenase
T	Testosterone

Review of Literature

Many environmental chemicals are known to exhibit estrogenic or androgenic activity some of these occur naturally in plants, while others are manmade by-products that are present in agricultural, industrial chemicals and domestic wastes (Ankley *et al.,* 2003; Falconer *et al.,* 2006; Fromme *et al.,* 2002; Lavado *et al.,* 2004; Petrovic *et al.,* 2002; Schwaiger *et al.,* 2002) These are chemicals that may interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife called Endocrine Disrupting Chemicals (Bateman *et al.,* 2004; Bjorkblom *et al.,* 2009; Cavaco *et al.,* 2001; Chikae *et al.,* 2004a; Mandich *et al.,* 2007; Schulz and Miura, 2002).

1.1 Different types of Endocrine Disrupting Chemicals (EDCs)

EDCs represent a broad class of molecules such as organochlorinated pesticides, organophosphate and industrial chemicals, plastics and plasticizers, fuels, and many other chemicals that are present in the environment or are in widespread use. Now a day's scientists make a number of recommendations to increase understanding the effects of EDCs on reproductive physiology on fishes (Caserta *et al.,* 2008).

1.1.1 Bis-phenol A

Polycarbonate plastics have many applications including use in some food and drink packaging such as water and baby bottles, compact discs, and medical devices including those used in hospital settings. Epoxy resins are used to coat metal products like bottle tops, food cans and water supply pipes. Bisphenol A (BPA), a compound widely used in industry, is present in polycarbonate plastics, epoxy resins, metal cans and water pipes, beverage and baby bottles, toys, sports equipment, dental sealants, medical equipment and tubing, and consumer electronics and also in ATM receipts (Mercola, 2014;

Vandenberg *et al.,* 2007). BPA waste has been shown to entering through handling loading heating or releasing in the aquatic environment (Murmur and Srivastava, 2011). Bisphenol A mimics the activity of estrogens such as 17-β estradiol (E2) via interaction with either an estrogen receptors (ERs) or androgen receptor (ARs) (Kang *et al.,* 2002; Sohoni *et al.,* 2001; Tijani *et al.,* 2013).

1.1.2 Organochlorine Hexachlorocyclohexanes (HCH)

Hexachlorocyclohexanes (HCHs) are a group of manufactured chemicals that do not occur naturally in the environment but reach through industrial runoff technical HCH is a mixture of various HCH isomers; alpha (α), beta (β), delta (δ) and gamma (γ) (also known as lindane (FAO, 2003). Due to lipophilic properties and persistence in the environment, β –HCH and α-HCH found excessive amount and γ –HCH less extent; it is bioaccumulated and shows biomagnifications through the food chain. In air, γ-HCH is photochemically converted to α-HCH. Both γ-HCH and α-HCH can be biologically transformed to the more persistent γ-HCH (Cacar, 1997).

1.1.3 Phthalic Acid Esters (PAEs)

Phthalic acid esters (PAEs) are familiar omnipresent environmental pollutants and used as plasticizers for the manufacturing of plastic products. Di (2-ethylhexyl) phthalate (DEHP) is a high production volume chemical used in the manufacture of a wide variety of food wrapping, some children's products like toys, and some polyvinyl chloride (PVC) medical devices. Zanotelli *et al.,* 2010 found that DEHP may pose a risk to fishes reproductive development Singh and Malik, 2005 was find out the concentration and distribution of five major PAEs, *viz.* di-methyl phthalate (DMP), di-ethyl phthalate (DEP), di-butyl phthalate (DBP), di-(2-ethylhexyl) phthalate (DEHP), and di-octyl

phthalate (DOP) in the sediment samples of Gomti River collected from 30 different locations. The mean concentration values of DMP, DEP, DBP, DEHP, and DOP were found as 10.54, 4.57, 10.41, 31.61, and 5.16 microg/kg, respectively. DEHP was the most frequently detected PAE (present in 93.3% samples) these authors concluded that PAE penetration into the food chain and the environments. Jarosova (2006) was determined the DEHP and DBP contents in packaging materials ranged from 0.1 to 4259 mg DEHP, and concentration from 0.1 to 1298 mg DBP per 1 kg in packaging material respectively. In all the food samples investigated, measurable levels of DEHP was less than 0.01–0.22 mg/kg sample and DBP was less than 0.01 to 1.31 mg/kg sample.

1.1.4 Heavy Metal

Endocrine disrupters are defined as chemical substances with either agonist or antagonist endocrine effects in human and animals. These effects may be accomplished by interferences with the biosynthesis or activity of several endogenous hormones. Recently, it was confirmed that heavy metals such as), arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn) may exhibit endocrine-disrupting activity in animal experiments. Emerging evidence of the intimate mechanisms of action of these heavy metals is accumulating (Georgescu *et al.,* 2011; Tan *et al.,* 2003).

1.1.5 Personal Care Products (PPCS)

Detailed information on different categories of EDCs, their primary pathways have been compiled and presented (Table1).

Table1: Different Categories of endocrine disrupting chemicals and their primary pathway to reach aquatic environment.

Category	**Primary pathway to waterways**	**Reference**
Alkyl-phenolic compounds non-ionic surfactants such as nonylphenol and octylphenol	Run off from agriculture waste and house hold waste in River.	(Braunbeck, 2002; Grizzle, 2002; Gronen *et al.,* 1999; Harris *et al.,* 2001; Kinnberg *et al.,* 2000; Knorr and; Schwaiger *et al.,* 2002; Shioda and Wakabayashi, 2000; Tanaka *et al.,* 2002)
fungicide vinclozolin	Extensively used in agriculture insecticides, herbicides and fungicides are included in this class.	(Baatrup and Junge, 2001; Bayley *et al.,* 2002; Makynen *et al.,* 2000; Tijani *et al.,* 2013)
Pharmaceuticals ethinylestradiol (EE2) used in oral contraceptives	Synthetic steroids mainly consist of oral Contraceptives as well as steroids.	(Tijani *et al.,* 2013; Zillioux *et al.,* 2001)
Plasticizers (Phthalates, Bisphenol A)	They are found in detergents, resins, some addictives, and monomers used in the production of plastics, Excessive use of plastics and dump anywhere in open field and wastage runoff from plastic industries in the form of by products	(Bhatia *et al.,* 2014; Haubruge *et al.,* 2000; Kang *et al.,* 2002; Kinnberg and Toft, 2003; Shioda and Wakabayashi, 2000; Sohoni *et al.,* 2001; Tijani *et al.,* 2013).
Metals (mercury)	Effluent from mills and industries	Mercury (Matta *et al.,* 2001; Tijani *et al.,* 2013).
Natural Hormones and Steroids sex hormone i.e. EE2,Estrone	Effluent from sewage treatment plants and run-off from animal farms.	(Ankley *et al.,* 2003; Jobling *et al.,* 2002; Singh and Srivastava, 2013; Tijani *et al.,* 2013)
personal care products encompassing cosmetics, fragrances, preservatives, and toiletries	Use of this compound i.e. Personal care product includes shampoos, toiletries, toothpaste, perfume, deodorants, etc. Pharmaceutical and personal care products (PPCPs) are	(Houtman, 2010; Tijani *et al.,* 2013).
Pharmaceuticals include antibiotics, anti-diabetics, anti-epileptic, antimicrobials etc.		Singh *et al.,* 2011

1.2 Effect EDCs on Aquatic life

Endocrine disrupting chemicals may interact with steroid hormones and their receptors, or other hormones and transcription factors in the biochemical pathway of hormonal activity. EDCs are able to cause abnormalities in invertebrate, fish, avian, reptilian, and mammalian species (Arditsoglou and Voutsa, 2008; Ferraz *et al.,* 2007; Hjelmborg *et al.,* 2006). EDCs can draw out estrogenic responses at very low concentrations there is need for concern as many of the phthalate esters and phenolic compounds have been found at measurable concentrations in wastewater, surface waters, sediments, groundwater, and even drinking water in many countries (Cai *et al.,* 2003; Cortazar *et al.,* 2005; Huang *et al.,* 2008; Sha *et al.,* 2007). Endocrine disruption in animals have been recognized by several researchers and now fetching an emerging topic to differentiate how these chemicals disturb the endocrine regulation in aquatic animal particular in fish (Harris *et al.,* 2001; Kinnberg and Toft, 2003). Researchers alerted aquatic contamination by endocrine-disrupting compounds through observation of a variety of reproductive changes in different species of fish, mollusks and amphibians sampled from contaminated river, drainage, downstream of sewage treatment plant (Gagne *et al.,* 2002; Jobling *et al.,* 2004; Folmar *et al.,* 2001; Tanaka *et al.,* 2002).

The examination of hermaphrodite fish in sewage treatment in UK during 1970s and 1980s initiated an attention of EDCs in effluents (Matthiessen and Sumpter, 1998). Purdom *et al.* (1994) reported estrogenic effects expressed as elevated levels of vitellogenin (VTG) in fish caged. Further sewage effulent has been suggested to be the causal factor for elevated levels of VTG and inhibition of gonadal growth observed in feral male bream (*Abramis brama*) (Hecker *et al.,* 2002; Vethaak *et al.,* 2005) and

depressed T levels in male carp (*Cyprinus carpio*) (Petrovic *et al.,* 2002; Lavado *et al.,* 2004). Howell *et al.,* 1980 first time showed the clearest case of androgenic disruption in the aquatic environment as the masculinisation of fish Androgens have, also been identified in sewage effluents with no secondary treatment (Thomas *et al.,* 2004). Circulating levels of hormones are usually determined in serum sample. The effect of changes in circulating levels of the sex hormones has been a subject of research in endocrine disruption. A decrease and increase in sex steroid concentrations in response to EDC exposure is reported, even though the mechanism of action is not clearly known (Björkblom, 2009; Snyder *et al.,* 2004).

In the earliest studies demonstrated that ant estrogenic endocrine disruptor tamoxifen has been reduced VTG and estradiol (E2) level in serum (Leanos-Castaneda *et al.,* 2007) intersex the fry in tilapia (Singh *et al.,* 2012) and 4 tert pentylphenol, diethylstilbestrol have also been showed intersex (Martyniuk *et al.,* 2011; Panter *et al.,* 2002) In another study 17α Methyltestosterone (MT) resulted the development atresia and hamper oocyte development in female fatherad minnow (Pawlowskin *et al.,* 2004). Dutta *et al.* (2006) conducted a histopathology study on the effect of endocrine disruptor endosulfan on testes of bluegill fish *Lepomis macrochirus* disrupted seminiferous tubules walls, structure of the testis was severely disorganized compared to control. In another chemical atrazine showed abnormal configuration of seminiferous tubules, deformed primary and secondary spermatocytes and a significant fall in Gonado Somatic Index (GSI) (Orton *et al.,* 2006). In the exposure of methyl parathion Narayana *et al.* (2006) reported sloughing of germ cells in seminiferous tubules, necrosis formation of multinucleated giant cells and cellular degeneration in rat testes. The common carp *Ciprinus carpio* was exposed to

pesticide BHC and Sumithion for 30 and 60 days after treatment the stage II oocytes was significantly lower atretic oocytes was higher in treated groups (Kaur and Virk, 1983). Mohan (2000) observed the subleathel concentration 0.05, 0.25, 0.5 ppm of melathion caused reduction in ovarian weight and growth of oocytes and rupture of follicular epithelium in fish *Glossogobius giuris* Kaur and Sexana (2002) reported significant decline in ovarian weight and occurrence of atersia in ovaries of fishes catched from highlypolluted area Budha Nallah Brook Satlaj River Punjab state.

Adverse effect of EDCs on GSI in common carp captured from polluted river due to contamination with Bis Pheonl-A (BPA) and other estrogenic chemicals nonphenol, octylphenol was observed by Hassanin *et al.* (2002); Patino *et al.* (2003).

In another rescent study on BPA showed delay in spermiation decrease sperm density, motility (Lohnsteiner *et al.,* 2005) Zha *et al.* (2008) detected ova-testes in male rare minnow during EE2 exposure. In the early development exposure of estrogenic and androgenic compound shown change the sex ratio towards more male or more female (Andersen *et al.,* 2003; Holbech *et al.,* 2006).

Das and Mukherjee (2000) reported a decline in muscle protein content in *L. Rohita* exposed to organophosphate pesticide. Another chemical diazinon also decline serum protein fraction in fish *Micropterus salmoides* (Pan and Dutta, 2000). Common carp exposed to the pesticide diazinon significantly reduced protein in blood (Luskova *et al.,* 2002) *Cyprinus carpio* was exposed to monocrotophos for 15 days a significant reduction in concentration of DNA and RNA and have opined that this decrease in RNA suggested reduced protein synthesis.

Rahman and Siddiqui (2006) exposed subchronic doses of phosphorothionate to male and female rat depressed the protein level in blood. Due to the contamination of EDCs in aquatic environment there are several studies reported abnormal Gonadal development, delayed maturation, high level atresia, intersexuality follicular atresia increased reproductive hormone level decreased GSI (Bateman *et al.,* 2004; Jobling *et al.,* 2002ab; Jobling and Tyler, 2003; Kiparissis *et al.,* 2003) Another frequently observed effect of exposure to environmental estrogens is the induction of the yolk precursor protein vitellogenin in male and sexually undeveloped fish, alternations in steroidogenesis, sperm production and quality (Andersen *et al.,* 2003; Denslow and Sepulveda, 2008; Folmar *et al.,* 1996; Hornung *et al.,* 2004; Orn *et al.,* 2003; Zerulla *et al.,* 2002). Short-term exposure to high concentrations of MT (µg/L range) decreased aromatase activity in male Japanese medaka (Melo *et al.,* 1999) and fathead minnow (Hornung *et al.,* 2004). Changes in external sex characteristics have been reported to be the most sensitive endocrine-disruption endpoint in fathead minnows exposed for 4 months to pulp mill effluents (Parrott *et al.,* 2003). Although some of these effects in other aquatics have been partly reversed over the last few years that high levels of deformities, defects found in fish egg and larvae from the North Sea (Dethlefsen *et al.,* 1996) masculinization of female gastropods in coastal waters produced by tributyltin (Vos *et al.,* 2000). In studies with Atlantic salmon (Salmo salar), showed that 4-nonylphenol, a model xenoestrogen compound can act as an estrogen mimic (Arukwe *et al.,* 1997a), as a steroid metabolism disruptor (Arukwe *et al.,* 1997b), and modulating estrogen receptor (ER) (Arukwe *et al.,* 2001).

There are a number of evidence exposure to estrogenic compounds has been verified by the presence of elevated levels of the female yolk protein precursor vitellogenin (VTG) in male fish (Folmar *et al.,* 2001). Estrogenic compounds may reduce the reproductive potential of fish populations by causing intersex, decreased gonad size, and altered sex ratio (Vigano *et al.,* 2001; Nolan *et al.,* 2001). In a different way Common carp exposed to 4-Nonphenol (NP) ranged 0.05 to 5.4 µg for 28-30 days in this study, exposure to NP did not elicit a statistically significant increase in plasma E2, plasma T, or plasma VTG concentrations. Furthermore, no-treatment related changes in gonad histology or morphological parameters were observed (Villeneuve *et al.,* 2002). Jyothi and Narayan (1996) reported that exposure to phorate caused reduced GSI of testis and ovary, thickened basement membrane and total degeneration and necrosis of germinal epithelium in *Clarias batrachus*. *Fathead minnow* (Pimephales promelas) have shown that exogenous E2 exposure can act both stimulatory and inhibitory on the expression of brain aromatase, depending on the concentrations (Halm *et al.,* 2002). The higher concentrations reduced the expression of cyp 19b in males while the lower ones stimulated it. Interestingly, in other study Guppy brain aromatase activity following short-term steroid and 4-Nonyl phenol exposures female brain aromatase was unaffected by the exogenous E2 treatment. However both ovarian and testicular aromatase expression increased with increasing E2 concentrations. In contrast to these examples, brain aromatase activity in female *Gambusia holbrooki* from downstream of a paper mill were elevated when compared to females sampled in the reference the females also exhibited masculinised anal fin development, indicating that the fish had been exposed to androgenic contaminants from the pulp industry, which may also have caused the

alterations in brain aromatase activity (Orlando *et al.,* 2002). Several studies have demonstrated that exposure of EDCs like Bisphenol-A exhibit dysfunctions in reproduction and development (Crain *et al.,* 2007) many of them suggested that these acts as an estrogen receptor agonist (Crain *et al.,* 2007; Vandenberg *et al.,* 2009).

Elzeinova *et al.,* 2008 observed Endocrine disruption compound Vinclozolin (VZ) effects are mediated by alternations in neuroendocrine regulation of reproduction via inhibiting the binding of the androgens to the AR. In fish, studies on VZ eff ects have been focused on gonadal diff erentiation and development (Makynen *et al.,* 2000; Kiparissis *et al.,* 2003) results disruption in testicular steroidogenesis and sperm quality.

Recent studies in Endocrine disruption in India

Sharma *et al.* (2014) reviewed the environmental and human exposure to persistent organic pollutants (POPs) in India. Sanghi *et al.* (2003) measured the organochlorines pesticide level in breast milk from Bhopal India. Devanathan *et al.* (2009) reviewed concentration of organochlorines in human breast milk from major metropolitan cities in India and effect of this pesticide. Kumar *et al.* (2008) showed a significant change change in the expression patterns of the major steroidogenic enzymes in adrenal and testis: cytochrome P450SCC, cytochrome P450C17, 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase. From wastewater treatment plant (WWTP) Northern region of India. This was further supported by increased enzymatic activities measured in vitro Serum hormone profile showed a decreased level of gonadotrophic hormones and increased testosterone level. Further, increase in the serum level of alkaline phosphatase, and histopathological changes in kidney and liver of treated animals, confirmed the toxic effects of contaminating chemicals.

From the literature it is evident that there are no studies on the interference of Phthalic acid ester and HCH are reported on carp reproductive phases. Many authers such as Akingbemi *et al.* (2004); Hatef *et al.* (2012); Latini *et al.* (2004) reported that DEHP is capable of disturbing the reproductive process by mimicking or antagonizing steroid hormone action and its effects on testosterone, luteinizing hormone or estrogen-like activity. DEHP exhibited both estrogenic and anti-estrogenic activity without affi nity to androgen receptor (AR) (Takeuchi *et al.,* 2005). Recent studies showed decrease in testosterone (T) hormone (Corton and Lapinskas, 2005; Foster, 2006; Swan, 2008). This information is necessary to establish the mechanisms of toxicity of phthalates and HCH in lower vertebrates and results will provide a more comprehensive understanding of the probable hazard phthalates to the reproductive health of fish in the environment.

DEHP has been shown to reduce activity of genes involved in testicular androgenesis (Thibaut and Porte, 2004). However, none of previous studies showed inhibition of reproductive hormone level (either T or 11-ketotestosteron, Estradiol Aromatase) (Crago and Klaper, 2012; Hafet *et al.,* 2012; Uren-Webster et al., 2010). Taken together, literature on DEHP effects on reproductive health is rare and somewhat contradictory and required more examination to establish the mechanisms of its toxicity. Therefore, in this study the effects of exposure to a range of concentrations of phthalic acid ester and HCH were investigated on the reproductive physiology of *Labeo rohita*.

1.3 Reproduction and Steroid hormone biosynthesis

Steroidogenic enzymes are responsible for the biosynthesis from cholesterol of various steroid hormones including glucocorticoids, mineralocorticoids, progestins, androgens, and estrogens. They consist of several specific cytochrome P450 enzymes (CYPs),

hydroxysteroid dehydrogenases (HSDs) and steroid reductases. Numerous organs are known to have the capability to synthesize biologically active steroids, including the adrenal gland, adipose tissue, brain, placenta, ovary and testis. Three organs that specialize in de novo steroid production, the adrenal gland, testis, and ovary. These are synthesized from the same cholesterol precursor via a series of biosynthetic steps catalyzed by different steroidogenic enzymes (Figure1). The cascade of hormone product depends on their respective gene. The first step in the synthesis of steroids is the conversion of cholesterol to pregnenolone. The enzyme system that catalyzes this reaction is known as P450-linked side chain cleaving system (P450scc) and is found in the mitochondria of the steroid-producing cells. There are conversion of Pregnenolone may be have two step progesterone, or undergo hydroxylation to yield 17α-hydroxypregnenolone by P45017α. Pregnenolone converted Progesterone by 3βHSD. Progesterone may also be hydroxylated, resulting in 17α-hydroxyprogesterone. The principal and most potent mineralcorticoid. 17α-hydroxypregnenolone and 17α-hydroxyprogesterone may also be converted to the androgens dehydroepiandrosterone (DHEA) and androstenedione. The conversion of androstenedione to testosterone is mediated by 17-hydroxysteroid dehydrogenase (17βHSD). Androgens are precursors for estrogens in the females and the aromatization of estrogenic steroids from them is mediated by P450arom. T and E2 are carried in the plasma and delivered to target tissue by specific gonadal-steroid binding globulins.

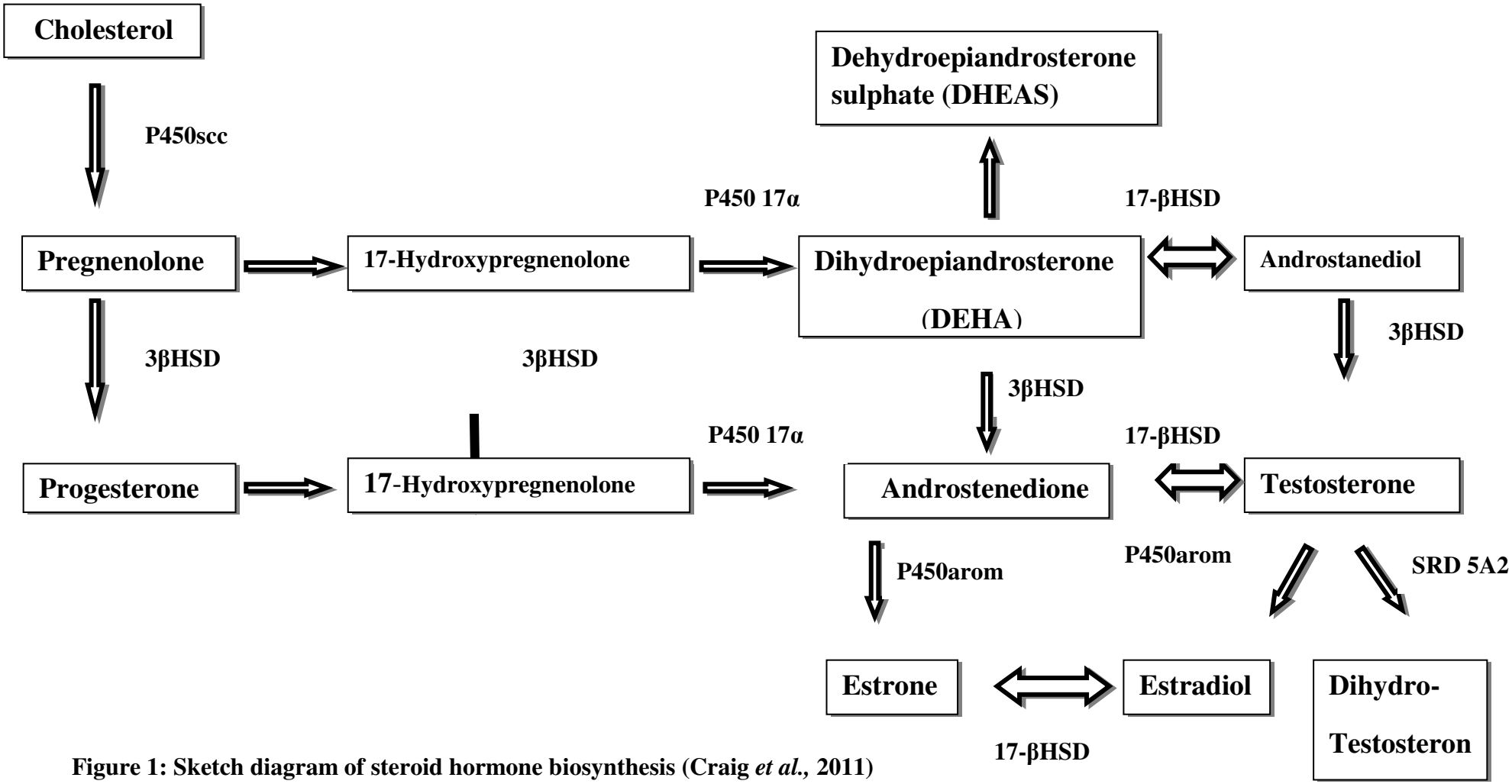


Figure 1: Sketch diagram of steroid hormone biosynthesis (Craig *et al.,* 2011)

Introduction

Rivers are vital carriers of water and nutrients to around the earth provide habitat, nourishment and means of transport to all organisms. Currently, residual pharmaceutical compounds, agricultural runoff, domestic effluents, livestock waste, personal care products, industrial waste are reported to be present in aquatic environment and are generally recognized as a source of environmental pollutants (Ankley *et al.,* 2003; Cespedes *et al.,* 2004; Gehring *et al.,* 2002; Harris *et al.,* 2005; Singh and Srivastava, 2013; Tijani *et al.,* 2013; Ying *et al.,* 2002). These natural and man-made compounds may interfere with the endocrine system and produce adverse effects in wildlife, humans and aquatic animals. Scientists frequently refer to these chemicals as "Endocrine Disruptor Chemicals (EDCs) (Candia, 2001; Lavado *et al.,* 2004; Verma and Singh, 2013). Many authors reported that Our Indian Rivers are contaminated with EDCs. Recently the Gomti River has been reported to be contaminated by many EDCs (Malik *et al.,* 2007; Nayak *et al.,* 1995; Singh *et al*., 2005; Singh and Singh, 2008; Srivastava, 2010). The muscle of the fish contaminated with photochemical ozone creation potential (POCPs (Organochlorine pesticide (OCPs) has been published in range between 2.58 and 22.56 ng g^{-1} (Malik *et al*., 2004; Singh *et al*., 2005a). Similar distribution patterns for aldrin, dieldrin and OCPs have also been reported in water and bed-sediments of the Gomti River (Malik, 2006) and in the surface water, groundwater and soil of the adjoining areas (Singh *et al*., 2005; 2007). Phthalate acid ester was found most often (94 %). The total phthalates in water samples ranged from 313 to 1,640 ng/l, whereas in sediments it was 2 to 1,438 ng/g DW (dry weight) with Di (2-ethylhexyl) phthalate (DEHP) having the highest concentration, this was the first study to describe the levels and ecotoxicological risks of phthalates in Kaveri River, India (Selvaraj *et al.,* 2015). Several other authors reported concentrations of DEHP are up to 100gL−1 in surface waters and

200mgkg−1 (wet weight) in sediments, heavily industrialized areas (Fromme *et al.,* 2002; Petrovic *et al.,* 2001). Today EDCs are ubiquitous in our environment available in air, water, soil (Jensen *et al.,* 2001; Kazeto *et al.,* 2004; Kinnberg and Toft, 2003; Schug, *et al.,* 2011; Zhang *et al.,* 2008) They are detected in food products due to packaging of plasticizers, occurring naturally in plants (phytoestrogens, EE2, Estrone), present in household products (Detergent, personal care products), pesticides (Lindane (Hexachlorocyclohexane isomers (α-HCH, β-HCH, γ-HCH))), DDT, endosulphan, atrazine), plastics (Bisphenol- A, phthalates like DMP(Di-methyl phthalate, DBP (Di-butyl phthalate) (Srivastava *et al.,* 2010; Verma and Singh, 2012), pharmaceutical wastes (birth control pills, industrial waste chemicals (PCBs, dioxin by-products of incineration, paper pulp mill and fuel combustion, and metals i.e. cadmium, lead, mercury (Propper *et al.,* 2005; Verma and Rana, 2009). Although limited scientific information is available on the permitted amount. These chemicals may turn on, shut off, or modify signals that hormones carry, which may influence the normal functions of tissues and organs. Many of these substances have been linked with developmental, reproductive, neural, immune, and other problems in wildlife and laboratory animals and the mechanism of action of these chemicals abnormalities are also reported in several aquatic animals (Bayley *et al*., 2002; Candia *et al*., 2001; Harris *et al*., 2001; Kinnberg *et al*., 2000; Kloas, 2002; Lavado *et al*., 2004; Schwaiger *et al*., 2002; Shioda and Wakabayashi, 2000). Sexual differentiation of developing gonads in fish is under the control of hormones of the hypothalamus-pituitary-gonadal axis. Sex steroids play a critical role in early differentiation of the gonads into the two sexual types and also subsequent maintenance of the differentiated types (Bjorkblom, 2009; Devlin and Nagahama, 2002). Cytochrome P450 aromatase is the enzyme complex accountable for the synthesis of estrogens by

the aromatization of androgens aromatase is the product of the Cyp19 gene, as a single copy per haploid genome. In teleosts there are two isoforms of the aromatase gene, Cyp19a and Cyp19b, which encode two structurally different proteins, P450 arom A and P450 arom B, respectively, with comparable catalytic activities. The promoter region of both genes has been characterized 20 different regulatory sites these include response elements for members of the nuclear receptor superfamily, sex steroid receptors and five transcription factors. These compound affect the regulation of CYP 19 gene family, there are number of genes that involved in the formation of steroidal hormone. These compounds indirectly affect the expression of the CYP 19 gene involving in aromatization process by up-regulate or down-regulate it. People are concerned because endocrine disrupting chemicals present in the environment at very low levels have been shown to have adverse effects in wildlife species as well as in laboratory animals. (Guilleette *et al*., 1994; Harris *et al*., 2001; Kinnberg *et al*., 2000; Kinnberg and Toft, 2003; Kloas, 2002; Lavado *et al*., 2004; Shioda and Wakabayashi 2000; Schwaiger *et al*., 2002). A decrease in reproduction success was one of the well-documented signs of endocrine disruption in fish. In India there is very less literature available the effect of EDCs on fishes, how these endocrine disruption chemicals works, what happen when it exposed to developing stage of fishes and what happen with sex steroid level and gonadal development during EDCs exposed fishes.

In view of the above facts, an experimental study was conducted to elucidate the effect of some EDCs on the gonadosomatic index, level of gonadal hormone, reproductive physiology and RNA/DNA ratio in *Labeo rohita* (Rohu) an Indian major carp commonly available in our rivers and streams all over the year. The fish *L. rohita* is very sensitive and requires healthy aquactic environment. However, presence of

EDCs in aquatic environments may result in several histopathological changes in different tissues (Das and Mukherjee, 2000) and also gonadosomatic index which is important parameter to reflect the effect of EDCs on the development of fishes (Ray, 2000). Further, balanced gonadal hormone level is the key requirement of the fish physiology so as to maintaint its population and sustain in nature. It is therefore, essentially required to assess this parameter in knowing the response of EDCs on fish reproduction physiology. Microtome application to know the histological changes is mostly used parameter in endocrine disruption studies because it analyses the development at tissue level so that we may find out clear cut idea about the impact of EDCs on specific tissue. We have also taken into account RNA/DNA ratio which shows the change in nucleic content in the EDCs exposed fishes. Gonadal hormone specially Vitellogenin level has been considered as a Biomarkers in rohu which has been used in monitoring as well as in risk assessment. Vitellogenin biomarker has been repeatedly used as early warning signals for recognition of effects of endocrine disrupting chemicals (Bjorkblom, 2009; Fossi *et al.,* 2002)

2.1 Experimental fish

Labeo rohita (Rohu) is the most important food fish in India in other major carp species, because the flesh or *L. rohita* is highly nutritive and very tasty. This graceful Indo-Gangetic riverine species is the natural inhabitant of the riverine system of northern and central India, and the rivers of Pakistan, Bangladesh and Myanmar. In India, it has been transfered into almost all riverine systems including the freshwaters of Andaman, where its population has effectively established. The traditional culture of this carp goes back hundreds of years in the small ponds of the eastern Indian states. Rohu is the principal species reared in carp polyculture systems along with the other two Indian major carps *viz., Catla catla* and mrigal, Cirrhinus *mrigala.* Due to

its wider feeding niche, which extends from column to bottom, rohu is usually stocked at relatively higher levels than the other two species. The three Indian major carps, rohu being the most important, are also the dominant species cultured in other countries such as Bangladesh, Pakistan, Myanmar, Vietnam, Nepal and Lao People's Democratic Republic. In all these countries, silver carp, grass carp and common carp are the most imperative species reared with the three Indian major carps in aquaculture. In view of the above important facts, an experimental study is proposed to elucidate the effect of some EDCs on the reproductive physiology of *L. rohita*. Because EDCs are reported in our Indian river and also several rivers all around the world. Many of these endocrine disrupting chemicals are the major cause of declination of *L. rohita* in our India Rivers.

Labeo rohita

Kingdom: ***Animalia***

Phylum: ***Chordata***

Class: ***Actinopterygii***

Order: ***Cypriniformes***

Family: ***Cyprinidae***

Genus: ***Labeo***

Species: ***rohita***

Plate 1: ***Labeo rohita***

Body Structure

Body fairly elongate, bilaterally symmetrical, its dorsal profile more arched than the ventral profile; body with cycloid scales, head without scale, snout depressed, projecting beyond mouth, without lateral lobe, eyes dorsolateral in position, not visible from outside of head, mouth small and inferior, lips thick and fringed with a

distinct inner fold to each lip, lobate or a pair of small maxillary barbels concealed in lateral groove, no teeth on jaws pharyngeal teeth in three rows, upper jaw not enlarge to front edge of eye, simple (unbranched) dorsal fin, lateral line and pelvic fin base, snout not truncate, without any lateral lobe, colour bluish on back, silvery on flanks and belly.

2.2 Objectives of The Work

There is a significant lack of research investigating the effects of phthalates on the reproductive health of aquatic animals. No information is available on sperm dysfunctions, but there are a few studies that show alternations of steroid receptors and steroidogenesis at relevant concentrations (Thibaut and Porte, 2004; Carnevali *et al.,* 2010; Uren-Webster *et al.,* 2010; Crago and Klaper, 2012). Hence an attempt has been made to fill this gap the following objectives which are outlined briefly as below:

1. To observe the effect of different doses of Phthalic acid ester and HCH on the maturation, gonado somatic index (GSI), survival and growth of *L. rohita.*
2. To observe the histopathological changes in gonad of *L. rohita* exposed to different concentration of EDCs.
3. To observe the changes in the level of reproductive hormones *viz.,* estrogen 17-β, testosterone, Aromatase and Vitellogenin in *L. rohita* exposed to different doses of EDCs.
4. To observe molecular changes (RNA/DNA ratio) in *L. rohita* exposed to different concentration of EDCs using some molecular tools.

The entire objectives aimed at finding out how the action of endocrine disrupting chemical Phthalic acid ester and Organochlorine HCH trigger the physiological and endocrine changes in fish reproduction resulting in adversities on sustainability of fish population.

Materials and Methods

3.1 Collection of Test animal:

L. rohita (rohu) was collected from private hatchery, Lucknow (Plate 2). Fish collected from the hatchery was identified using taxonomic keys and maintained and acclimatised in aquaria in laboratory conditions.

Plate 2 Test Fish Species, *Labeo rohita*

3.1.1 Feed for fish:

The experimental fishes were fed with healthy commercially available palette diet (Tyio Pvt. Ltd.) containing 32% protein daily twice a day (morning and evening) as per given details (Table 2) below:

Table 2: Total ingredients of artificial feed used.

Bio- ingredients	**Total percent**
Crude protein	32%
Crude fat minimum	4%
Crude fiber maximum	5%
Moister maximum	10%

3.2 Procurement/ chemicals/ hormones and other materials:

Phthalic acid ester Dimethyl phthalate (DMP), dibutyl phthalate (DBP) and Di (2-ethylhexyl) phthalate (DEHP) were purchased from Sigma Aldrich, USA. Testosterone and 17-β Estradiol estimation kit were purchased from Enzo-Life Sciences, India. Aromatase estimation kit was purchased from Uscn Life Science Inc., (Catalogue no E931Hu). ARO and Vitellogenin kits were procured from Blue Gene (Catalogue no E10V0059). Fish Vitellogenin (VG) other chemicals used in this study were purchased from Merck and Fisher Scientific.

3.3 Techniques Used:

3.3.1 Physiological Parameter

3.3.1.1 Length –Weight:

Length and weight of each fish was measured before and after the experiment using Vernier calliper while the weight of fish was taken by digital electronic balance (Denver Instrument Germany).

3.3.1.2 Gonado-somatic Index:

Gonado-Somatic Index (GSI) is the ratio of gonad weight to body weight used to estimate reproductive condition. Reproduction is the most critical stage in the life cycle of a species, which determines its fitness and survival. GSI is generally the indication of reproductive success. For determining gonado-somatic-index, the gonad were removed and taken on a blotting paper to remove excess moisture, then weighed. GSI was calculated according to the following equation (Singh *et al.,* 2013)

$$GSI = \frac{W1}{W2} \times 100$$

Where, W_1 = total weight of gonad

W_2 = total wet weight of fish

3.3.2 Biochemical Test:

3.3.2.1 17-β Estradiol estimation:

Estradiol-17β level in the serum was measured using kit provided by Enzo -Life Sciences, India (Catalogue No. 900-174).

Materials Needed:

1. Estradiol-17 β standard to allow extraction efficiency to be accurately determined.
2. Diethyl Ether
3. Speed vac.
4. dH2O

Procedure:

1. Added diethyl ether to serum or plasma samples at 5:1 (v/v) ether: sample ratio.
2. Mixed solutions by vortexing for 2 minutes.
3. Allowed phases to separate for 2 minutes.
4. Transferred organic phases to a glass test tube containing 1mL dH2O.
5. Vortexed the mixture for 2 minutes, and allowed phases to separate for 2 minutes.
6. Transferred organic phase to a clean glass test tube, and dry samples down using a speed vac. for 2-3 hours.

7. Rehydrated samples at room temperature in the assay buffer. A minimum of 250uL of the assay buffer is recommended for reconstitution to allow for duplicate sample measurement.

Reagent Preparation:

1. Wash Buffer

Prepared the wash buffer by diluting 5mL of the supplied washed buffer then concentrate with 95mL of deionised water. This was stored at room temperature until the kit expiration, or for 3 months, whichever is earlier.

2. Estradiol -17 β Standard

Allowed the 300,000 pg/mL 17 β- Estradiol Standard to come to room temperature and vortex prior to use. Lable seven 12 X 75m tubes 1 through 7 and one tube1. Pipet 500µL assay buffer into tubes 2 through 7. Remove 10 µL from the stock vial and add to use "initial" and vortex thoroughly. Remove 400 µL from tube "initial" and add to tube 1 and vortex thoroughly. Remove 500 µL from tube 2. Vortex thoroughly, continues this from tubes 3 through 7.

This diluted standard was used within 60 minutes of preparation. The concentrations of Estradiol-17 β in the tubes were labeled above.

3. Conjugate 1:2 Dilution for Total Activity Measurement

Prepared the Conjugate 1:2 Dilution by diluting 50 µL of the supplied conjugate with 50uL of Assay Buffer. The dilution was used within three hours of preparation. This 1:2 dilution is indented for use in the Total Activity wells only.

Assay Procedure:

As referred to the Assay layout sheet to determine the number of wells was used. Removed the wells not needed for the assay and returned them with the desiccant to the Mylar bag and seal. Stored the unused wells at 4º C.

- Pipetted 150µl of the assay buffer into the SNB non specific binding wells.
- Pipetted 100 µl of the assay buffer into the Bo (0 pg/ml standard) wells.
- Pipetted 100µl of standards 1through 7 to the bottom of the appropriate wells.
- Pipetted 100 µl of the samples to bottom of the bottom the appropriate wells.
- Pipetted 50 µl of the blue conjugate into each well except the TA and blank wells.
- Pipetted 50 µl of the yellow antibody into each well except the blank TA and NSB wells.

Note: Every well used was green in colour except the NSB wells which was blue the blank and TA wells were empty at this point and have no colour.

- Sealed the plate incubate at room temperature with shaking (- 500rpm*) for two hours.
- Empted the contents of the wells and wash by adding 400 µl of wash buffer to every well. Repeated 2 more times for a total of 3washes. After the final wash empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
- Pipetted 5 µl of the blue conjugate (diluted 1:2) to the TA well.
- Added 200 µl of the substrate solution into each well.
- Incubated for 1hour at room temperature without shaking
- Pipetted 50 µl stop solution into each well.

- After blanking the plate reader against the substrate blank .read optical density at 405nm. If plate reader is not capable of adjusting for the blank manually subtract the mean OD of the substrate blank from all readings.
- The optimal speed for each shaker varied and the range was from 120-700 rpm the speed must be set to ensure adequate mixing of the wells but not so vigorously the contents of the wells splash out and contaminate other wells.

Detailed for making standard solution and flow chart for the estradiol assay discussed in Table 3 and 4.

Table 3: Dilution table for making 17-β estradiol dilution standards 1-7

Std.	Assay Buffer Vol. (µL)	Vol. Added (µL)	17β-Estradiol Conc. (pg/mL)
Int.	990	10, Stock	3000
1	800	400, Initial.	1000
2	500	500, Std. 1	500
3	500	500, Std. 2	250
4	500	500, Std. 3	125
5	500	500, Std. 4	62.5
6	500	500, Std. 5	31.3
7	500	500, Std. 6	15.6

Table 4: 17-β estradiol assay protocol flow chart

Well I.D.:	Blank A1, B1	TA C1, D1	NSB E1, F1	Bo G1, H1	Stds. G1, H1	Samples G3-H12
Assay Buffer	-------	-------	150µL	100µL	-------	-------
Std. and/or Sample	-------	-------	-------	-------	100uL	100 µL
Conjugate	-------	-------	50µL	50µL	50 µL	50 µL
Antibody	-------	-------	-------	50µL	50 µL	50 µL
Incub.2hrs. @ RT, shaking (sealed)				--------		
Asp. & Wash 3x 400 µL						
Conjugate	-------	5µL*	-------		-------	-------
Substrate	200µL	200µL	200µL	-------	200 µL	200µL
Incub. 1 hour @ RT				200µL		
Stop Solution	50µL	50µL	50µL	50µL	50 µL	50 µL
Read @ 405 nm						

Calculation of Results:

The concentration of estradiol-17β was calculated as follow:

1. Calculated the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

   Average Net OD = Average OD – Average NSB OD

2. Calculated the binding of each pair of standard well as a percentage of the maximum binding wells (Bo), using the following formula;

   Percentage Bound = Net OD/Net Bo OD x 100

3. Using logit- log paper, plotted the percentage Bound (B/Bo) versus concentration of Estradiol-17 β for the standards. Approximate a straight line through the points. The concentration of Estardiol-17 β of the unknowns can be determined by interpolation. Samples with concentrations outside of the standard curve range will need to be re-analysed using a different dilution.

3.3.2.2 Testosterone Estimation

Serum testosterone level in the fish was estimated by Testosterone EIA kit, provided by Enzo Life Sciences, Catalog Number - ADI- 900- 065. The procedure of testosterone estimation is following:

Reagent Preparation:

1. **Testosterone Standard**

   Allowed the 50, 000 pg/mL Testosterone standard solution to warm to room temperature. Labelled five 12 x 75 mm glass tubes 1 through 5. Pipetted 1mL of standard diluents (Assay Buffer 3 or Tissue Culture Media) into tube 1. Pipetted 750µL of standard diluents into tubes 2 through 5. Removed 40µL of

the 50,000 pg/L standard to tube 1.Vortex thoroughly. Added 250 µL of tube 1 to tube 2 and vortex thoroughly. Add 250 µL of tube 2 to tube 3 and vortex. Continue this for tubes 4 and 5. The concentration of Testosterone in tubes 1 through 5 was 2,000, 500, 125, 31.25 and 7.81 pg mL respectively. Diluted standards were used within 60 minutes of preparation.

2. Wash Buffer

Prepare the wash buffer by diluting 5mL of the supplied wash buffer concentrate with 95mL of deionized water. This can be stored at room temperature until the kit expriration, or for 3 months, whichever is earlier.

3. Assay Procedure:

Brought all reagents to room temperature for at least 30 minutes prior to opening. All standards and samples should be run in duplicate.

1. Refer to the Assay layout sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the zip. Store unused wells at 4º C.
2. Pipetted 100µl of standard diluent (Assay Buffer 3 or Tissue Culture Media) into the NSB and Bo (0 pg/mL standard) wells.
3. Pipetted 100µl of standards 1through 5 to into the appropriate wells.
4. Pipetted 100 µl of the samples into the appropriate wells.
5. Pipetted 50 µl of Assay Buffer 3 into the NSB wells.
6. Pipetted 50 µl of the yellow antibody into each well except the blank TA and NSB wells.
7. Incubated the plate at room temperature on a plate shaker for 1 hour at ~ 500rpm.

8. Pipetted 50 µl of the blue conjugate into each well except the Total activity (TA) and blank wells.

   NOTE: Every well used should be Green in colour except the NSB wells which should be Blue. The blank and TA wells are empty at this point and have no color.

9. Incubated the plate at room temperature on a plate shaker for 1 hour at ~ 500rpm.
10. Emptied the contents of the wells and wash by adding 400 µl of wash buffer to every well. Repeated the wash 2 more times for a total of 3washes.
11. After the final wash emptied or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
12. Added 5 µl of the blue conjugate to the TA wells.
13. Added 200 µl of the pNpp substrate solution into each well. Incubate at 37 ºC for 1 hour without shaking. The plate was covered with the plate sealer provided.
14. Added 50 µl stop solution into each well. This stops the reaction and the plate was read immediately.
15. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the blank wells, manually subtract the mean optical density of the Blank wells from all readings

    Detailed for making standard solution and flow chart for the testosterone assay discussed in Table 5 and 6.

Table 5: Dilution table for making testosterone standards 1-5:

Std.	**Diluent Vol. (µL)**	**Vol. Added (µL)**	**Testosteron (pg/mL)**
1	960	40, Stock	2000
2	750	250, Std. 1	500
3	750	250, Std. 2	150
4	750	250, Std. 3	31.25
5	750	250, Std. 4	7.8

Table 6: Testosterone assay protocol flow chart:

Well I.D.:	**Blank A1, B1**	**TA C1, D1**	**NSB E1, F1**	**Bo G1, H1**	**Stds. A2- B3**	**Samples C3- H12**
Standard Diluents	-------	-------	100µL	100µL	-------	-------
Assay Buffer 3	-------	-------	50 µL	-------	-------	------
Std. and/or Sample	-------	-------	-------	-------	100 µL	100 µL
Antibody	-------	-------	-------	50µL	50 µL	50 µL
Incub.1hrs.@ RT shaking	--------	--------	-------	-------	--------	--------
Asp. & Wash 3 x 400 µL						
Conjugate	-------	5µL	-------		-------	-------
Substrate	200 µL	200µL	200 µL	200 µL	200 µL	200µL
Incub.1 hr @ 37ºC, sealed	-------	--------	--------	-------	--------	--------
Stop Solution	50µL	50µL	50µL	50µL	50 µL	50 µL

Calculation of Results:

Several options are available for the calculation of concentration of Testosterone in the samples. We recommend that the data be handled by an immunoassay soft-ware package utilizing a 4 parameter logistic curve fitting program. If data be handled by an immunoassay soft reduction software was not readily available, the concentration of Testosteron can be calculated as follow:

1. Calculated the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

   Average Net OD = Average OD – Average NSB OD

2. Calculated the binding of each pair of standard well as a percentage of the maximum binding wells (Bo), using the following formula;

   Percentage Bound = Net OD/Net Bo OD x 100

3. Using logit- log paper, plotted the percentage Bound (B/Bo) versus concentration of Testosteron for the standards. Approximate a straight line through the points. The concentration of Testosteron of the unknowns was determined by interpolation.

3.3.2.3 Aromatase Estimation

The Standard diluted with 1.0 mL of Standard Diluent and kept for 10 minutes at room temperature, shake gently(not to foam). The concentration of the standard in the stock solution is 20ng/mL Please firstly dilute the stock solution to 10ng/mL and the diluted standard serves as the highest standard (10ng/mL). Then prepare 7 tubes containing 0.5mL Standard Diluents and use the diluted standard to produce a double dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.625ng/mL, 0.312ng/mL, 0.156ng/mL, and the last eppendorf tubes with Standard Diluent is the blank as 0ng/mL.

1. **Assay Diluent A and Assay Diluent B -** Diluted 6mL of Assay Diluent A or B Concentrate(2×) with 6mL of deionized or distilled water to prepare 12 mL of Assay Diluent A or B. The prepared working dilution was not frozen**.**
2. **Detection Reagent A and Detection Reagent B -** Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute to the working concentration with working Assay Diluent A or B, respectively (1:100).

3. **Wash Solution** - Dilute 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600 mL of Wash Solution (1×).
4. **TMB substrate** - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Assay procedure

1. Determined wells for diluted standard blank and sample. Prepare 7 wells for standard, 1 well for blank. Add 100µL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at 37oC.
2. Removed the liquid of each well, don't wash.
3. Added 100µL of Detection Reagent A working solution to each well. Incubate for 1 hour at 37oC after covering it with the Plate sealer.
4. Aspiradte the solution and wash with 350µL of 1× Wash Solution to each well using a squirt bottle, Multi-channel pipette, manifold dispenser or auto washer, and let it sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Repeat 3 times. After the last wash; remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
5. Added 100µL of **Detection Reagent B** working solution to each well. Incubate for 30 minutes at 37oC after covering it with the Plate sealer.
6. Repeated the aspiration/wash process for five times as conducted in step 4.
7. Added 90µL of **Substrate Solution** to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37oC (Don't exceed 30 minutes).

Protect from light. The liquid will turn blue by the addition of Substrate Solution.

8. Added 50µL of **Stop Solution** to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Removed any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the micro plate reader and conduct measurement at 450nm immediately

Calculation of results

Averaged the duplicate readings for each standard, control, and samples and subtractd the average zero standard optical density. Created a standard curve on log-log graph paper, with ARO concentration on the y-axis and absorbance on the x-axis. Drew the best fit straight line through the standard points and determined it by regression analysis. Using some plot software, such as curve expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

3.3.2.4 Vitellogenin Procedure:-

7 standered of Vitellogenin from 0 ng/ml to 10 ng/ml

1. Determined wells for diluted standard blank and sample. Prepare 7 wells for standard, 1 well for blank. Add 100µL blank each of dilutions of standard and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at 37oC.
2. Removed the liquid of each well, don't wash.

3. Added 100µL of Detection Reagent A working solution to each well. Incubate for 1 hour at 37oC after covering it with the Plate sealer.
4. Aspirated the solution and wash with 350µL of 1× Wash Solution to each well using a squirt bottle, Multi-channel pipette, manifold dispenser or auto washer, and let it sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Repeat 3 times. After the last wash; remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
5. Added 100µL of Detection Reagent B working solution to each well. Incubate for 30 minutes at 37oC after covering it with the Plate sealer.
6. Repeated the aspiration/wash process for five times as conducted in step 4.
7. Added 90µL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37oC (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.
8. Added 50µL of Stop Solution to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Removed any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the micro plate reader and conduct measurement at 450nm immediately

Calculation of results

Average the duplicate readings for each standard, control, and samples and subtract the average zero standard optical density. Create a standard curve on log-log graph paper, with VTG concentration on the y-axis and absorbance on the x-axis. Draw the

best fit straight line through the standard points and it can be determined by regression analysis. Using some plot software, such as curve expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

3.3.3 Histological Studies:

The microscopic anatomy of the cellular changes in gonadal tissues was done using histological techniques. The morphological and cellular changes was studied with the help of a microscope as per methodology given by Coolidge & Howard (1979) and Humason (1979) and described as below:

Tissue Fixation:

- The desired tissue of fish was cut into small pieces preferably 4 to 5 mm square size.
- These pieces were put in vials and fixed in 10% formalin. The volume of the fixative was at least 20 times the sample size.
- The tissue was fixed for more than 24 hours.

Embedding:

- The samples were washed thoroughly in tap water.
- The tissue was trimmed into smaller size of 0.5x 0.4x 0.4 cm3 size.
- The hard tissue (gill and muscle) were decalcified in decalcifying solution and soaked them in 5% sodium sulphate (Na2So4) for 1-5 hours and rinsed in tap water for 10-30 minutes.
- The tissue was dehydrated through ascending alcohol grades 30, 50, 70, 90 and 100%.
- Put the tissue in xylene to clear opacity from the dehydrated tissue making them transparent.

- Put the tissue in half xylene half paraffin for half an hour.
- Put the tissue in full paraffin for one hour for proper infiltration.
- The tissue was then embedded in paraffin which was allowed to solidify round the tissue.

Preparation of tissue blocks for sectioning:

- Tissues were embedded in paraffin and were trimmed into square blocks for sectioning.
- The blocks were fixed in the block holder of the microtome (P1 XXa).

Sectioning:

- The prepared paraffin blocks containing sample tissue were cut at 4-5 mm thickness in the microtome (P1. XX b).
- The ribbon containing tissue was put into a box.
- The required size of ribbon was carefully transferred.

Stretching of tissue:

The ribbon was put in warm water (45- 50ºC) placed in a big Petridish or water bath to stretch the tissue of wrinkles and make it flat.

Preparation of slide:

- The required size of ribbon I cut and transferred to a microscope slide covered with Mayer's albumen and containing a little water. The ribbon strip is then oriented on the slide as desired.
- The water is then drained out and the ribbon on the slide is then air dried overnight.
- The slide is now ready for staining.

Staining Hematoxylin and Eosin:

- Put the slide in xylene to dissolve the paraffin.
- Then hydrate the tissue in descending grades of alcohol starting from 100, 90, 70, 50, 30% and water with each step of 5 minutes duration.
- Rinsed in water for 5 minutes.
- Stained in Hematoxylin for 5-10 minutes.
- Rinsed in water.
- Dehydrated through 50%, 70%, 90% alcohol.
- Counter stained in Eosin.
- Washed in 90% alcohol.
- Dipped in Absolute alcohol.
- Cleared in xylene.
- Mounted in Canada balsam or DPX.
- Observed the section under the microscope.

3.3.4 RNA/DNA Ratio:-

First, the muscle samples (100 mg ml^{-1}, w/v) were homogenized for 5 min in 5% trichloroacetic acid (TCA) at 90°C and then centrifuged at 5000 rpm for 20 min. For the determination of RNA, 2.0 ml of distilled water and 3.0 ml of orcinol reagent (1gm orcinol +100 ml HCL+ 0.5gm Ferric chloride) was added in 1.0 ml of supernatant. The reaction mixture was kept in boiling water bath for 20 min. The greenish-blue colour thus developed was read at 660 nm in a spectrophotometer. For DNA determination, 1.0 ml of distilled water and 4.0 ml of freshly prepared diphenylamine reagent (1 gm Diphenylamine reagent + 100 ml glacial acetic acid + 2.75 ml con H_2 SO_4) were added to 1.0 ml of the supernatant. The reaction mixture was kept on a boiling water bath for 10 min. The blue colour developed was measured

at 600 nm. Standard curves for RNA and DNA were drawn using different concentrations of yeast RNA and calf thymus DNA, respectively. The values were expressed as µg/100 mg fish muscle tissue on dry basis.

1. DNA

Table: 7: DNA Dilution for making standard Stock- 2.5mg/ml (if more stocks then take 10mg add dissolve in 4ml)

S.No	Standard	Concentration	Reagent	H2O	Total
1	Blank -----	---	4ml	2.0ml	6 ml
2	**0.1**	**0.25mg**	**4ml**	**1.9 ml**	**6 ml**
3	0.2	0.50mg	4ml	1.8 ml	6 ml
4	**0.3**	**0.75 mg**	**4ml**	**1.7 ml**	**6 ml**
5	0.4	1.0 mg	4ml	1.6 ml	6 ml
6	**0.5**	**1.25 mg**	**4ml**	**1.5 ml**	**6 ml**
7	0.6	1.50 mg	4ml	1.4 ml	6 ml
8	0.8	2.00 mg	4ml	1.2 ml	6 ml
9	1.0	2.50 mg	4ml	1.0 ml	6 ml

Red one are optional (incase if chemical is less)

2. RNA

Table 8: RNA Dilution table for making standard, Stock – 5 mg/ml Take 25mg or 0.025g of RNA to be dissolved in 5ml

S.No	Standard	Concentration	Reagent	H2O	Total
1	Blank -----	---	3ml	3.0 ml	6 ml
2	0.1	0.5mg	3ml	2.9 ml	6 ml
3	0.2	1.0mg	3ml	2.8 ml	6 ml
4	0.3	1.5 mg	3ml	2.7 ml	6 ml
5	0.4	2.0 mg	3ml	2.6 ml	6 ml
6	0.5	2.5 mg	3ml	2.5 ml	6 ml
7	0.6	3.0 mg	3ml	2.4 ml	6 ml
8	0.7	3.5 mg	3ml	2.3 ml	6 ml
9	0.8	4.0 mg	3ml	2.2 ml	6 ml
10	0.9	4.5 mg	3ml	2.1 ml	6 ml
11	1.0	5.0 mg	3ml	2.0 ml	6 ml

Unknown

Three replications (optional)

S.No	NA extract/ Homogenate	Concentration	Reagent	H2O	Total
1	1.0 ml	Unknown	3 (RNA) /4 (DNA) ml	2 (RNA) /1 (DNA) ml	6 ml
2	1.0 ml		3/4ml	2/1.0 ml	6 ml
3	1.0 ml		3/4ml	2/1.0 ml	6 ml

Calculation of Results:-

Average the duplicate readings for each standard, control, and samples and subtract the average zero standard optical density. Create a standard curve on log-log graph paper, with concentration on the y-axis and absorbance on the x-axis. Draw the best fit straight line through the standard points and it can be determined by regression analysis.

3.3.5 Water Analysais:

Water analysis (temperature, pH, hardness, conductivity and dissolved oxygen) was carried out by using a water analysis kit provided by Multi 340i, Weiheim, 2004, Germany. Temperature of experimental water tested with the help of digital thermometer. All these water analysis parameters were tested after a week of experiment.

3.3.6 Statistical analysis:

All data of this study were calculated and expressed as mean and standard error means (SD±SEM). One way analysis of variance (ANOVA) is assessed by using a computer programmed software package, SPSS (Statistical Package for Social Science) version-

16.0. For all statistical tests, values were considered significantly different with *P* values of less than 0.05 ($P<0.05$).

3.3.7 Experimental Design:

3.3.7.1 Effect of Phthalic acid ester (DMP, DBP and DEHP) on *L. rohita*

Test Species:

To study the effect of phthalic acid ester on the gonad development of *L. rohita*, we conducted an experiment in glass aquaria in triplicates. About 100 fishes were measuring 134±4.2-141±2.2gm weight and 20.4±1.5cm length equally distributed and kept into four glass aquaria. Water in the aquaria was continuously filtered (thermo-controlled filters), aerated and the temperature in the aquaria was maintained between 25 and 27^0C by using good quality thermostat heaters.

Test Chemical:

Three concentration of phthalic acid ester {0.2mg/L, 0.3mg/L, 0.5mg/L} was dissolved into 1% of DMSO and then diluted with distilled water added into rearing waters so as study the effect of phthalic acid ester on gonad development for control only same concentration of DMSO diluted in to water.

Test Procedure:

Fishes were fed two times a day with commercial flaked food Taiyo grow (Taiyo Pet Productions PVT.LTD). Subsequently, fishes were fed plankton collected from the local ponds and fish farms, once daily at noon. In addition Soya powder and corn powder were also given to the maturing fishes. This experiment continued for 90 days.

Water analysis test was done after every one week of experiment. After 30 days of experiment both control as well as phthalic acid ester treated fishes (one third of sum) were gently netted out from aquarium and measured length and weight. Fishes from each group was anesthetized by putting then in water containing 100 mg tricaine methanesulfonate (MS-222, Sigma chem. Ltd. USA) per liter buffered 1:1 with sodium bicarbonate. Then blood was drawn from the caudal fin of fish and collected in efendoef tubes. About 2 ml of the blood was centrifuged at 5000 rpm for 5 minute and the serum so obtained was used for analysis of reproductive hormone. Near about 1ml serum also collected for each 17-β estradiol, testosterone vitellogenin and aromatase estimation. At the same time, gonads collected from the control as well as experimental fishes were also subjected for RNA/DNA estimation of gonad.

Anesthetized fishes were dissected after collecting blood and gonads were removed immediately afterwards and fixed in 10% natural buffer formalin solution, dehydrated in different grades (30%, 70%, 90% and absolute) ethanol and embedded in paraffin wax. Sections (5-7 µm thick) were cut, dewaxed dehydrated with different grades of ethanol, stained with Haematoxylin and Eosin, and dehydrated and mounted in Canada balsam (Merk, Darmstadt, Germany). Sections were examined under a compound microscope (Olympus co., Japan) and phase contrast microscope (Nikon co., Japan).

3.3.7.2 Effect of γ- HCH (Hexachlorocyclohexanes) on *L. rohita*

Test Species

For study of effect of HCH on *L. rohita*, 100 numbers of maturing fish (weight 140 ± 5.5gm and length 22.5 ± 0.22cm) were collected and kept in four different glass aquaria. Fishes were equally distributed in to aquaria and acclimatized into laboratory

condition for one week, then experiment conducted. Water in the aquaria was recirculated and continuously filtered (thermo-controlled filters), aerated and the temperature in the aquaria was maintained between 25 and 27^0C by heating elements of the pumps.

Fish food and Test Chemicals:

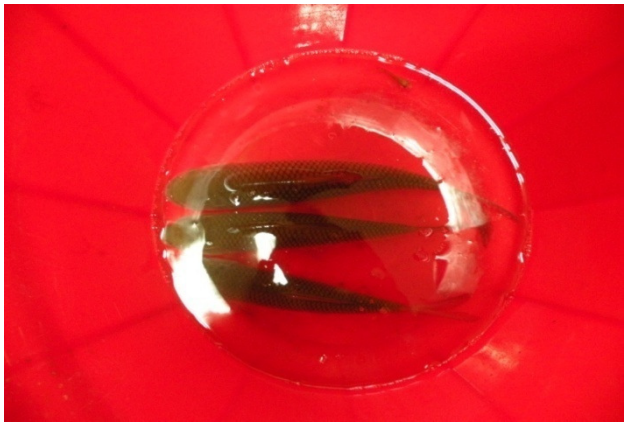
Fishes were fed two times a day with commercial flaked food Taiyo grow. Subsequently, fishes were fed plankton collected from the local ponds and fish farms, once daily at noon. This experiment continued for 90 days.

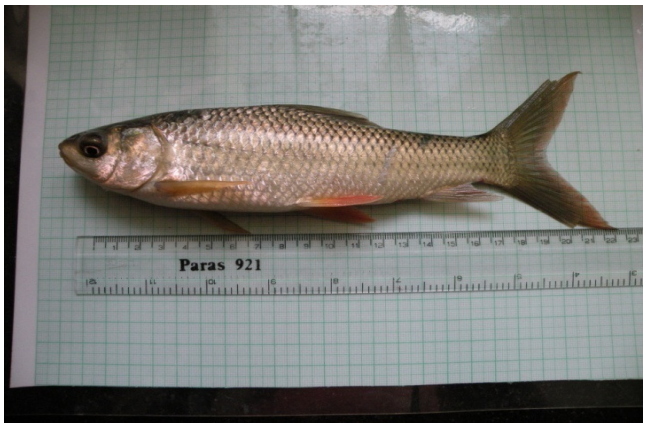
Different concentration of HCH (0.2mg/L, 0.3mg/L, and 0.5mg/L) dissolved in to 95% acetone firstly dissolved into ethanol to prepared stock solution. By diluting this solution with distilled water, the required dose was added in the aquarium water in different treatment groups. The water was changed reguraly at the interval of 15 days and again test chemical mixed in water from prepared test chemical stock solution.

Test Procedure:

The effect of HCH on gonadal development and sex differentiation was studied. In this study after each 30 days of experiment, each fish was measured for length and weight. The all physiological, biochemical and histological parameter of the experimental as well as control fish was determined by method described earlier. In this experiment HCH treatment was given to the *L. rohita* by immersion method. For this study stock solution of different dose of HCH (0.2mg/L , 0.3mg/L and 0.5mg/L) was prepared and added into aquaria water, while in the control group, acetone added into the aquaria water. This experiment was also run for three months, after one month one third was of each group of fish netted out from aquarium and all physiological, biochemical and histological parameters were determined. Fish from

each group was anesthetized by putting then in water containing 100 mg tricaine methanesulfonate (MS-222, Sigma chem. Ltd. USA) per liter buffered 1:1 with sodium bicarbonate. Fish were cut ventrally, using a scalpel, from the genital papilla to the base of the pectoral fin. A window on the lateral side was opened and the viscera were removed, leaving gonads, swim bladder and kidneys in place. Once the pair of gonads was identified, a macroscopic observation based on the morphology, location, and insertion of the gonadal tissue was made. Then, a few drops of Bouin's solution were applied topically to the gonads. This procedure hardened the gonadal tissue facilitating its removal both gonads were removed using a forceps and placed on a glass slide and weight it regular after 30 days interval due to find out GSI of control and EDCs exposed fish. The tissue was fixed in Bouin's solution for 6 hours, dehydrated, embedded in paraffin, stained with haematoxylin and eosin and sectioned (5μm) transversely.





Some Photograph during Experimental setup

Results

4.1 Effect of Phthalic Acid ester DMP on *L. rohita*

Effect of DMP on Gonado somatic index (GSI) of *L. rohita*:

The experimental fishes were found healthy throughout the experimental period. DMP exposed fishes did not show any changes on GSI compare to control group. The observed GSI value in control group ranged from 0.18 ± 0.03 - 0.32±0.01 and in DMP treated group ranged from 0.19±0.01-0.316 ±0.015 in the dose of 0.2mg/L, 0.19±0.01-0.3±0.016 in dose of 0.3mg/L and 0.17±0.01-0.33±0.05 in dose of 0.5mg/L after 30, 60 and 90 days (Figure 2). The DMP treated group of fish did not show any significant (P >0.5) variation (Table no.11) and there was a slight reduction in the weight (150±2.6g) after 90 days of DMP treatment 0.5mg/L but it was found 154 ± 4.4g in control group after 90 days.

Effect of DMP on biochemical Parameters of *L. rohita*:

Testosterone level in serum of DMP treated fish was analyzed and found non-significant (P>0.5) compared with control group (Table 11.1). In high dose of DMP 0.5mg/L the testosterone value was 1493.3±16.6pg/ml while in control group of fish it was 1497.7±14.pg/ml; in dose of 0.3mg/L it was 1523.7±5.35pg/ml and in 0.2mg/L DMP the testosterone value was 1523±5.3pg/ml after 30 days of experiment. Again after 60 days of experiment in high dose of DMP 0.5mg/L the testosterone value was 1472.6±13pg/ml while in control group of fish it was 1482±14pg/ml, in the treatment group of 0.3mg/L it was 1523±13pg/ml and in 0.2mg/L the testosterone value was 1510±12.6pg/ml. After 90 days of experiment in high dose of DMP 0.5mg/L the testosterone value was 1470.1± 16.4pg/ml while in control group of fish it was 1523.7±5.3pg/ml, in dose of 0.3mg/L it was 1530.1±12pg/ml and in 0.2mg/L the testosterone value was 1535± 14.6pg/ml shown in Figure 3.

Serum 17-β estradiol was measured there was no significant (P>0.5) changes in Di-methyl phthalate (DMP) treated fish groups as compared to the control group shown in Table 11.1. Control group estradiol level was ranged from 227.3±8.3 to 251.3±13.12pg/ml after 30, 60 and 90 days of experiment respectively. In 0.2mg/L dose of DMP it was 218.9±5.8pg/ml; in the treatment group of 0.3mg/L it was measured 253.5±15.3pg/ml and in 0.5mg/L it was found 237.5± 11.4pg/ml at the end of 30 days. After 60 days in dose of 0.2mg/L it was 223.9± 7.1pg/ml, in 0.3mg/L dose of DMP it was found 261.6±14.pg/ml and in 0.5mg/L dose of DMP it was 231.5±10.1pg/ml. At the end of experiment after 90 days in 0.2mg/L dose of DMP it was 233±12.6pg/ml; in dose of 0.3mg/L it was 262.1±13pg/ml and in 0.5mg/L dose of DMP it was found 262±13.9pg/ml shown in Figure 4. Serum Vitellogenin had also no chang compare to control group, the data was insignificant (P>0.5) in any group of treatment. In control group it was recorded 1083.9±17.1, 1017±16.6 and 1113±12.3 after 30, 60 and 90 respectively shown in Table no 11.1. In 0.2mg/L dose of DMP it was 1060.8±13pg/ml, 1189.7± 16.2pg/ml, 1157.6± 16.2pg/ml after 30, 60, 90 days respectively, in 0.3mg/L dose of DMP it was found 1078.4±18pg/ml, 1190.9±15.2pg/ml, 1104±17.5pg/ml after 30, 60, 90 days respectively. In high dose of DMP treatment it was found 1103.8± 14.5pg/ml, 1133.8± 11.5pg/ml and 1070± 14.5pg/ml 11.5pg/ml after 30, 60 and 90 days of treatment shown in Figure 5.

Serum aromatase level was insignificant compare to control thought the experiment period shown in Table no 11.1. In control group it was recorded 75.74±3.5, 77.3±2.3 and 79±3 after 30, 60, 90 days. Figure 6 shown that in experimental exposure group it was recorded 79.6±2.12, 83.45±2.5, 86.8±2.1 in 0.2mg/L DMP after 30, 60 and 90 days and in 0.3mg/L concentration it was measured 89.8±2.4, 84.6±2.1, 89.3±3.2, and in high concentration 0.5mg/L it was recorded 84.9±2.5, 81.7±2.2, 85.5±2.6 after 30, 60 and 90 days respectively.

Effect of DMP on RNA/DNA Ratio of *L. rohita*

The group of fish treated with DMP was shows insignificant difference between treatment and control group. Table 11.2 shows RNA/DNA ratio value in control group ranged from 2.2±0.06-2.12±0.06 and in DMP treated group ranged from 2.1±0.06-2.09±0.05 in dose of 0.2mg/L, 2.15±0.01-2.04±0.01 in dose of 0.3mg/L and 1.93±0.04-1.94±0.04 in dose of 0.5mg/L (Figure 7).

Table no 11.3 described Physio-chemical analysis of water during DMP experiment on the *L. rohita*. The gonad developmental stages are described below.

Different Stage of Maturation of gonads in *L. rohita* control condition up to one year from December 2014-Deceber 2015.

Ovarian Development:

Histological characteristics of ovarian tissues of Rohu (*L. rohita*) at the age of 1 year were investigated. Developmental stages of oocytes were determined by the histological technique and stages characteristics classified by Gadekar, 2014.

1. Resting phase (November to January)
2. Preparatory phase: (February to March)
3. Prespawning phase (April to June)
4. Spawning phase (July to August)
5. Postspawning phase: (September to October)

The observation was done by phase contrast microscope the microphotograph shows different structure of gonadal developmental stages of *L. rohita*. In ovary, each ovarian follicle consisted of the developing oocyte surrounded by two layers of somatic cells, the inner granulose cells and the outer thecal cells. Table 9 explain all these stages were found in this study differentiated by the following characteristics.

Table: 9: Different stages of ovarian development of *L. rohita*.

Characteristic Structure of Ovary	**Maturation Stage**	**Phase**
Early stage of oocyte maturation the ovary is mainly composed of small oogonia Figure 26.	Chromatin nucleoli stage	I
Oocytes protoplasm side is growing, chromatin material is visible inside the oocytes, nucleolus to many small size and proximity internal layer nuclear membrane are put and vacuoles thin layer around the core made up of follicular intensity has decreased basophile oocytes Figure 27.	Perinucleolar stage	II
Vesicles find yolk oocytes increased in size, around a few core vesicles row is visible, follicular cells and increased thickness of layers formed radius, amount acidosis ovuplasm increased Figure 28.	Cortical alveoli formation stage	III
Nucleolus scattered in various parts of the core and the number of decreases, making the last stage of vacuoles reached, increased follicular layer and two layers of granulosa cell layer Teca radius is more specific. Acidophilic oocytes are completely Figure 29.	Vitellogenic (yolk) stage	IV
Gonad, eggs and mature oocyte diameter is increased; Yolk accumulation, with vacuoles also merged and formed large vacuoles gives Intake oocytes and nuclear migration toward the animal pole. Layer around the ovarian follicular developed Figure 30.	Ripe (mature) stage	V
Spontaneous spawning, the spent ovary showed many newly recruited oogonia, some developing oocytes and few atretic follicles.	Spawned or spent stage	VI

Testicular Development:

The testes of the control fish were flat and elongated, while the testes of groups were bean-shaped and rounded. Previtellogenic cells and somatic cells in the testis were still few in number and Previtellogenic cells were distinguishable from somatic cells by their bigger size, larger nuclei round to oval contour, and light cytoplasm.

Spermatogenesis:

These processes followed formation of haploid spermatids, and were prominent in the dramatic increase in dimension of the spermatocytes. Concomitantly, the enclosed sex cells decrease in dimensions and increased in numbers with final maturation of

spermatozoa within the cysts. The maturation stages were divided into stages characterized by the condensation of chromatin in the nuclei that formed the sperm head, a decrease in dimensions, discarding of the residual cytoplasm (residual bodies), and development of the middle piece and flagella complex of the mature sperm. Table 10 explain all these stages were found in this study differentiated by the following characteristics.

Table: 10: Different stages of testes development of *L. rohita***.**

S.No.	Sperm cell types	Histological Characteristics
1	Primary spermatogonium (sperm mother cell)	Largest among spermatogenic cell types, spherical in shape, present in cystic form, large nucleus with prominent eccentric nucleolus, less cytoplasmic affinity with (i.e. Hematoxylin - Eosin) dyes Figure 31.
2	Secondary spermatogonium	Produced as a result of mitotic division of spermatogonium primary spermatogonia, smaller, rounded in shape, present in groups, centrally placed nucleus with visible chromatin threads and nucleolus, less cytoplasmic content Figure 32.
3	Primary spermatocytes	Produced by multiplication of secondary spermatogonia, saller in size, eccentrically placed nucleus with chromatin threads gathered on one side, forming nucleolus in invisible condition Figure 33.
4	Secondary spermatocytes	Produced as a result of reduction division of primary spermatocytes nucleus with dark clumps of chromatin nucleus not visible less cytoplasmic content Figure 34
5	Spermatid	Produced as a result of second meiotic division of the secondary spermatocytes structure deeply stained with haematoxylin, rounded nucleus cytoplas scanty Figure 35
6	Spermatozoa	Produced in the metamorphic changes in spermatids by orientation and reorganization of nucleoplasm and cytoplasm together with the development of flagellum small rounded deeply stained structure present in clusters Figure 35.

Effect of DMP on gonadal development and structure of *L. rohita*:

During experimental period it was found that ovary of control *L. rohita* was I and II maturity stages. After the first 30 days of experiment in 0.2mg/L, 0.3mgL, 0.5mg/L dose of DMP the ovary development at resting phase they were small in size with large nuclei. They were separated from the germinal epithelium and are known as oogonia. The immature oocytes remain unstained, but later they are stained deeply with hematoxylin. The nucleus was large with many prominent nucleoli. Oocytes protoplasm side was growing, Chromatin material was visible inside the oocytes, Nucleolus to many small size and proximity internal layer nuclear membrane are put and vacuoles thin layer around the core made up of follicular intensity has decreased basophile oocytes Figure 36.

After 90 Days of experiment the ovarian development shifted resting stage to preparatory phase. The oocytes were larger in size with prominent nuclei. During maturation, yolk deposition took place. At a perinucleolar stage, the yolk nucleus was located close to the nuclear membrane; however, yolk nucleus migrates to the periphery of the ooplasm during further maturation.

The development of ovary same as in control condition. There is no lesion, or altered structure seen in histological observation compare to control Figure 37.

4.2 Effect of Phthalic acid ester (DBP) on *L. rohita*

Effect of DBP on Gonado somatic index (GSI) of *L. rohita*:

DBP exposed fish has no effect on GSI. Figure 8 shows that GSI value in control group ranged from 0.19±0.01-0.34±0.02 and in DBP treated group had no effect 0.16±0.01-0.32 ±0.02 in 0.2mg/L, 0.16±0.02-0.322±0.02 in 0.3mg/L and 0.17±0.01-

0.34±0.02 in 0.5mg/L after 90 days. The group of fish which treated with DBP did not show significant (P >0.5) variation Table 12.

Effect of DBP on biochemical Parameters of *L. rohita*:

DBP treated fish was analyzed and found significant (P<0.5) reduction in testosterone level in serum when data was compared with the control fish (Table 12.1). In high dose of DBP the testosterone serum level was significantly (p<0.5) the testosterone value was 1174.3±13.3pg/ml after 30 day, while in control group of fish it was 1523.7±5.3pg/ml while in 0.3mg/L the testosterone value was 1428.7±9.2pg/ml and in 0.2mg/L the testosterone value was 1523±12.8pg/ml after 30 days of experiment. After 60 days in high dose of DBP 0.5mg/L the testosterone value was 988.7±13.7pg/ml (p<0.05), in 0.3mg/L it was 1082±12.5pg/ml and 0.2mg/L the testosterone value was 1513±5.35pg/ml, while in control group of fish it was 1513±8.5pg/ml. After 90 days of experiment in high dose of DBP 0.5mg/L the testosterone value was 961± 10.4pg/ml it was highly significant at (P<0.001) while in control group of fish it was 1533.7±8.5pg/ml, in 0.3mg/L the testosterone value was 1093.1±19.3pg/ml and in 0.2mg/L it was 1258± 13.6pg/ml shown in Figure 9.

Serum 17-β estradiol found significantly (P<0.05) increase in DBP treated fish group as compared to the control fish group it was 227.3±8.3-251.3±13.12pg/ml (Table 12.1). In 0.2mg/L dose of DBP it was 359.9±14.6pg/ml; in 0.3mg/L dose of DBP it was found 394.5±16.6pg/ml and in 0.5mg/L dose of DBP it was found 394± 16.1pg/ml at the end of 30 days all data was significant at P<0.05. After 60 days of experiment in 0.2mg/L dose of DBP estradiol value was 340±13pg/ml; in 0.3mg/L dose of DBP it was found 438±21.6pg/ml and in 0.5mg/L dose of DBP it was found 438±13pg/ml and end of experiment after 90 days in 0.2mg/L dose of DBP estradiol value was 360.8±14pg/ml; in 0.3mg/L dose of DBP it was found 524±7pg/ml and in

0.5mg/L dose of DBP it was found 524±9.7pg/ml. data was significant at P<0.01 figure 10. Serum Vitellogenin has slightly increased compare to control group, the data was non-significant (P>0.5) In 0.2mg/L dose of DBP it was 1038.8±11pg/ml; in 0.3mg/L dose of DBP it was also found non-significant (P>0.05) 1128.4±14p.3g/ml and in 0.5mg/L dose of DBP it was found (P<0.05) 1383.8± 15.3pg/ml at the end of 30 days. After 60 days in 0.2mg/L dose of DBP vitellogenin was 1155± 12.6pg/ml (P>0.05); in 0.3mg/L it was found 1445.9±15.4pg/ml (P<0.05) and in 0.5mg/L dose of DBP it was found 1383±17.8pg/ml. and after 90 days of experiment in 0.2mg/L dose of DBP vitellogenin level was 1258.6±12.2pg/ml; in 0.3mg/L dose of DBP it was found 1450±12.8pg/ml and in 0.5mg/L dose of DMP it was found 1404±18.9pg/ml. all data are significant (P<0.05) compare to control group. Control fish serum vitellogenin ranges, 1018±16.1; 959±14.5; 999±19.6 30, 60, 90 respectively Figure 11.

Serum aromatase level in control was recorded 77.8±2.5, 79.5±3.5 and 84.3±2.5 pg/ml after 30, 60, 90 days respectively (Table 12.1) In experimental DBP exposure in the dose of 0.2mg/L it was slightly increase in the aromatase level which were 81.6±2.1, 105.3±4.5, 108.5±4.5 in 0.2mg/L after 30, 60 and 90 days respectively and was insignificant (P>0.05). Treatment in the dose of 0.3mg/L DBP concentration it was measured 94.4±5.6, 105.5±5.4, 116.4±2.5 after 30, 60 and 90 days respectively, and in higher concentration 0.5mg/L there was a further increase in aromatase level 105.5±5.2, 125.9±6.3, 136.2±4.2 after 30, 60 and 90 days respectively (Figure 12) The aromatase level was significantly increase (P<0.05) after 90 days of treatment.

Effect of DBP on RNA/DNA Ratio of *L. rohita:*

The group of fish treated with DBP showed significant decrease of RNA/DNA ratio in high dose of the experimental period. Table 12.2 shows RNA/DNA ratio value in

control group which ranged from 2.2±0.01-2.12±0.09 and in DBP treated group it did not significantly (P>0.05) affect any changes in 0.2mg/L, 0.3mg/L and 0.5mg/L where it was 2.1±0.09, 2.15±0.01 and 2.1±0.06 after 30 days respectively. After 60 days it was also insignificant (P>0.05) where in the dose of 0.2mg/L where the value was 2.09±0.4, but it showed significantly declined (P<0.05) in 0.3mg/L and 0.5mg/L it was significantly (P>0.05) reduced 1.5±0.04 and 1.52±0.05. After 90 days of experiment it was also not significant (P>0.05) in 0.02mg/L 2.09±0.04 but it show significant declined (P<0.5) value in treatment groups of 0.3mg/L and 0.5mg/L where it was 1.7±0.08 and 1.4±0.02 (Figure 13)

Table no 12.3 Described Physio-chemical analysis of water during DBP experiment exposed *L. rohita* in aquarium.

Effect of DBP on gonadal development and structure of *L. rohita*:

During experimental period it was found that maturity stages of ovary of *L. rohita* were I and II in control condition. However, after the first 30 days of experiment in 0.2mg/L, 0.3mgL, 0.5mg/L dose of DBP the ovary development was in resting phase and they were small in size with large nuclei. They were as similar as in control group known as oogonia. The immature oocytes remained unstained, but later they are stained deeply with hematoxylin. The nucleus was large with many prominent nucleoli. Oocytes Protoplasm side was found growing, chromatin material was visible inside the oocytes, nucleolus to many small size and proximity internal layer nuclear membrane were put and vacuoles thin layer around the core made up of follicular intensity had decreased basophile oocytes Figure 37.

After 90 Days of experiment the ovarian development shifted from resting stage to Preparatory phase in control and experiment condition. The oocytes were larger in

size with prominent nuclei. During maturation, yolk deposition took place. At a perinucleolar stage, the yolk nucleus was located close to the nuclear membrane (Figure 13b) however, yolk nucleus migrates to the periphery of the ooplasm during further maturation in controlled condition, but in 0.5mg/L dose of DBP, histological analysis of the gonads confirmed that the gonads visually identified as intersex, Intersex population was the intermediate form of ovary and testes. Histological structure showed many spermatids surrounded by the ovarian cavity in all group of intersex population. Developing ovary showed perinucleolar oocytes (POC) Spermatocytes (ST) imposex condition figure 15a. There were a significantly lower proportion of spermatozoa in the testes of males in the treatment group of 0.5mg/L compared to control Figure 38.

4.3 Effect of Phthalic Acid ester (DEHP) on *L. rohita*

Effect of DEHP on Gonado somatic index (GSI) of *L. rohita*:

The experimental fishes were found unhealthy throughout the experimental period. DEHP exposed fish had no significant effect on GSI only few groups of exposure showed significant decrease. Table 13 Shows that GSI value in control group ranged from 0.1±0.01-0.33±0.02 after 90 days and in DEHP treated group 0.2 mg/L had not significant ($P>0.5$) changes in GSI it was 0.13±0.01, in 0.3mg/L it was 0.16±0.01 and high does 0.5mg/L it was 0.18±0.02 after 30 days of experiment. After 60 days in treatment dose of 0.2mg/L the GSI was 0.22±0.01, in 0.3mg/L it was 0.23±0.01 and in high dose of DEHP 0.5mg/L it was 0.22±0.01 all data were non-significant ($P>0.05$). After 90 days DEHP exposed *L. rohita* GSI was 0.31±0.02, 0.33±0.01 in dose of 0.2mg/L and 0.3mg/L respectively. There was significant ($P<0.5$) reduction in GSI at high dose of DEHP exposed fish it was measured 0.28±0.01 after 90 days. (Figure 14)

Effect of DEHP on biochemical Parameters of *L. rohita*:

DEHP treated fish was analyzed and found significant (P<0.5) reduction in testosterone level in serum when data was compared with the control fish (Table 13.1). In high dose of DEHP the testosterone serum level was significantly (p<0.05) decrease (P<0.05) the value was 1106±19.8pg/ml after 30 day, while in control group of fish it was 1525.7±5.38pg/ml; in 0.3mg/L the testosterone value was 1523.7±13pg/ml and 0.2mg/L the testosterone value was 1525±12.5pg/ml after 30 days of experiment. After 60 days in high dose of DEHP 0.5mg/L the testosterone value was 613.2±10.9pg/ml (P<0.01), in 0.3mg/L treatment dose it was 1511±6.1pg/ml and in 0.2mg/L the testosterone value was 1508±14.9pg/ml, while in control group of fish it was 1508±1.83pg/ml. At the end of experiment after 90 days in high dose of DEHP 0.5mg/L the testosterone value was 586.5± 19.9pg/ml (P<0.01) while in control group of fish it was 1528.8±12.7pg/ml , in 0.3mg/L the testosterone value was 1445.1±17.5pg/ml and in 0.2mg/L it was 1408± 14.9pg/ml (Figure 15).

Serum 17-β estradiol found significantly (P<0.05) increase in DEHP treated fish group as compared to the control fish group it was ranged from 263±13.7-282 ±14.9pg/ml shown in Table 13.1. In 0.2mg/L dose of DEHP it was 307±8.16pg/ml; in 0.3mg/L dose of DEHP it was found 333.3±12.2pg/ml and in 0.5mg/L dose of DEHP it was found 370±16.4pg/ml at the end of 30 days. After 60 days in 0.2mg/L dose of DEHP estradiol level was 363± 12.2pg/ml; in 0.3mg/L dose of DEHP it was found 413.8±20.2pg/ml and in 0.5mg/L dose of DEHP it was found 406.7±13.5pg/ml all data were significant at (P<0.05). After 90 days of experiment in 0.2mg/L dose of DEHP it was measured 324.3±10.2pg/ml; in 0.3mg/L dose of DEHP it was found 529±17.7pg/ml and in 0.5mg/L dose of DEHP it was found 529.32±17.7pg/ml Figure 16. Serum Vitellogenin had slightly increased compare to control group, the data was

insignificant (P>0.5) in 0.2mg/L dose of DEHP it was 970.7±12.2pg/ml; in 0.3mg/L dose of DEHP vitellogenin level was significantly increased (P<0.05) it was 1116.7±18.7pg/ml and in 0.5mg/L dose of DEHP it was measured (P<0.05) 1436.3± 18.7pg/ml at the end of 30 days; After 60 days in 0.2mg/L dose of DEHP vitellogenin level was 1139± 18.1pg/ml; in 0.3mg/L dose of DEHP it was measured 1447.4±13.9pg/ml and in 0.5mg/L dose of DEHP it was found 1628±13.8pg/ml all were significant (p<0.05) and end of experiment after 90 days in 0.2mg/L dose of DEHP vitellogenin was measured 1229.8±17.5pg/ml; in treatment group of 0.3mg/L it was 1536.5±18.7pg/ml and in 0.5mg/L dose of DEHP it was 1701±20pg/ml all data were significant (P<0.05) compare to control group. Control fish serum vitellogenin ranged 1018±16.1; 919.8±20.1; 1039.8±19.5pg/ml after 30, 60, 90 respectively Figure 17.

Serum aromatase level in control was recorded 79.74±2.5, 79.1±3.1 and 79.7±2.1pg/ml after 30, 60 and 90 days respectively. In experimental DEHP exposure in the dose of 0.2mg/L it was slightly increased in aromatase level which were 92.35±3.5, 95.45±3.5, 106.1±2.1 after 30, 60 and 90 days respectively. Treatment in the dose of 0.3mg/L of DEHP concentration it was measured 97.7.5±2.4, 111.6±4.5, 126.3±4.2 after 30, 60 and 90 days respectively, and in higher concentration 0.5mg/L there was significant (P<0.05) increase in aromatase level 113.9±3.4, 153.3±4.2, 213.3±2.3 after 30, 60 and 90 days respectively Figure 18 (Table 13.1).

Effect of DEHP on RNA/DNA Ratio of *L. rohita*

The group of fish treated with DEHP showed significant decrease of RNA/DNA ratio in high dose of the experimental period Table 13.2 shows RNA/DNA ratio value in control group which ranged from 2.6±0.09-2.74±0.05 and in DEHP treated groupit did not significantly (P>0.05) affect any changes in 0.2mg/L, 0.03mg/L and 0.5mg/L

where it was 2.6±0.06, 2.1±0.04, 2.1±0.04 respectively after 30 days respectively. But After 60 days of experiment it was significantly reduced in all group of treatment ($P<0.05$) in 0.02mg/L DEHP it was 2±0.07, in 0.3mg/L it was 1.99±0.07 and in 0.5mg/L it was 1.95±0.05. After 90 days of experiment it was also significant decrease in RNA/DNA ratio in the dose of 0.02mg/L where the value was 1.9±0.05 and in 0.3mg/L and 0.5mg/L treatment dose it were measured 2.08±0.05 and 1.8±0.08 respectively (Figure 19)

Table 13.3 includes all water parameter during experimental work.

Effect of DEHP on gonadal development and structure of *L. rohita*:

Exogenously DEHP exposure to fish was observed significant changes in gonad structure and their development. Histological analysis of the gonads confirmed that the gonads visually identified as intersex, Intersex population was the intermediate form of ovary and testes. There were a significantly lower proportion of spermatozoa in the testes of males in 0.5mg/L treatment group compared to control group figure 39, but there were significantly more spermatocytes in the testes of control fish. Histological structure showed many spermatids surrounded by the ovarian cavity in all group of intersex population. (Figure 40, 41). At the high concentration of DEHP the microphotograph shows that the oocytes going to degenerate due to endocrine disruption Figure 41.

4.4 Effect of Organochlorine γ-HCH on *L. rohita*

Effect of γ-HCH on Gonado somatic index (GSI) of *L. rohita*:

The experimental fishes were found deleterious throughout the experimental period. γ-HCH exposed fish had significantly ($P<0.5$) decreased GSI compare to control group. Table 14 shows that GSI value in control group ranged from 0.17±0.01-

0.35±0.01 after 30, 60 and 90 respectively. HCH treated group it did not significantly (P>0.05) affect any change in 0.2mg/L, 0.3mg/L and 0.5mg/L where it was 0.14±0.01, 0.16±0.01 and 0.16±0.01 after 30 days respectively. After 60 days it was also insignificant (P>0.05) where in the dose of 0.02mg/L it was 0.21±0.01. In 0.3mg/L and 0.5mg/L doses it was 0.22±0.01 and 0.26±0.01. After 90 days of experiment in the treatment group of 0.2mg/L and 0.3mg/L GSI value was 0.27±0.01 and 0.24±0.01 respectively. But in high dose 0.5mg/L HCH was significantly (P<0.05) decrease GSI compare to control group of fish where it was 0.29±0.01 and there was a slight reduction in the weight (143±4.6g) after 90 days of γ-HCH treatment. The control fish obtained 151± 6.04g in 90 days (Figure 20).

Effect of γ-HCH on biochemical Parameters of *L. rohita*:

HCH treated fish was analyzed and found that serum testosterone level significantly (P<0.5) increased compared to control group (Table 14.1). After 30 Days of experiment, the serum testosterone level was showed no changes in any group of experiment exposure. in high dose of HCH (0.5mg/L) testosterone value was insignificant (P>0.05) it was 1683.7±14.1 pg/ml, while in control group of fish it was 1523.7±5.3pg/ml; in the treatment dose of 0.3mg/L the testosterone value was 1578.7±9.2pg/ml and in dose of 0.2mg/L the testosterone value was 1513±5.8 pg/ml. After 60 days of experiment in high dose of HCH the testosterone value was significantly (P<0.05) increased 1844.7±17.7pg/ml, in dose of 0.3mg/L HCH the testosterone value was 1693±6.5 pg/ml and in 0.2mg/L it was 1510±5.35pg/ml, while in control group of fish it was 1493.7±16.2 pg/ml. After 90 days in high dose of HCH 0.5mg/L the testosterone value was 1933.7± 16.7pg/ml (P<0.05) while in control group of fish it was 1503.7±14.13 pg/ml ; in 0.3mg/L the testosterone value was

1781.1±11.4 pg/ml and in dose of 0.2mg/L HCH the testosterone value was 16.2± 15.6 pg/ml Figure 21.

Serum 17-β estradiol (E2) was measured significant (P<0.05) decreased in HCH treated fish group as compared to the control fish group. In control group it was ranged from 227.3±8.3-251.3±13.12pg/ml after 90 days (Table 14.1). In 0.2mg/L dose of HCH E2 was measured 211.4±6.6pg/ml; in 0.3mg/L dose of HCH it was found 109.5±6.6pg/ml and in 0.5mg/L dose of HCH it was measured 108± 6.15 pg/ml at the end of 30 days all data were insignificant (P>0.05). After 60 days of experiment in 0.2mg/L dose of HCH it was measured 218± 14.2 pg/ml; in 0.3mg/L dose of HCH it was found 115±12.2 pg/ml and in 0.5mg/L dose of HCH it was found significant (P<0.05) decreased estradiol value was 88.5±3.2 pg/ml. After 90 days of experiment in 0.2mg/L dose of HCH it was 246±15.4 pg/ml; but serum estradiol level was significantly (P<0.05) decreased in 0.3mg/L dose of HCH it was found 79.7±3.9 pg/ml and in 0.5mg/L dose of HCH it was found 84±3.5 pg/ml Figure 22. Serum Vitellogenin has slightly decreased compare to control group after 90 days of treatment. All data was insignificant (P>0.5) in 0.2mg/L dose of HCH it was 1286±14.2 pg/ml, 1235± 19.8pg/ml and 1345±14.6 pg/ml after 30 60 and 90 days respectively; in dose of 0.3mg/L ther was found significant (P<0.05) reduction in vitellogenin level it was 1123.6±9.1 pg/ml, 1109.2±8.1 pg/ml and 1109.2±15.1 pg/ml after 30 60 and 90 days respectively. In the treatment group of 0.5mg/L it was measured 1115.7± 18.9pg/ml, 1123±16.3 pg/ml and 1136.5±13.4pg/ml after 30 60 and 90 days respectively all data were significant (P<0.05) decreased compare to control group. Control fish serum vitellogenin ranges, 1341±14; 1312±19.8; 1320±16.9 after 30, 60, 90 respectively Figure 23.

Serum aromatase level did not show significant variation (P>0.05) in any treatment group compare to control shows in Table 14.1. In control group it was 79.5±4.5, 89.5±2.3 and 87.6±4.5 pg/ml after 30, 60 and 90 days respectively. In experimental group it was recorded 83.5±3.5, 79.5±3.5, 87.5±2pg/ml in 0.2mg/L dose of HCH after 30, 60 and 90 days and in 0.3mg/L concentration it was measured 74.5±2.4, 70.2±3.5, 79.5±2.6 pg/ml, and in high concentration 0.5mg/L dose of HCH it was 70 ±2.5, 71.5±4, 70.5±2pg/ml after 30, 60 and 90 days respectively Figure 24.

Effect of γ-HCH on RNA/DNA Ratio of *L. rohita*

The group of fish treated with HCH showed a gradually significant decrease of RNA/DNA ratio in all group of exposure. Table 14.2 shows RNA/DNA ratio value in control group ranged from 2.6±0.09-2.74±0.05 and in dose of 0.2mg/L, 0.03mg/L and 0.5mg/L HCH ratio it was 1.9±0.13, 1.3±0.14 and 1.04±0.07 after 30 days respectively. It was gradually decreased after long time exposure and significant (P<0.05) reduction showed in dose of 0.02mg/L it was measured 1.6±0.22, in treatment group 0.3mg/L it was 1.8±0.17 and in dose of 0.5mg/L it was 1.45±0.07 after 60 days. It was also significant (P<0.05) reduction after 90 days in 0.02mg/L it was 1.9±0.13, in dose of 0.3mg/L and 0.5mg/L it was measured 1.4±0.18 and 0.89±0.05 respectively (Figure 25).

Table 14.3 includes all the water parameter during experiment.

Effect of γ-HCH on gonadal development and structure of *L. rohita*-

After 90 Days of experiment in control group the ovarian development shifted resting stage to preparatory phase. The oocytes were larger in size with prominent nuclei. During maturation, yolk deposition took place. At a perinucleolar stage, the yolk nucleus was located close to the nuclear membrane; however, yolk nucleus migrates

to the periphery of the ooplasm during further maturation Figure 42. Gonads of HCH treated *Labeo rohita* showed intersex condition developing oocyte, arrow showed degenerated follicular connective tissue (FTC), ovules goes to atresia, showed intersex condition structure showed number of spermatids (ST) stage surrounded the mature oocytes (MOC) Bar scale = 20µm.. There was lesion, or altered structure seen in histological observation compare to control Figure 43, 44 and 45.

Table: 11. Effect of different dose of Phthalic acid ester (DMP) on the Morphological parameters of maturating stage of *L. rohita* (All Data are presented as $\bar{X} \pm$ SEM).

Physiological Parameters	Control (C)			0.2mg/L			0.3mg/L			0.5mg/L		
	30 days ($\bar{X} \pm$ SEM)	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**
Weight (g)	141.6 ±2.22	144.5 ±2.6	154 ± 4.4	143.2 ± 3.4$^{n.s.}$	143.9 ± 3.6$^{n.s.}$	150.8 ± 2.9$^{n.s.}$	141.7 ± 4.1$^{n.s.}$	149.2 ± 2.1$^{n.s.}$	150.1 ± 2.3$^{n.s.}$	147.6 ± 3.5$^{n.s.}$	146 ± 4.8$^{n.s.}$	150 ± 2.6$^{n.s.}$
Length (cm)	20.87 ±0.3	21.65 ± 0.39	22.05 ±0.3	21.97 ±0.14$^{n.s.}$	21.8 ±0.20$^{n.s.}$	22.23 ±0.29$^{n.s.}$	20.75 ±0.27$^{n.s.}$	22.5 ±0.22$^{n.s.}$	22.14 ±0.33$^{n.s.}$	21.3 ± 0.24$^{n.s.}$	21.65 ± 0.39$^{n.s.}$	22.35 ±0.21$^{n.s.}$
Gonad weight	0.260 ±0.02	0.395 ±0.01	0.500 ±0.02	0.273 ±0.01$^{n.s.}$	0.387 ±0.02$^{n.s.}$	0.474 ±0.01$^{n.s.}$	0.274 ±0.02$^{n.s.}$	0.393 ±0.02$^{n.s.}$	0.491 ±0.02$^{n.s.}$	0.261 ±0.01$^{n.s.}$	0.370 ±0.01$^{n.s.}$	0.500 ±0.06$^{n.s.}$
GSI	0.182 ±0.03	0.270 ±0.013	0.327 ±0.01	0.190 ±0.01$^{n.s.}$	0.271 ±0.07$^{n.s.}$	0.316 ±0.05$^{n.s.}$	0.193 ±0.01$^{n.s.}$	0.263 ±0.04$^{n.s.}$	0.327 ±0.06$^{n.s.}$	0.178 ±0.015$^{n.s.}$	0.253 ±0.01$^{n.s.}$	0.334 ±0.05$^{n.s.}$

Significance between the control and experimental group: a= P< 0.001, b= P<0.01, c= P< 0.05, n. s= not significant

Table: 11.1 Effect of different dose of DMP on the biochemical parameters of maturating stage of *L. rohita* (All Data are presented as $\bar{X} \pm$ SEM).

Parameters	**Control (C)**			**0.2mg/L**			**0.3mg/L**			**0.5mg/L**		
	30 days ($\bar{X} \pm$ SEM)	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**
Testosterone **(pg/ml)**	1497.7 ±14.5	1482 ±14.0	1523.7 ±5.3	1523 ±5.3 n.s.	1510 ±12.6 n.s.	1535 ±14.6 n.s.	1523.7 ±5.35 n.s.	1523 ±13.5 n.s.	1530 ±12.0 n.s.	1493.3 ±16.6 n.s.	1472.6 ±13 n.s.	1470.1 ±16.4 n.s.
17 β Estradiol **(pg/ml)**	227.3 ±8.3	235.9 ±9.8	251 ±13.1	218.9 ±5.8 n.s.	223.9 ±7.1 n.s.	233 ±12.6 n.s.	253.58 ±15.3 n.s.	261.6 ±14.5 n.s.	262.1 ±13 n.s.	237.2 ±11.4 n.s.	231.7 ±10.1 n.s.	262 ±13.9 n.s.
Vitellogenin **(pg/ml)**	1083.9 ±17.1	1017 ±16.6	1113 ±12.3	1060.8 ±13.0 n.s.	1189.7 ±16.2 n.s.	1157.6 ±16.2 n.s.	1078.4 ±18 n.s.	1190.9 ±15.2 n.s.	1104 ±17.5 n.s.	1103.8 ±14.5 n.s.	1133.8 ±19.3 n.s.	1070 ±19.8 n.s.
Aromatase **(pg/ml)**	75.74 ±3.5	77.3 ±2.1	79 ±3	79.6 ±2.12 n.s.	83.45 ±2.5 n.s.	86.8 ±2.1 n.s.	89.7 ±3 n.s.	84.6 ±21 n.s.	89.3 ±3.2 n.s.	84.9 ±2.5 n.s.	81.7 ±2.2 n.s.	85.5 ±2.6 n.s.

Significance between the control and experimental group: a= P< 0.001, b= P<0.01, c= P< 0.05, n. s= not significant.

Table: 11.2 Effect of different dose of Phthalic acid ester (DMP) on the RNA/DNA ratio of *L. rohita* (All Data are presented as X±SEM).

Physiological Parameters	**Control (C)**			**0.2mg/L**			**0.3mg/L**			**0.5mg/L**		
	30 days ($\bar{X}$ ± SEM)	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**
DNA/RNA ratio	2.2 ±0.06	2 ±0.03	2.12 ±0.06	2.1 ±0.06$^{n.s.}$	2 ±0.07$^{n.s.}$	2.09 ±0.05$^{n.s.}$	2.15 ±0.1 $^{n.s.}$	1.95 ±0.02$^{n.s.}$	2.04 ±0.1 $^{n.s.}$	1.93 ±0.04 $^{n.s.}$	1.92 ±0.01 $^{n.s.}$	1.94 ±0.04 $^{n.s.}$

Significance between the control and experimental group: a= P< 0.001, b= P<0.01, c= P< 0.05, n. s= not significant.

Table: 11.3 Physio-chemical analysis of water during DMP exposued *L. rohita* in aquuarium

Water parameters	**Control (C) ($\bar{X}$ ± SEM)**	**Range**	**10µg/L (M_1) ($\bar{X}$ ± SEM)**	**Range**	**20µg/L (M_2) ($\bar{X}$ ± SEM)**	**Range**	**25µg/L (M_3) ($\bar{X}$ ± SEM)**	**Range**
Temperature (ºC)	27.32 ± 0.29	26-28.9	27.4 ± 0.28	26-28.9	27.35 ± 0.30	26-29	26.98 ± 0.39	26.1-29.8
pH	7.22 ± 0.25	6.2-8.1	7.8 ± 0.19	6.8 -8.6	7.5 ± 0.19	6.9-8.5	7.14 ± 0.18	6.5-8.2
Dissolve Oxygen (mg/L)	4.96 ± 0.39	3.2-6.7	4.26 ± 0.33	2.6-5.6	5.09 ± 0.39	2.9-7.1	3.86 ± 0.31	2.9-5.7
Electrical Conductivity (µS/cm)	274.9 ± 6.89	255-310	273.6 ± 5.60	255-296	274.8 ± 5.96	245-298	269.2 ± 5.62	240-295
Hardness (mg/L)	201.5 ± 3.17	180-211	199.3 ± 5.50	160-215	198.2 ± 5.12	165-215	195.9 ± 5.13	165-216

Table: 12 Effect of different dose of Phthalic acid ester (DBP) on the Morphological parameters of maturating stage of *L. rohita* (All Data are presented as $\bar{X}$ ± SEM).

Physiological Parameters	Control (C)			0.2mg/L			0.3mg/L			0.5mg/L		
	30 days ($\bar{X}$ ± SEM)	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**
Weight (g)	130.6 ± 8.3	141.3 ± 7.2	143.5 ±5.9	131.4 ±8.8	134.8 ± 8.0	139 ±7.6	138.3 ±5.2	141.3 ±4.8	142.9 ±4.8	140.4 ±5.5	138.4 ±6.7	144.8 ±5.3
Length (cm)	20.42 ±0.2	21.2 ±0.2	21.95 ±0.19	20.65 ±0.13	21.8 ±0.20	22.23 ±0.29	20.85 ±0.21	21.75 ±0.19	22.25 ±0.17	20.65 ±0.28	20.7 ±0.s2177	20.7 ±0.21
Gonad Weight	0.247 ±0.023	0.367 ±0.02	0.46 ±0.03	0.243 ±0.02	0.356 ±0.027	0.508 ±0.01	0.221 ±0.02	0.313 ±0.02	0.451 ±0.02	0.240 ±0.02	0.316 ±0.02	0.486 ±0.01
GSI	0.19 ±0.01	0.272 ±0.02	0.341 ±0.02	0.161 ±0.01$^{n.s.}$	0.236 ±0.02$^{n.s.}$	0.321 ±0.02$^{n.s.}$	0.165 ±0.02$^{n.s.}$	0.227 ±0.02$^{n.s.}$	0.322 ±0.02 $^{n.s.}$	0.171 ±0.01 $^{n.s.}$	0.231 ±0.01 $^{n.s.}$	0.341 ±0.02 $^{n.s.}$

Significance between the control and experimental group: a= P< 0.001, b= P<0.01, c= P< 0.05, n. s= not significant.

Table: 12.1 Effect of different dose of DBP on the biochemical parameters of maturating stage of *L. rohita* (All Data are presented as $\bar{X} \pm$ SEM).

Parameters	**Control (C)**			**0.2mg/L**			**0.3mg/L**			**0.5mg/L**		
	30 days ($\bar{X} \pm$ SEM)	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**
Testosterone **(pg/ml)**	1523.3 ±5.3	1513.7 ±9.01	1533.7 ±8.5	1523.7 ±12.8$^{n.s.}$	1513 ±5.35^{n,s,}	1258.7 ±13.0^{c}	1428 ±9.2$^{n.s.}$	1082.7 ±12.5^{c}	1093.7 ±19.3^{c}	1174.3 ±13.3^{c}	988.7 ±13.7^{c}	961 ±10.0^{b}
17 β Estradiol **(pg/ml)**	227.3 ±8.3	235.9 ±9.8	251 ±13.12	359.8 ±14.6^{c}	340 ±23^{c}	360.8 ±14.8^{c}	394 ±16.1^{c}	438.1 ±21.6^{c}	524.3 ±7^{c}	394 ±16.1^{c}	438 ±21^{c}	524 ±9.7^{c}
Vitellogenin **(pg/ml)**	1018 ±16.0	959 ±14.5	999 ±19.6	1038 ±11.6$^{n.s.}$	1155.7 ±12.1$^{n.s.}$	1258.9 ±12.6$^{n.s.}$	1128.3 ±24.3$^{n.s.}$	1445.5 ±15.4^{c}	1450.8 ±12.8^{c}	1383.4 ±15.3^{c}	1478.4 ±17.1^{c}	1404.8 ±18.9^{c}
Aromatase **(pg/ml)**	77.8 ±2.5	79.5 ±3.5	84.3 ±2.5	81.6 ±2.1$^{n.s.}$	105.3 ±4.5$^{n.s.}$	108.5 ±4.5$^{n.s.}$	94.4 ±5.6$^{n.s.}$	105.5 ±5.4$^{n.s.}$	116.4 ±2.5$^{n.s.}$	105.5 ±5.2$^{n.s.}$	125.9 ±6.3$^{n.s.}$	136.2 ±4.2^{b}

Significance between the control and experimental group: a= P< 0.001, b= P<0.01, c= P< 0.05, n. s= not significant.

Table: 12.2 Effect of different dose of Phthalic acid ester (DBP) on the RNA/DNA ratio of *L. rohita* (All Data are presented as X±SEM).

Physiological Parameters	**Control (C)**			**0.2mg/L**			**0.3mg/L**			**0.5mg/L**		
	30 days ($\bar{X}$ ± SEM)	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**
DNA/RNA ratio	2.2 ±01	2 ±0.02	2.12 ±0.02	2.1 ±0.09$^{n.s.}$	2 ±0.02$^{n.s.}$	2.09 ±0.04^{c}	2.15 ±01 $^{n.s.}$	1.95 ±0.04^{c}	1.7 ±0.08^{c}	2.1 ±0.06 $^{n.s.}$	1.92 ±0.05 $^{n.s.}$	1.4 ±0.02 ^{c}

Significance between the control and experimental group: a= P< 0.001, b= P<0.01, c= P< 0.05, n. s= not significant.

Table: 12.3 Physio-chemical analysis of water during DBP exposued *L. rohita* in aquarium.

Water parameters	**Control © ($\bar{X}$ ± SEM)**	**Range**	**10µg/L (T_1) ($\bar{X}$ ± SEM)**	**Range**	**20µg/L (T_2) ($\bar{X}$ ± SEM)**	**Range**	**25µg/L (T_3) ($\bar{X}$ ± SEM)**	**Range**
Temperature (°C)	25.39 ± 0.36	26-28.9	25.24 ± 0.36	26-29.0	27.98 ± 0.33	26-29.0	25.19 ± 0.33	26.1-29.0
pH	6.5 ± 0.24	6.2-8.4	6.38 ± 0.28	6.3-8.9	6.45 ± 0.19	6.8-8.8	7.52 ± 0.20	6.8-8.9
Dissolve Oxygen (mg/L)	3.51 ± 0.27	3.2-6.7	4.26 ± 0.33	2.6-5.6	5.09 ± 0.39	2.9-7.1	4.86 ± 0.31	2.9-5.7
Electrical Conductivity (µS/cm)	672.7 ± 4.93	659-710	658.0 ± 3.63	654-726	642.6 ± 9.04	640-710	679.1 ± 9.64	615-710
Hardness (mg/L)	152.5 ± 4.43	160-205	180.1 ± 4.64	160-210	185.4 ± 6.002	160-210	181.1 ± 6.10	160.210

Table: 13. Effect of different dose of Phthalic acid ester (DEHP) on the Morphological parameters of maturating stage of *L. rohita* (All Data are presented as $\bar{X} \pm$ SEM).

Physiological Parameters	**Control (C)**			**0.2mg/L**			**0.3mg/L**			**0.5mg/L**		
	30 days ($\bar{X} \pm$ SEM)	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**
Weight (g)	134.5 ±4.2	138 ±4.3	140.5 ±4.7	130 ±5.2	136.6 ±5.3	145.9 ±6.9	132.9 ±2.4	135 ±5.3	137.6 ±3.2	135.9 ±4.5	138.2 ±5.3	37.8 ±4.7
Length (cm)	20.51 ±0.4	21.35 ±0.4	21.25 ±0.35	20.6 ±0.2	20.82 ±0.2	21.32 ±0.28	20.75 ±0.27	2105 ±0.16	21.35 ±0.27	20 ±0.2	20.7 ±0.2	20.7 ±0.2
Gonad weight	0.20 ±0.01	0.50 ±0.03	0.54 ±0.3	0.17 ±0.02	0.43 ±0.02	0.44 ±0.03	0.22 ±0.02	0.31 ±0.02	0.42 ±0.01	0.25 ±0.03	0.31 0.02±	0.46 ±0.01
GSI	0.128 ±0.01	0.31 ±0.02	0.33 ±0.02	0.13 $\pm 0.01^{n.s.}$	0.22 $\pm 0.01^{n.s.}$	0.31 $\pm 0.02^{n.s.}$	0.16 $\pm 0.01^{n.s.}$	0.23 $\pm 0.01^{n.s.}$	0.33 $\pm 0.01^{n.s.}$	0.18 $\pm 0.02^{n.s.}$	0.22 $\pm 0.01^{n.s.}$	0.28 $\pm 0.01^{C.}$

Significance between the control and experimental group: a= P< 0.001, b= P<0.01, c= P< 0.05, n. s= not significant.

Table: 13.1 Effect of different dose of DEHP on the biochemical parameters of maturating stage of *L. rohita* (All Data are presented as $\bar{X} \pm$ SEM).

Parameters	**Control (C)**			**0.2mg/L**			**0.3mg/L**			**0.5mg/L**		
	30 days ($\bar{X} \pm$ SEM)	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**
Testosterone **(pg/ml)**	1525.7 ± 5.38	1508 ±1.83	1528.8 ± 12.7	1525 $\pm 12.5^{n.s.}$	1508 $\pm 14.9^{n.s.}$	1408.3 $\pm 14.9^{n.s.}$	1523.7 $\pm 13^{n.s.}$	1511.9 $\pm 6.1^{n.s.}$	1445 $\pm 17.5^{n.s.}$	1106 $\pm 19.8^{n.s.}$	613.2 $\pm 10.9^{b}$	586.5 $\pm 19.9^{b}$
17 β Estradiol **(pg/ml)**	263 ±13.7	233.3 ±15.3	282 ±14.9	307 $\pm 8.16^{n.s.}$	363 $\pm 12.2^{c}$	324.3 $\pm 10.2^{c}$	333.3 $\pm 12.2^{n.s.}$	413.8 $\pm 20.2^{c}$	529 $\pm 17.7^{c}$	370 $\pm 16.4^{n.s.}$	406.7 $\pm 13.5^{c}$	529.32 $\pm 17.7^{c}$
Vitellogenin **(pg/ml)**	1018 ±16.1	919.8 ±20.1	1039.8 ±29.5	970.7 $\pm 122^{n.s.}$	1139 $\pm 18.1^{c}$	1229.8 $\pm 17.5^{c}$	1116.7 $\pm 18.7^{n.s.}$	1447.4 $\pm 13.9^{c}$	1536.5 $\pm 18.7^{c}$	1436.3 $\pm 18.7^{c}$	1628.6 $\pm 13.8^{c}$	1701 $\pm 20^{c}$
Aromatase **(pg/ml)**	79.74 ±2.5	79.1 ±3.1	79.7 ±2.1	92.35 $\pm 2.12^{n.s.}$	95.45 $\pm 3.5^{n.s.}$	106.1 $\pm 2.1^{n.s.}$	97.7 $\pm 2.5^{n.s.}$	111.6 $\pm 4.5^{c}$	126.3 $\pm 4.2^{c}$	113.9 $\pm 3.4^{n.s.}$	153.3 $\pm 4.2^{c}$	213.3 $\pm 2.3^{c}$

Significance between the control and experimental group: a= P< 0.001, b= P<0.01, c= P< 0.05, n. s= not significant.

Table: 13.2 Effect of different dose of Phthalic acid ester (DEHP) on the RNA/DNA ratio of *L. rohita* (All Data are as X±SEM).

Physiological Parameters	**Control (C)**			**0.2mg/L**			**0.3mg/L**			**0.5mg/L**		
	30 days ($\bar{X}$ ± SEM)	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**
DNA/RNA ratio	2.66 ±0.09	2.63 ±0.06	2.74 ±0.05	2.6 ±0.06$^{n.s.}$	2 ±0.07 n.s.	1.9 ±0.05 n.s.	2.1 ±0.04$^{n.s.}$	1.99 ±0.07 n.s.	2.08 ±0.05 n.s.	2.1 ±0.04 n.s.	1.95 ±0.05 ^{b}	1.8 ±0.08 ^{b}

Significance between the control and experimental group: a= P< 0.001, b= P<0.01, c= P< 0.05, n.s. = not significant.

Table: 13.3 Physio-chemical analysis of water during DEHP exposued *L. rohita* in aquarium.

Water parameters	**Control (C) ($\bar{X}$ ± SEM)**	**Range**	**10µg/L (T_a) ($\bar{X}$ ± SEM)**	**Range**	**20µg/L (T_b) ($\bar{X}$ ± SEM)**	**Range**	**25µg/L (T_c) ($\bar{X}$ ± SEM)**	**Range**
Temperature (ºC)	25.31 ± 0.28	26-28	26.19 ± 0.26	26.5-29.0	26.58 ± 0.26	26-29.0	25.78 ± 0.43	26.4-29.0
pH	6.24 ± 0.24	6.8-8.17	7.51 ± 0.38	6.3-8.3	7.25 ± 0.19	6.8-8.1	7.32 ± 0.31	6.8-8.0
Dissolve Oxygen (mg/L)	5.84 ± 0.25	3.2-6.5	5.26 ± 0.32	3.6-5.6	4.19 ± 0.94	4.9-7.1	3.86 ± 0.41	4.2-5.9
Electrical Conductivity (µS/cm)	612.7 ± 4.31	640-720	618.0 ± 3.67	690-756	615.6 ± 9.07	640-710	679.5 ± 9.22	655-700
Hardness (mg/L)	175.5 ± 5.43	160-215	192.4 ± 4.77	162-210	199.3 ± 6.10	179-210	161.1 ± 5.10	160.195

Table: 14. Effect of different dose of Hexachlorocyclohexanes (γ-HCH) on the Morphological parameters of maturating stage of *L. rohita* (All Data are as $\bar{X} \pm$ SEM).

Physiological Parameters	Control (C)			0.2mg/L			0.3mg/L			0.5mg/L		
	30 days ($\bar{X} \pm$ SEM)	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**	**30 days ($\bar{X} \pm$ SEM)**	**60 days ($\bar{X} \pm$ SEM)**	**90 days ($\bar{X} \pm$ SEM)**
Weight (g)	140 ±5.5	142 ±5.19	151 ±6.04	151 ±2.2	154 ±1.4	148.4 ±4.1	149 ±2.2	153.7 ±1.04	153 ±1.34	139 ±5.5	142 ±5.5	143 ±4.6
Length (cm)	22.5 ±0.22	22.5 ±.22	23.5 ±0.2	22.5 ±0.22	21.9 ±0.4	22.09 ±0.31	22.5 ±0.22	21.9 ±0.4	22.09 ±0.3	22.5 ±0.22	21.65 ±0.39	22.05 ±0.3
Gonad weight	0.240 ±0.02	0.37 ±0.01	0.51 ±0.01	0.25 ±0.02	0.34 ±0.01	0.40 ±0.01	0.231 0.02	0.34 ±0.01	0.38 ±0.02	0.23 ±0.01	0.37 ±0.01	0.44 ±0.01
GSI	0.17 ±0.01	0.26 0.01	0.35 ±0.01	0.14 ±0.01 $^{n.s}$	0.21 ±0.01 $^{n.s}$	0.27 ±0.01 $^{n.s}$	0.15 ±0.01 $^{n.s}$	0.22 ±0.01 $^{n.s}$	0.24 ±0.01 $^{n.s}$	0.16 ±0.01 $^{n.s}$	0.26 ±0.01 $^{n.s}$	0.29 ±0.01 ^{c}

Significance between the control and experimental group: a= P< 0.001, b= P<0.01, c= P< 0.05, n.s. = not significant.

Table: 14.1. Effect of different dose of γ-HCH on the biochemical parameters of maturating stage of *L. rohita* (All Data are as X± SEM).

Parameters	Control (C)			0.2mg/L			0.3mg/L			0.5mg/L		
	30 days ($\bar{X}$ ± SEM)	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**
Testosterone **(pg/ml)**	1523.7 ±5.3	1493.7 ±16.2	1503.7 ±14.13	1513.7 ±5.3 n.s.	1510.4 ±6.9 n.s.	1602 ±15.6 n.s.	1578.7 ±24.6 n.s.	1693.7 ±12 n.s.	1781 ±11.4 c	1683 ±14.1 n.s.	1844 ±27.8 c	1933.7 ±26.7 c
17 β Estradiol **(pg/ml)**	263 ±10.5	303.8 ±13.3	259.8 ±13.5	211.4 ±6.6 n.s.	218 ±14.2 n.s.	246 ±15.4 n.s.	109 ±6.6 n.s.	115 ±12.2 n.s.	79.7 ±3.9 c	108 ±6.15 n.s.	88.5 ±3.2 c	84 ±3.5 c
Vitellogenin **(pg/ml)**	1341.8 ±14	1312.5 ±19.8	1320 ±16.9	1286 ±14.2 n.s.	1235 ±19.8 n.s.	1345 ±14.6 n.s.	1123.6 ±9.1 c	1109.2 ±8.1 c	1110.9 ±15.2 c	1115.7 ±18.9 c	1123.1 ±16.3 c	1136.5 ±13.4 c
Aromatase **(pg/ml)**	79.5 ±4.5	89.5 ±2.3	87.6 ±4.5	83.5 ±3.5 n.s.	79.5 ±3.5 n.s.	87.5 ±2 n.s.	74.5 ±2.4 n.s.	70.2 ±3.5 n.s.	79.5 ±2.6 n.s.	70 ±2.5 n.s.	71.5 ±4 n.s.	70.5 ±2 n.s.

Significance between the control and experimental group: a= P< 0.001, b= P<0.01, c= P< 0.05, n.s. = not significant.

Table: 14.2 Effect of different dose of γ-HCH on the RNA/DNA ratio of *L. rohita* (All Data are as X±SEM).

Physiological Parameters	Control (C)			0.2mg/L			0.3mg/L			0.5mg/L		
	30 days ($\bar{X}$ ± SEM)	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**	**30 days ($\bar{X}$ ± SEM)**	**60 days ($\bar{X}$ ± SEM)**	**90 days ($\bar{X}$ ± SEM)**
DNA/RNA ratio	2.66 ±0.9	2.63 ±0.6	2.74 ±0.5	1.9 ±0.13 n.s.	1.6 ±0.22 n.s.	1.91 ±0.13 n.s.	1.3 0.14 n.s.	1.83 ±0.1 n.s.	1.47 ±0.18 n.s.	1.04 ±0.07 c	1.45 ±0.07 c	0.89 ±0.05 c

Significance between the control and experimental group: a= P< 0.001, b= P<0.01, c= P< 0.05, n.s. = not significant.

Table: 14.3 Physio-chemical analysis of water during γ-HCH exposued *L. rohita* in aquarium.

Water parameters	**Control (C) ($\bar{X}$ ± SEM)**	**Range**	**10µg/L (T_a) ($\bar{X}$ ± SEM)**	**Range**	**20µg/L (T_b) ($\bar{X}$ ± SEM)**	**Range**	**25µg/L (T_c) ($\bar{X}$ ± SEM)**	**Range**
Temperature (ºC)	27.31 ± 0.28	26-28	27.19 ± 0.26	26.5-29.0	28.58 ± 0.26	26-29.0	25.78 ± 0.43	26.4-29.0
pH	7.24 ± 0.24	6.8-8.17	6.51 ± 0.38	6.3-8.3	6.25 ± 0.19	6.8-8.1	7.32 ± 0.31	6.8-8.0
Dissolve Oxygen (mg/L)	5.84 ± 0.25	3.2-6.5	4.26 ± 0.32	3.6-5.6	4.19 ± 0.94	4.9-7.1	3.86 ± 0.41	4.2-5.9
Electrical Conductivity (µS/cm)	602.7 ± 4.31	640-720	688.0 ± 3.67	690-756	645.6 ± 9.07	640-710	659.5 ± 9.22	655-700
Hardness (mg/L)	205.5 ± 5.43	160-215	182.4 ± 4.77	162-210	169.3 ± 6.10	179-210	171.1 ± 5.10	160.195

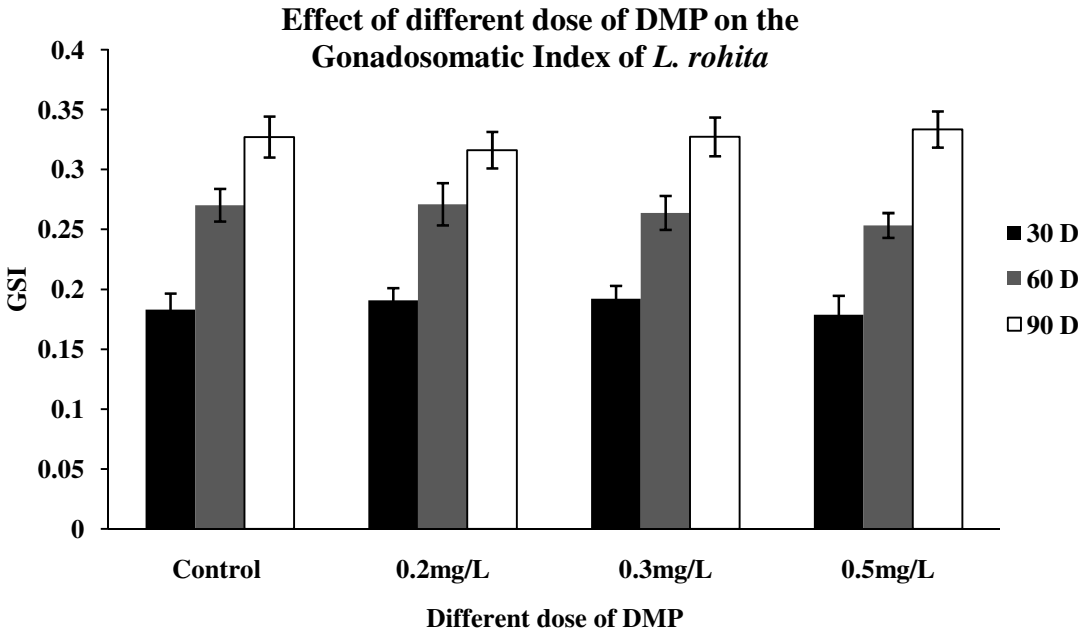


Figure 2: Effect of different dose of DMP on the Gonadosomatic Index of *L. rohita*

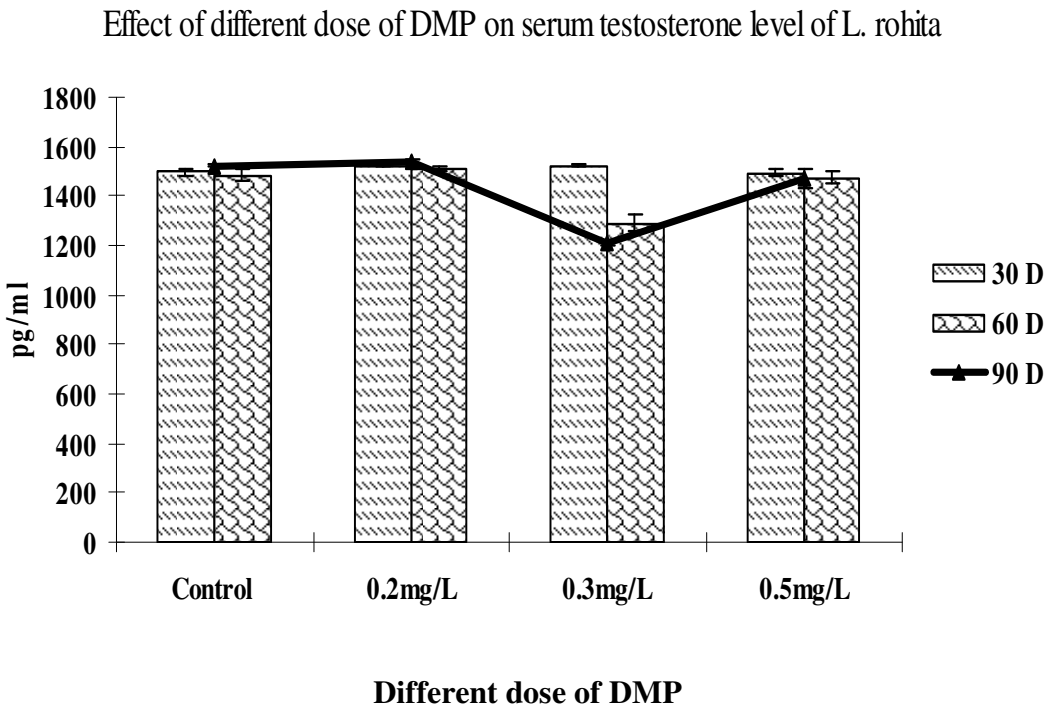


Figure 3: Effect of different dose of DMP on serum testosterone level of *L. rohita*

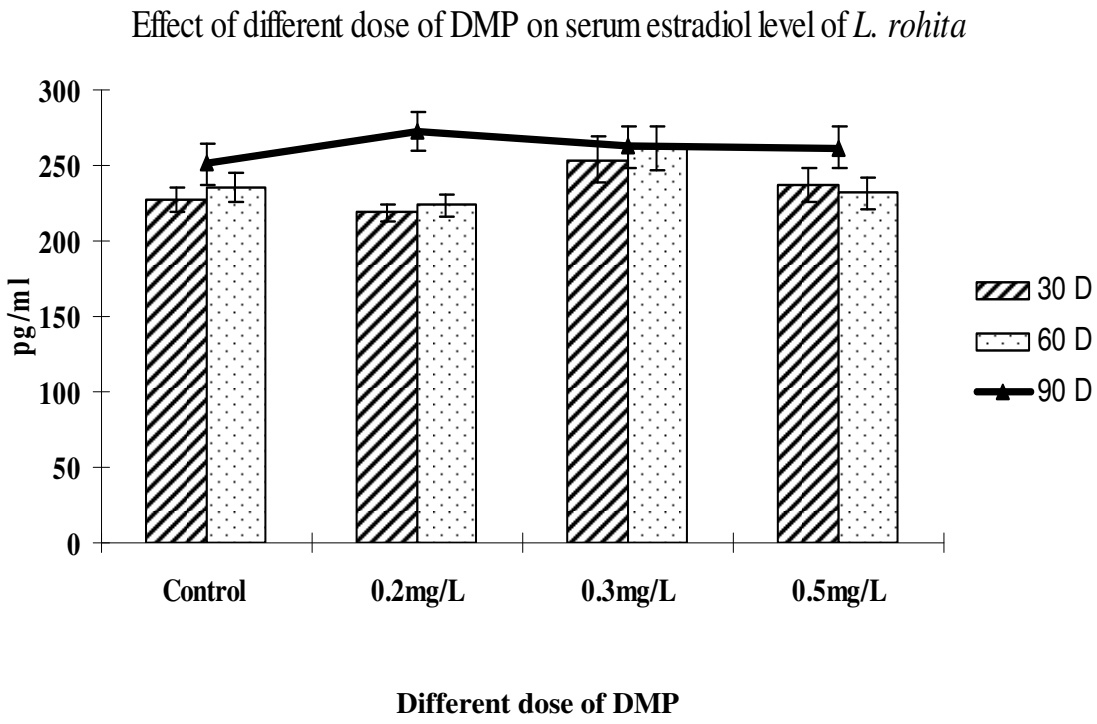


Figure 4: Effect of different dose of DMP on serum estradiol level of *L. rohita*

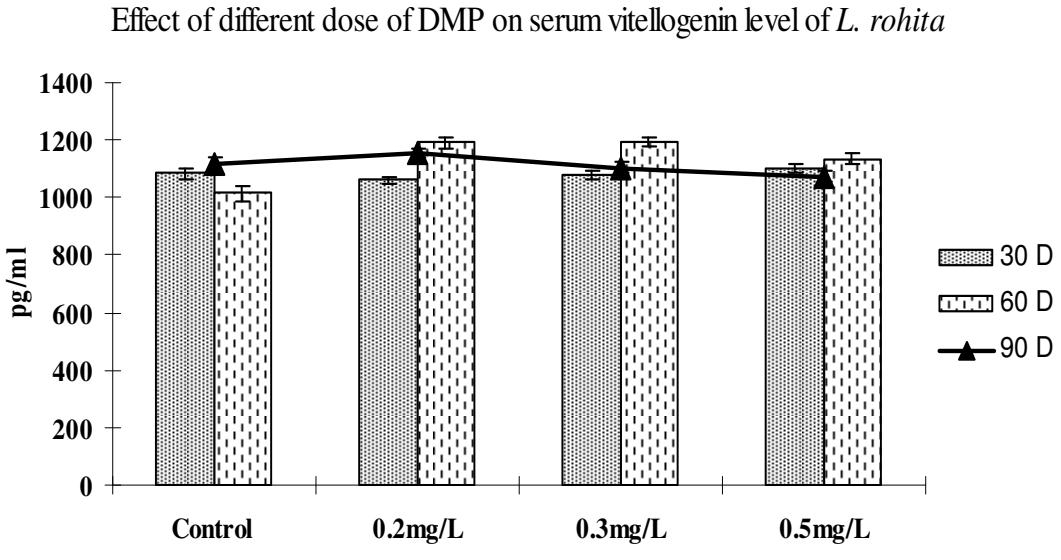


Figure 5: Effect of different dose of DMP on serum vitellogenin level of *L. rohita*

Effect of different dose of DMP on serum aromatase level of *L. rohita*

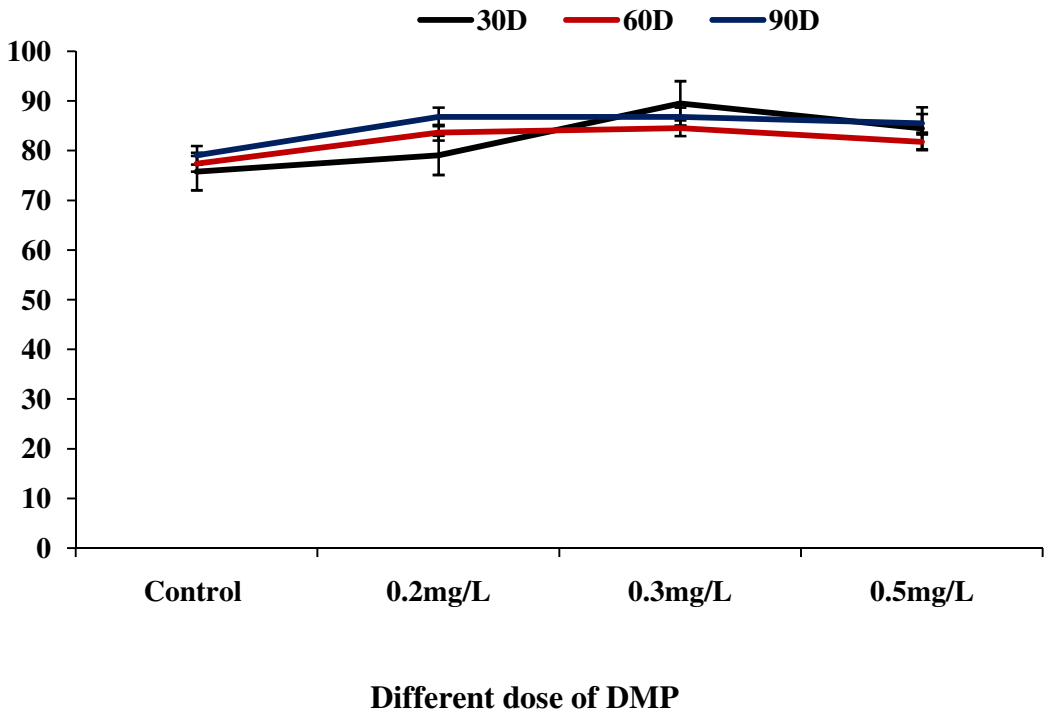
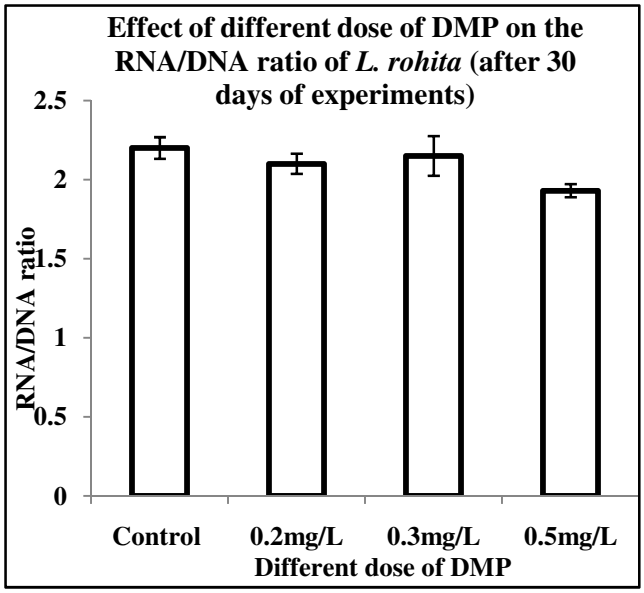
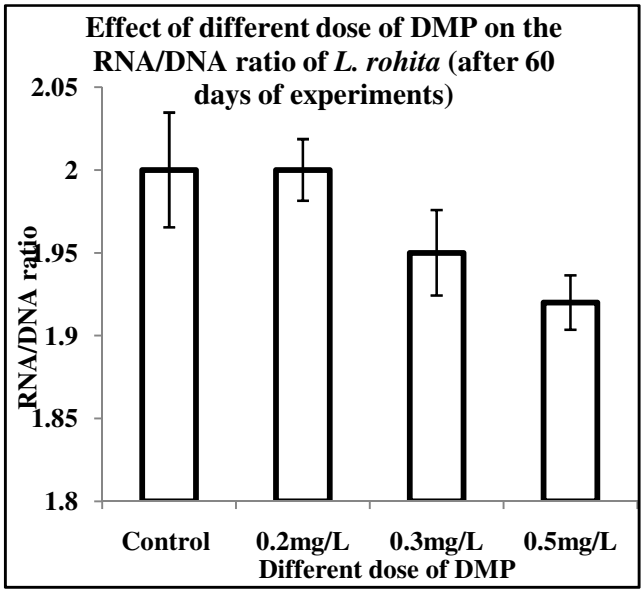


Figure 6: Effect of different dose of DMP on serum aromatase level of *L. rohita*





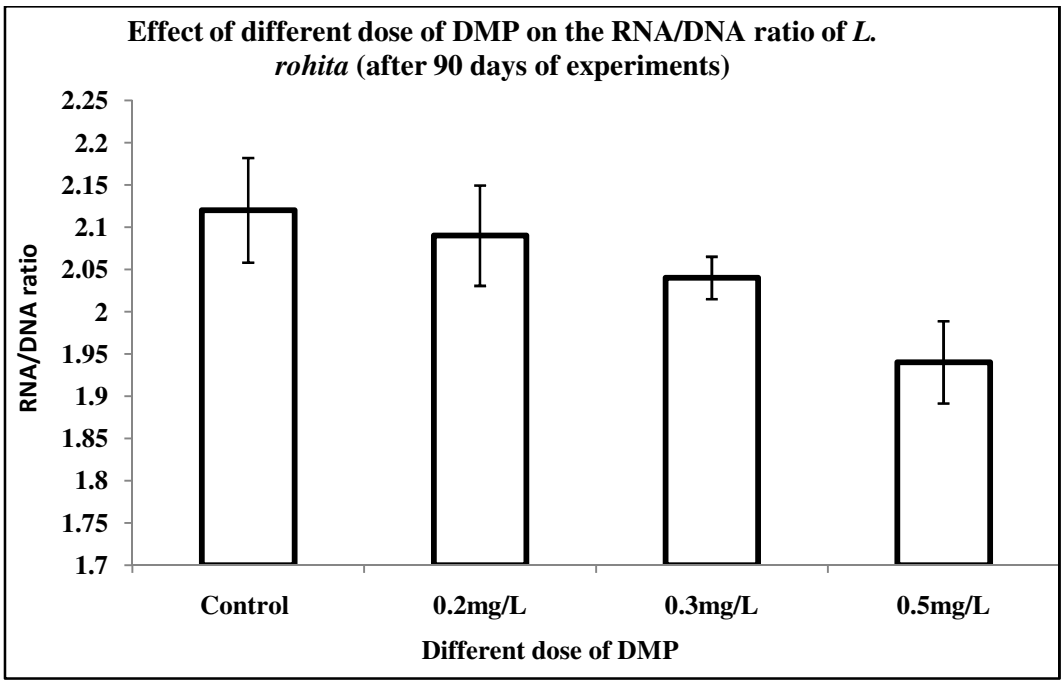


Figure 7: Effect of different dose of DMP on the RNA/DNA ratio of *L. rohita.*

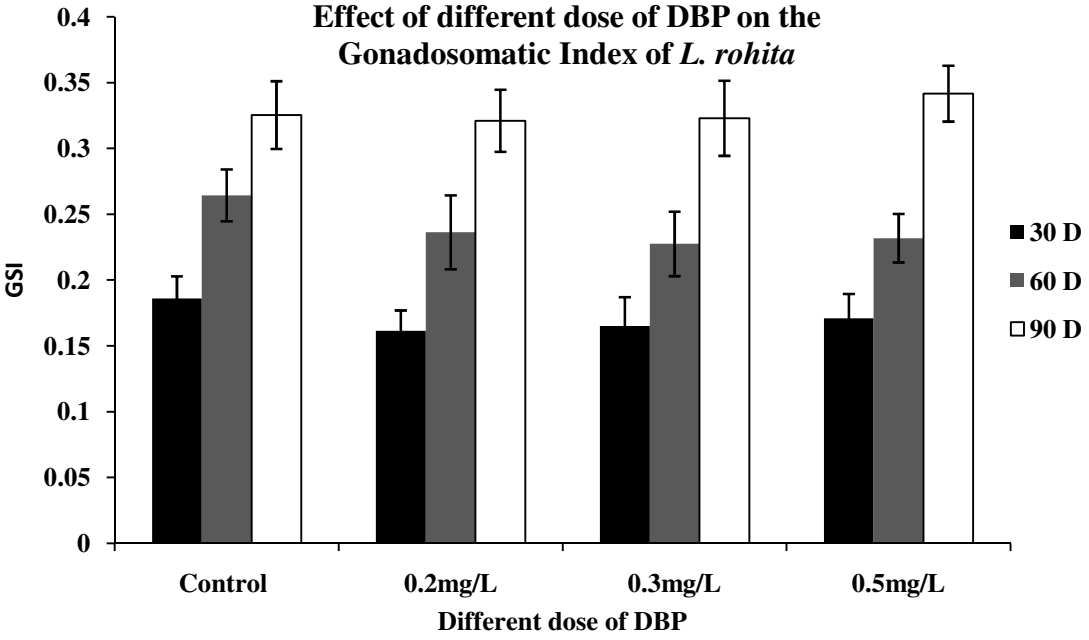


Figure 8: Effect of different dose of DBP on the Gonadosomatic Index of *L. rohita*

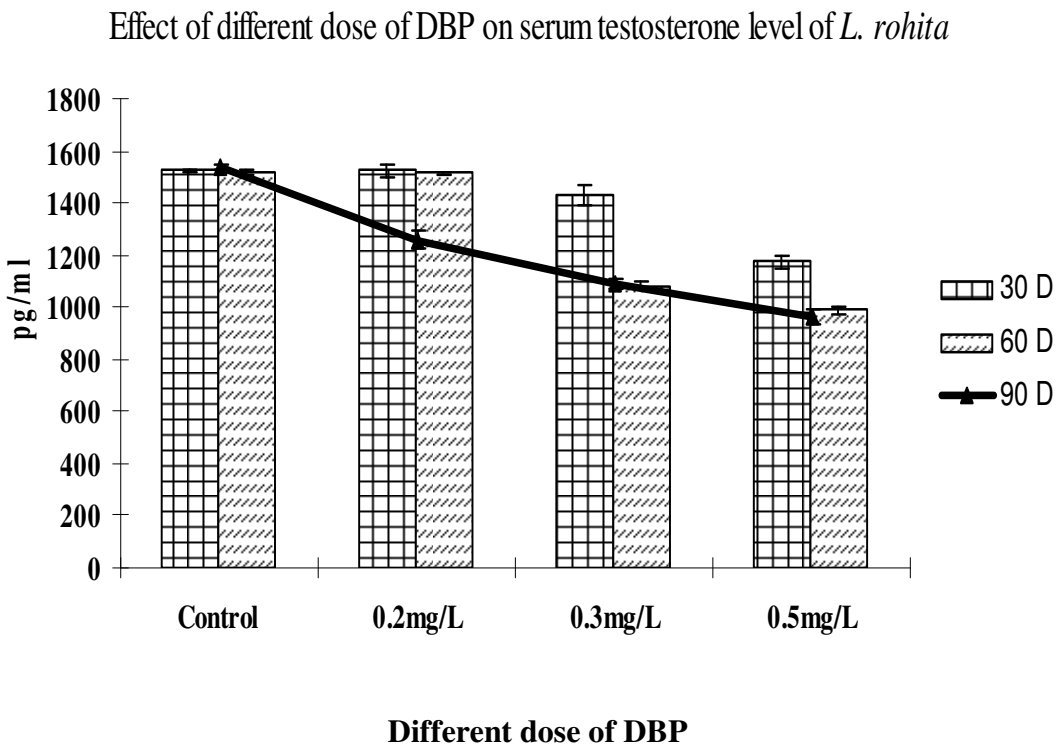


Figure 9: Effect of different dose of DBP on serum testosterone level of *L. rohita*

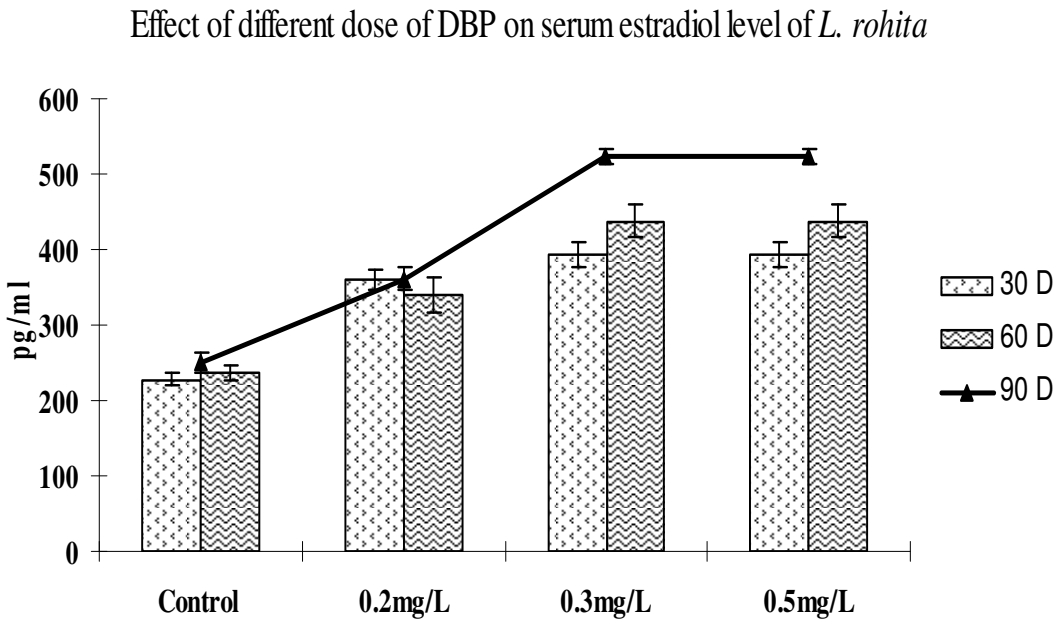


Figure 10: Effect of different dose of DBP on serum estradiol level of *L. rohita*

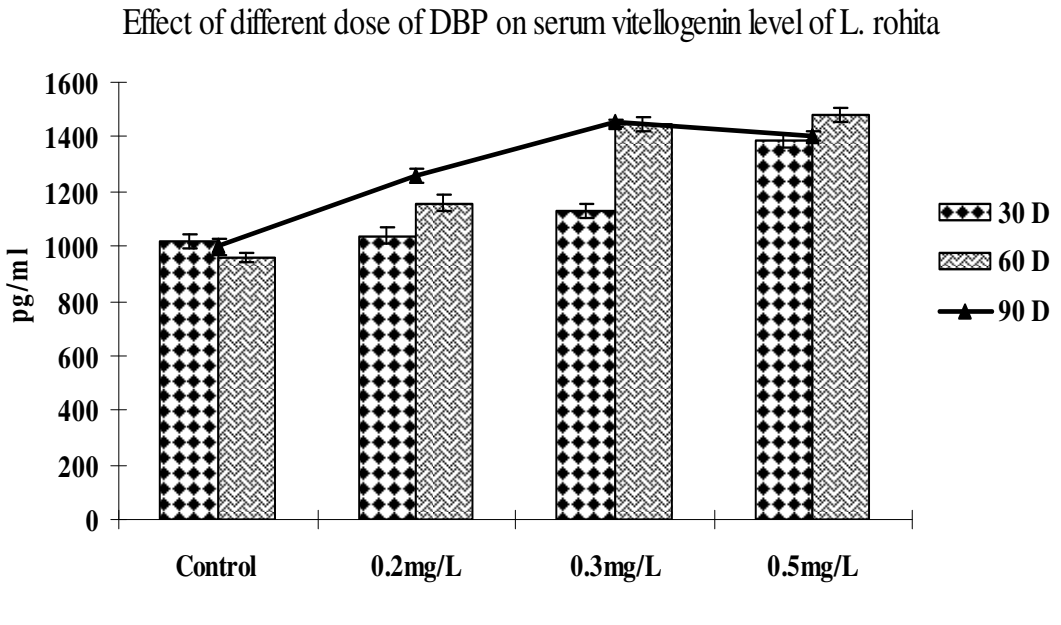


Figure 11: Effect of different dose of DBP on serum vitellogenin level of *L. rohita*

Effect of different dose of DBP on serum aromatase level of *L.rohita*

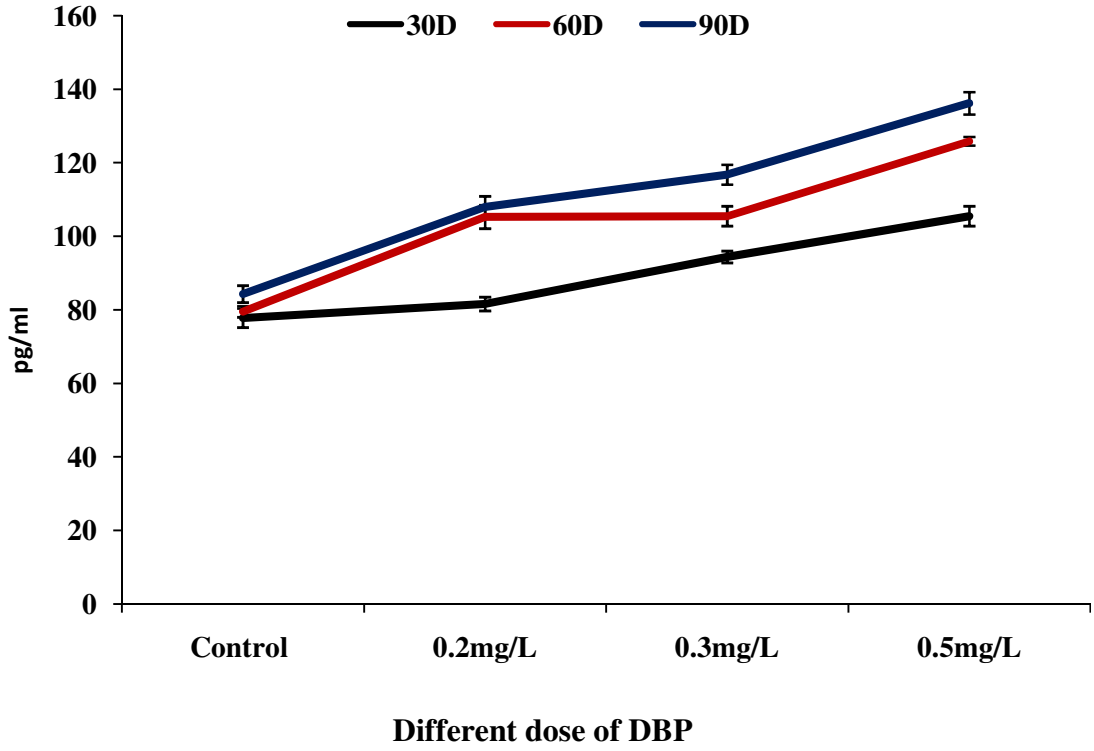
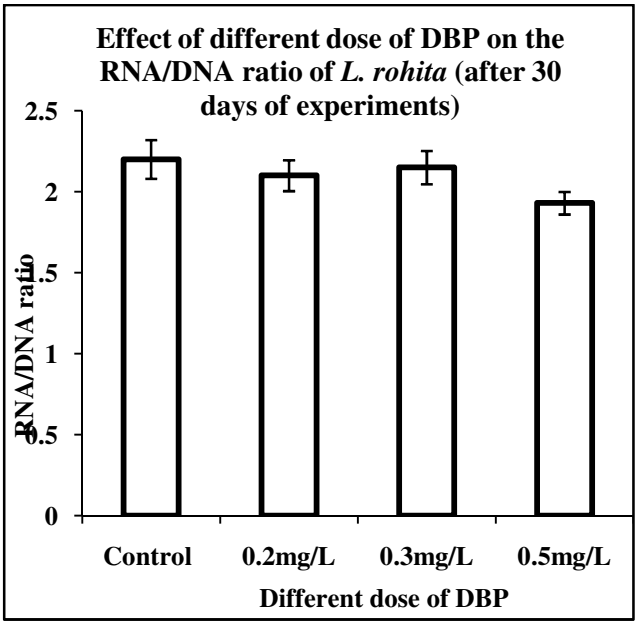
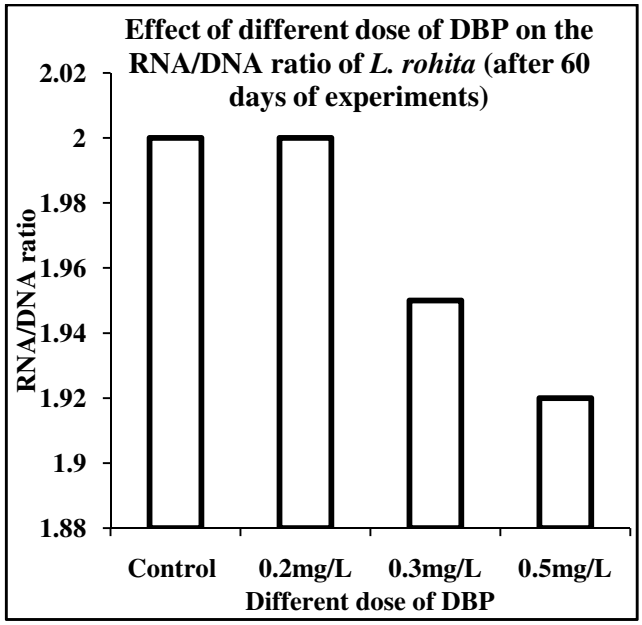


Figure 12: Effect of different dose of DBP on serum aromatase level of *L. rohita*





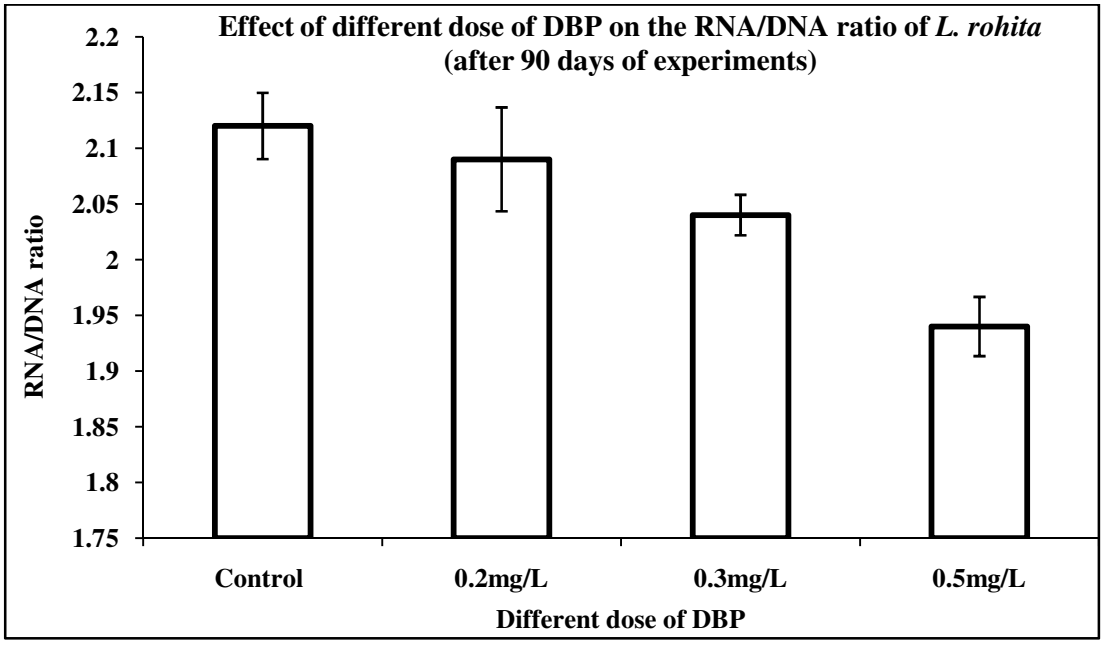


Figure 13: Effect of different dose of DBP on the RNA/DNA ratio of *L. rohita.*

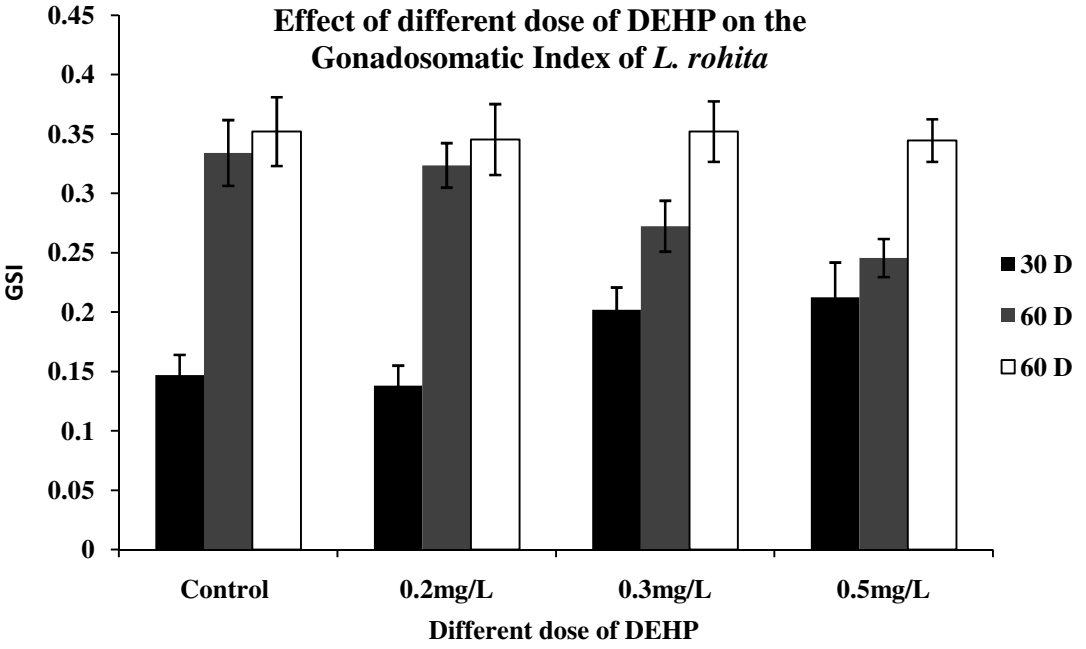


Figure 14: Effect of different dose of DEHP on the Gonadosomatic Index of *L. rohita*

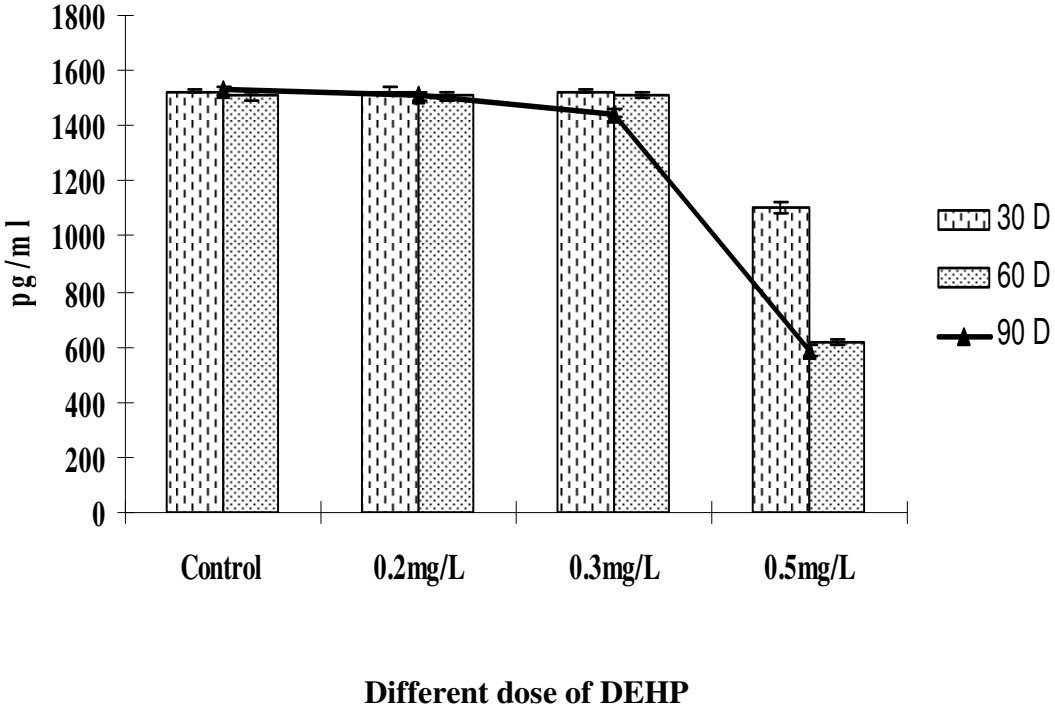


Figure 15: Effect of different dose of DEHP on serum testosterone level of *L. rohita.*

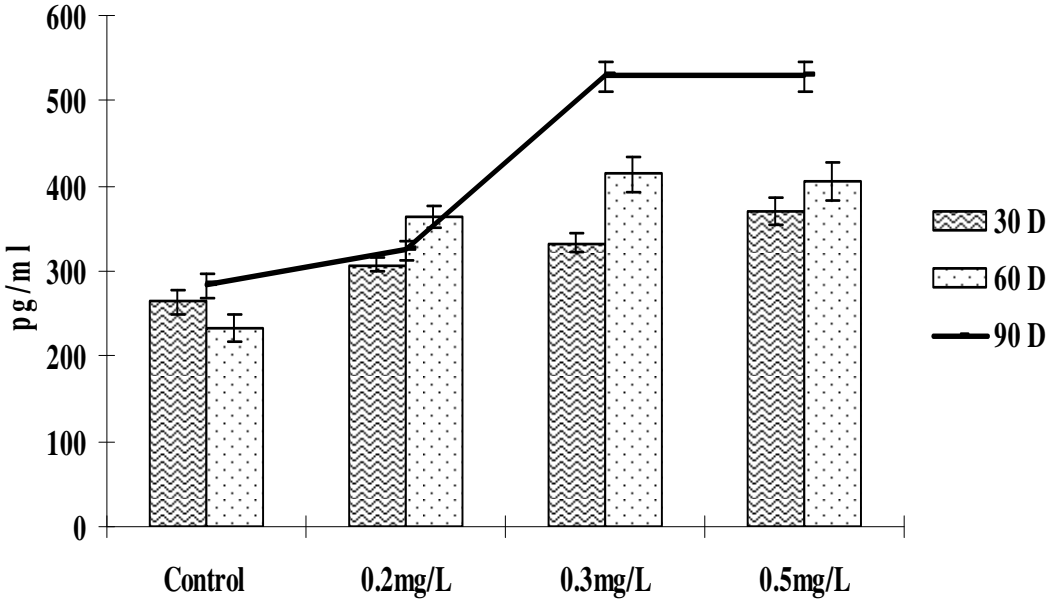


Figure 16: Effect of different dose of DEHP on serum estradiol level of *L. rohita*

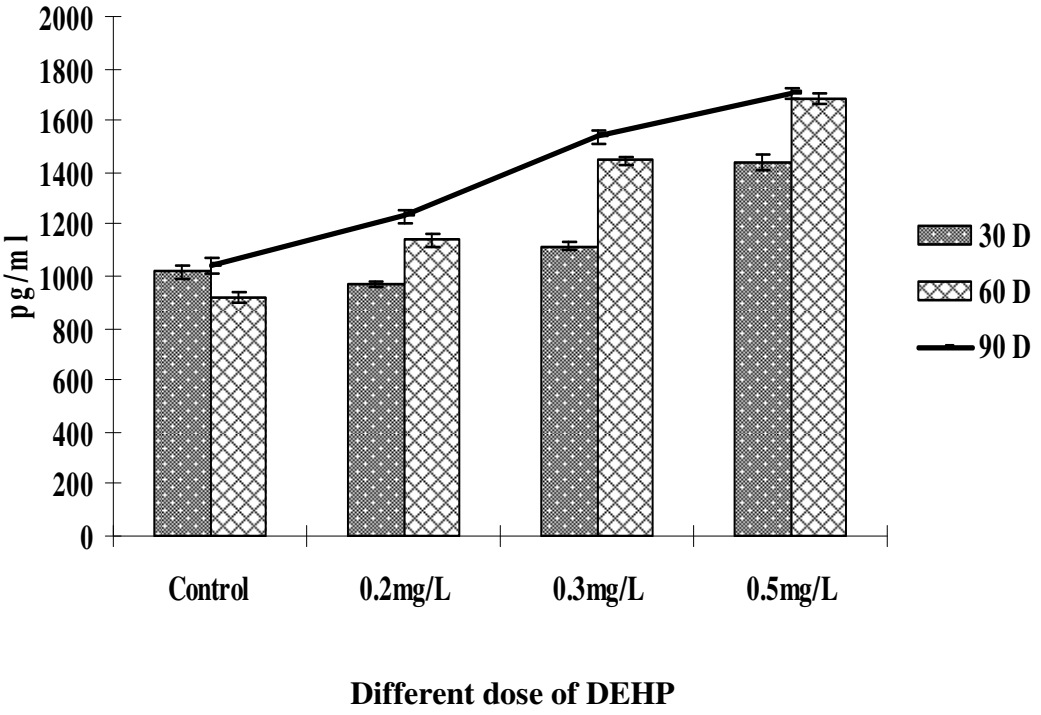


Figure 17: Effect of different dose of DEHP on serum vitellogenin level of *L. rohita*

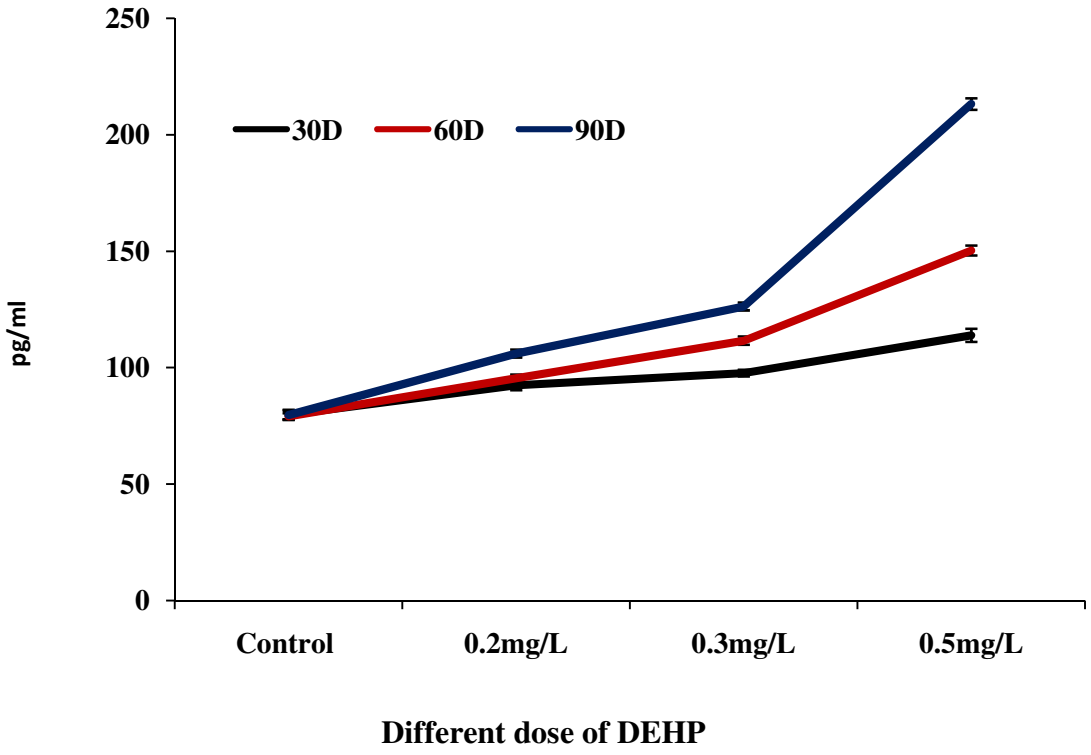
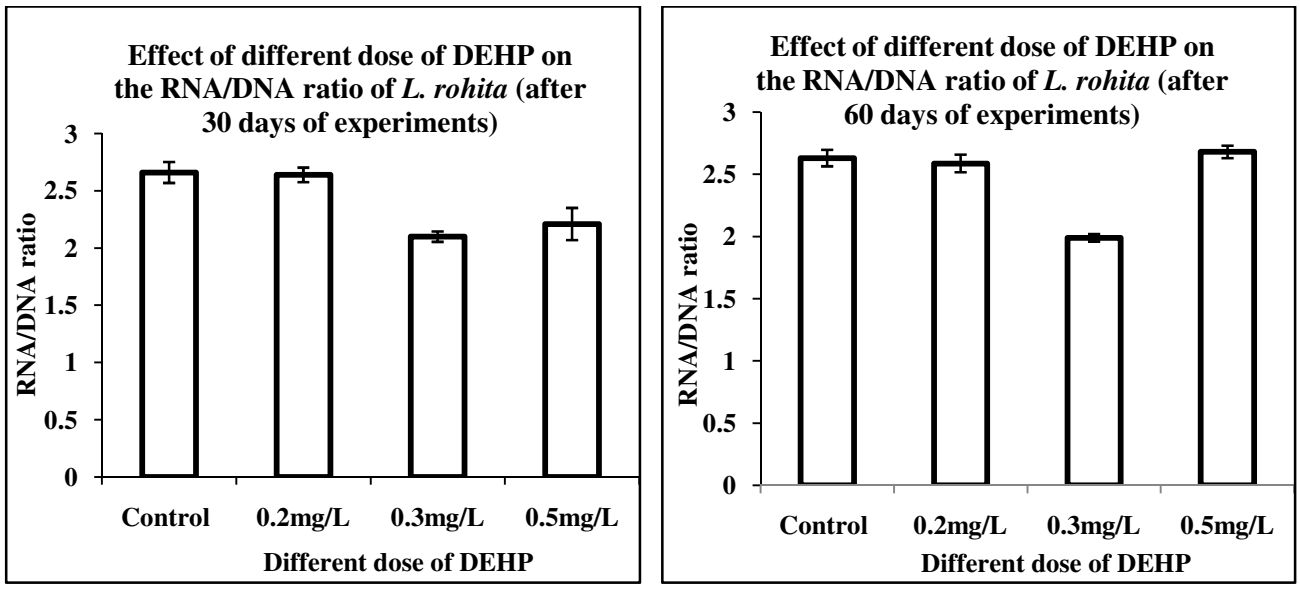


Figure 18: Effect of different dose of DEHP on serum aromatase level of *L. rohita*



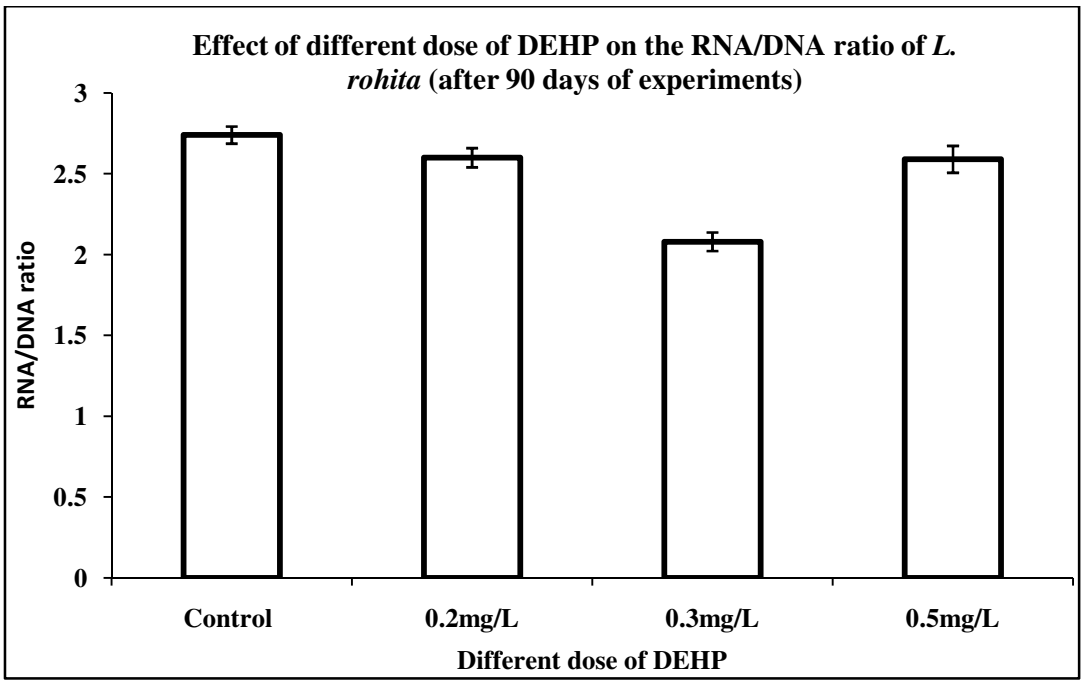


Figure 19: Effect of different dose of DEHP on the RNA/DNA ratio of *L. rohita.*

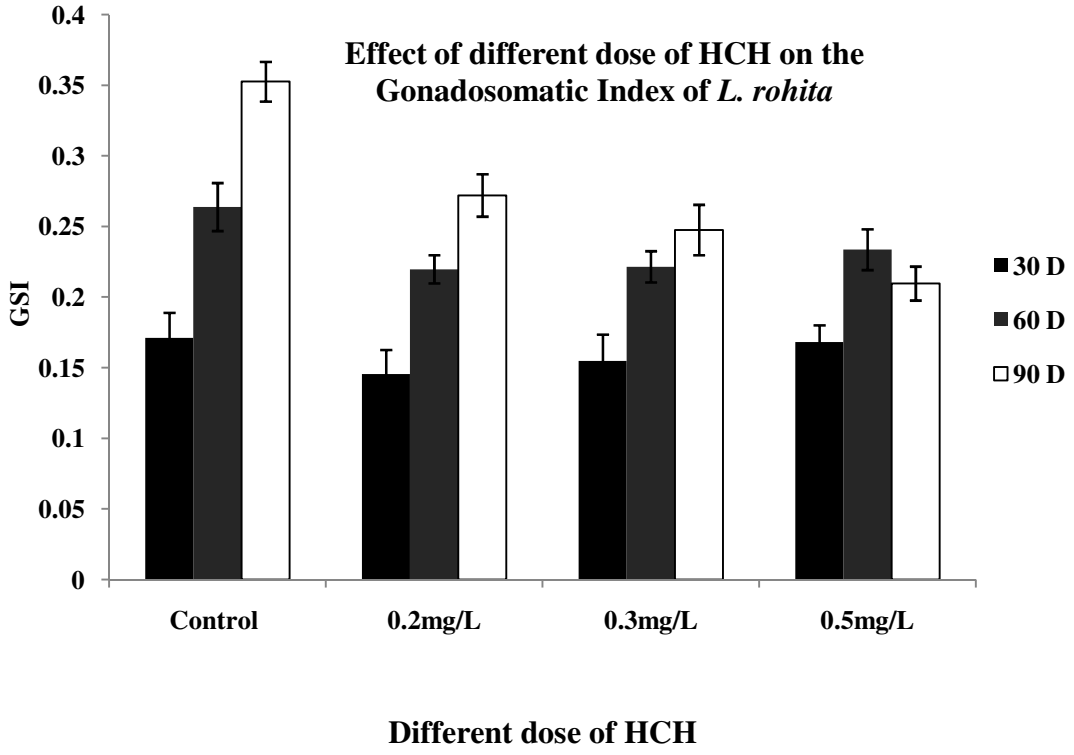


Figure 20: Effect of different dose of HCH on the Gonadosomatic Index of *L. rohita*

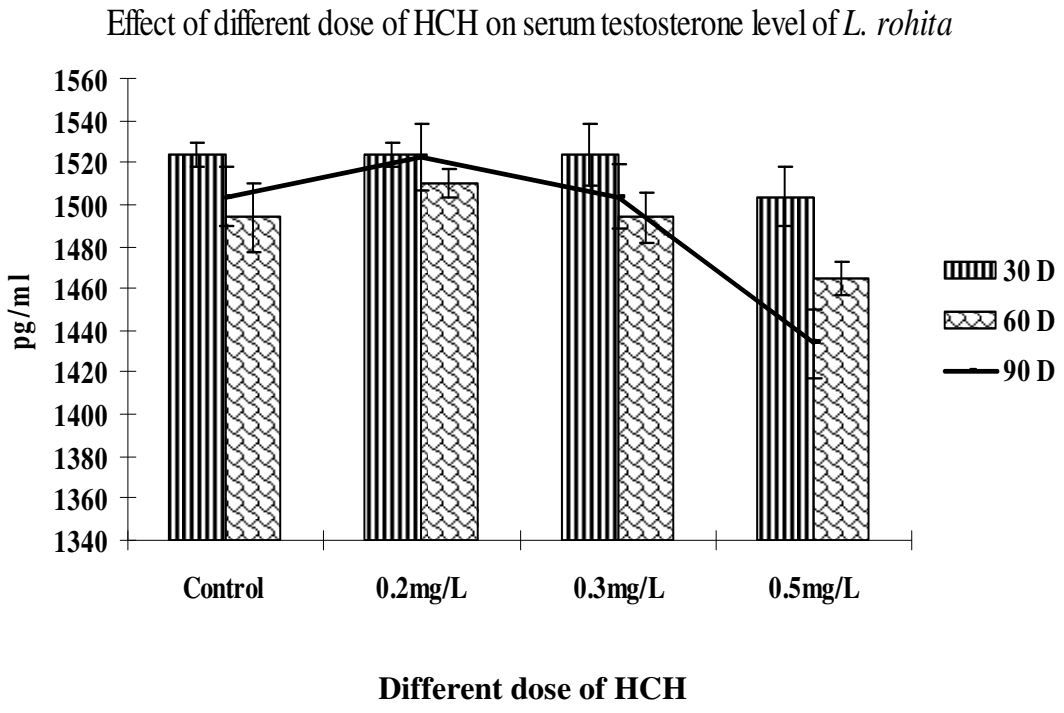


Figure 21: Effect of different dose of HCH on serum testosterone level of *L. rohita*

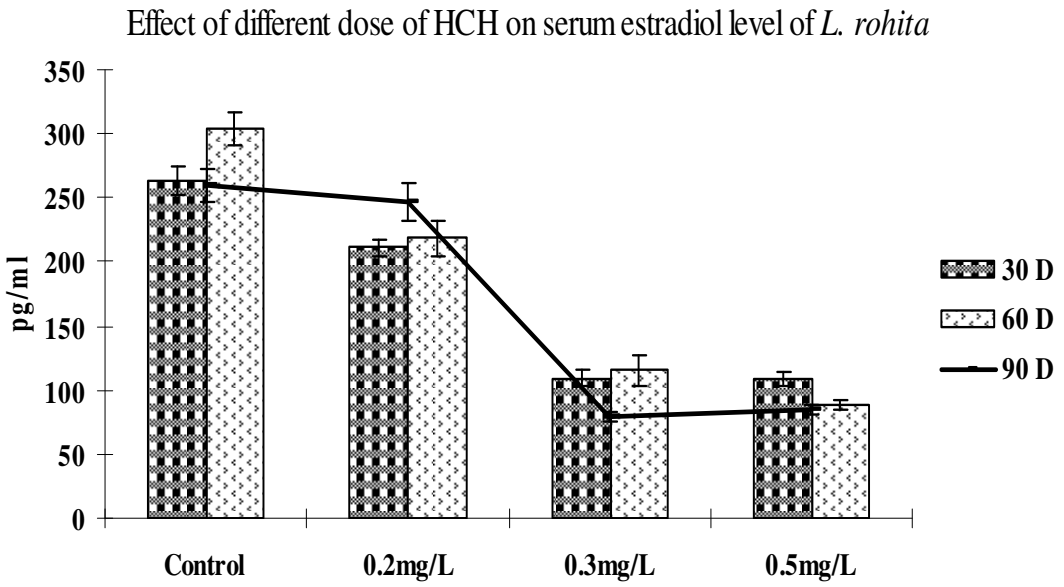


Figure 22: Effect of different dose of HCH on serum estradiol level of *L. rohita*

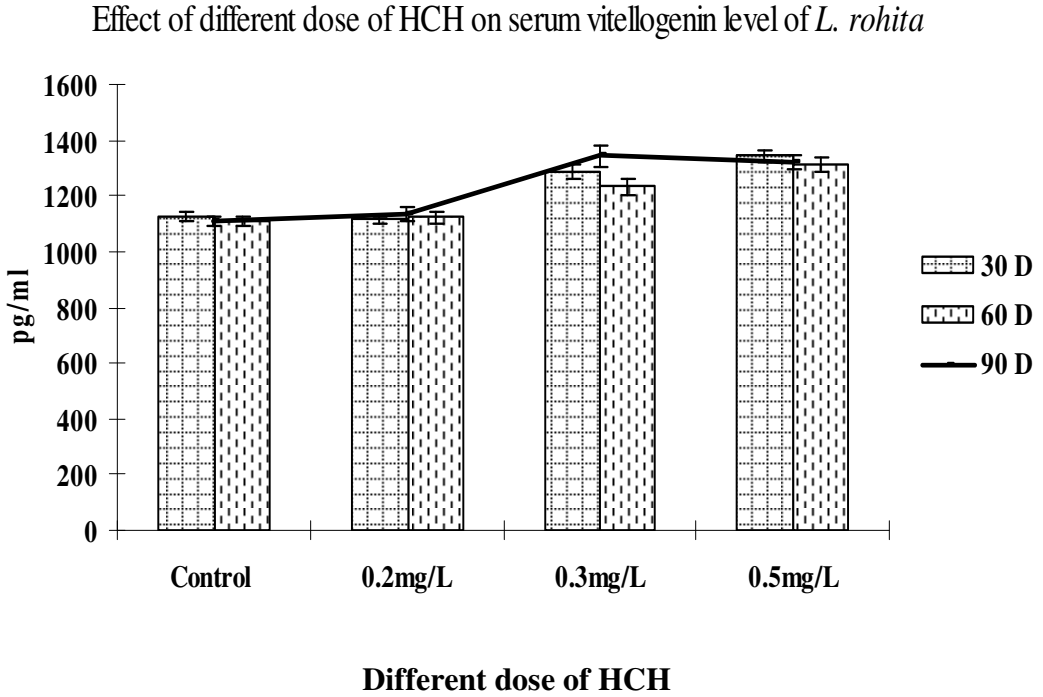


Figure 23: Effect of different dose of HCH on serum vitellogenin level of *L. rohita*

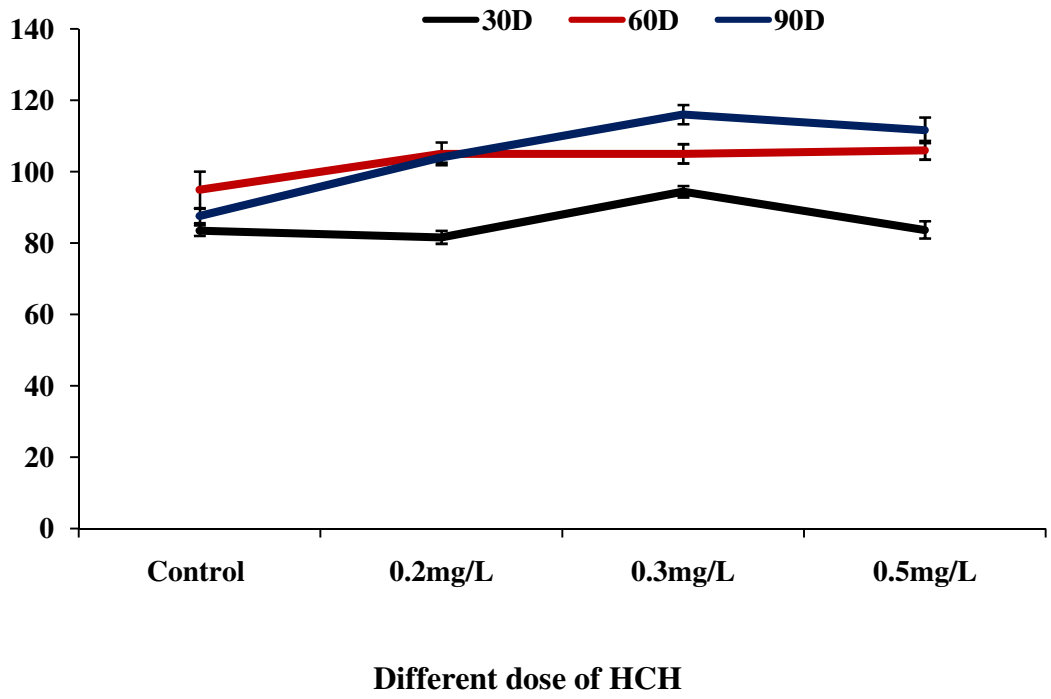


Figure 24: Effect of different dose of HCH on serum aromatase level of *L. rohita*

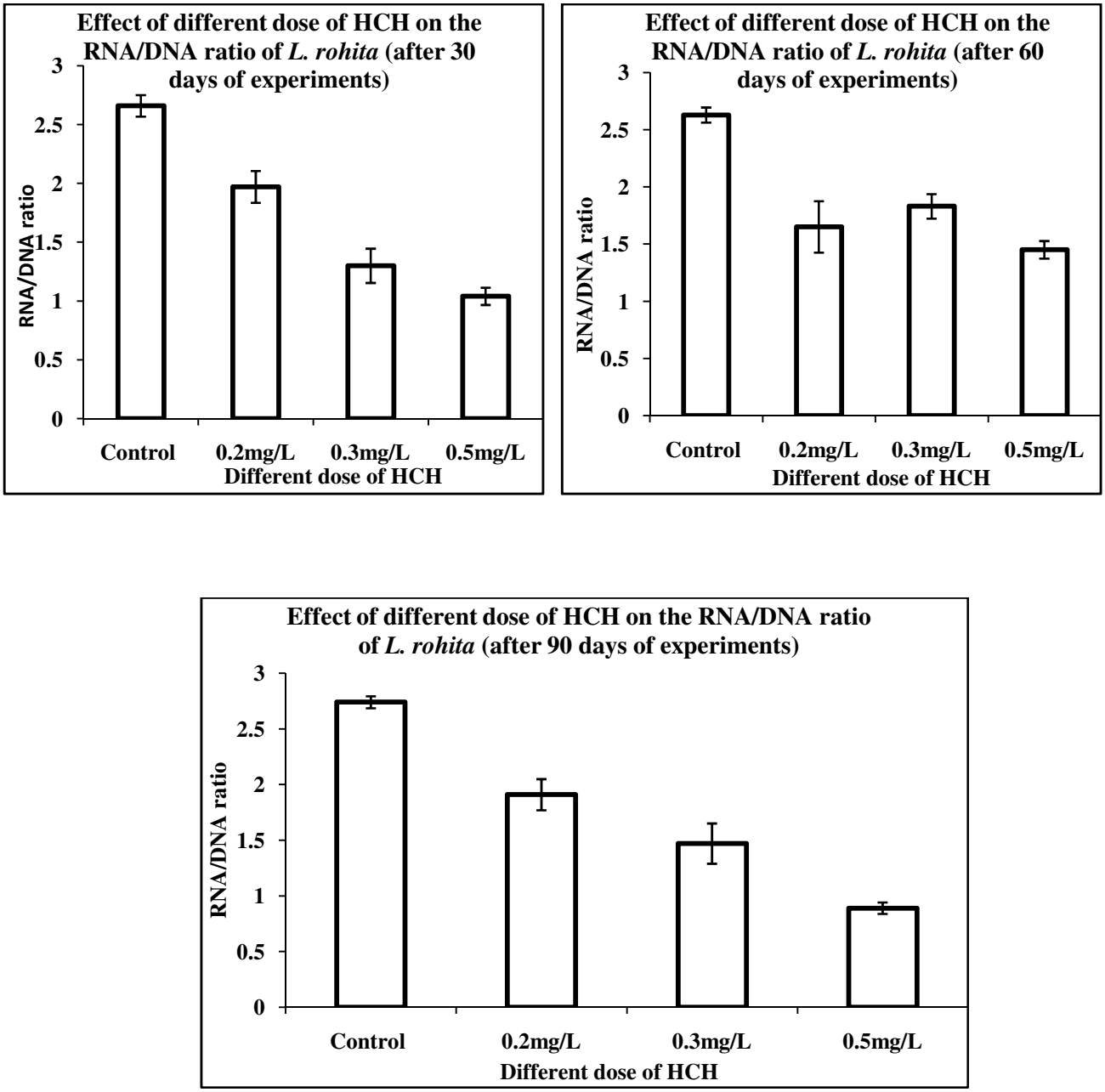


Figure 25: Effect of different dose of HCH on the RNA/DNA ratio of *L. rohita.*

Result- Histological Study

Photomicrograph of developmental stage of ovary during experimental work throughout whole year in control group of *L. rohita* (H&E stain)

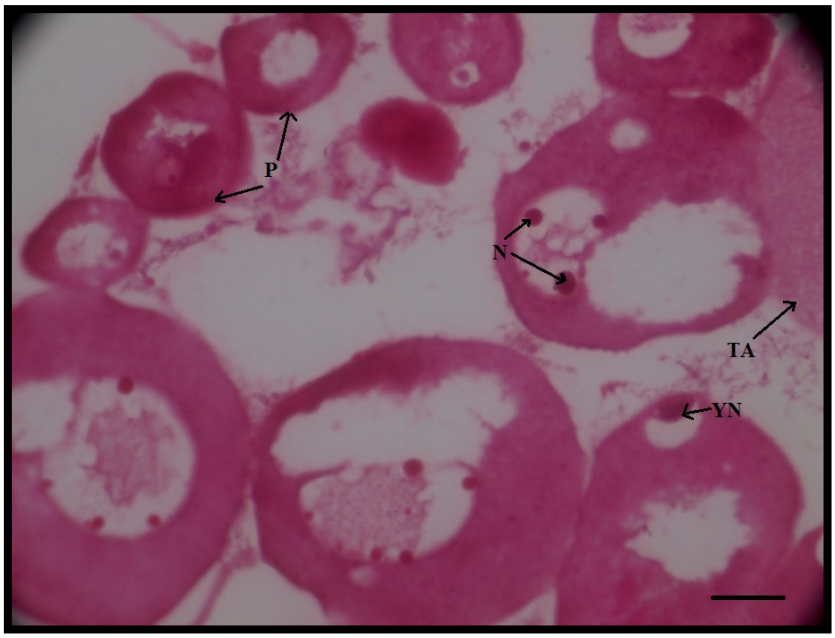


Figure 26. Ovary of *L. rohita* showed perinucleolar stage of oocyte (P) with several small nucleoli (N) attached to nuclear membrane and yolk particles (YP). Bar scale = 20 µm.

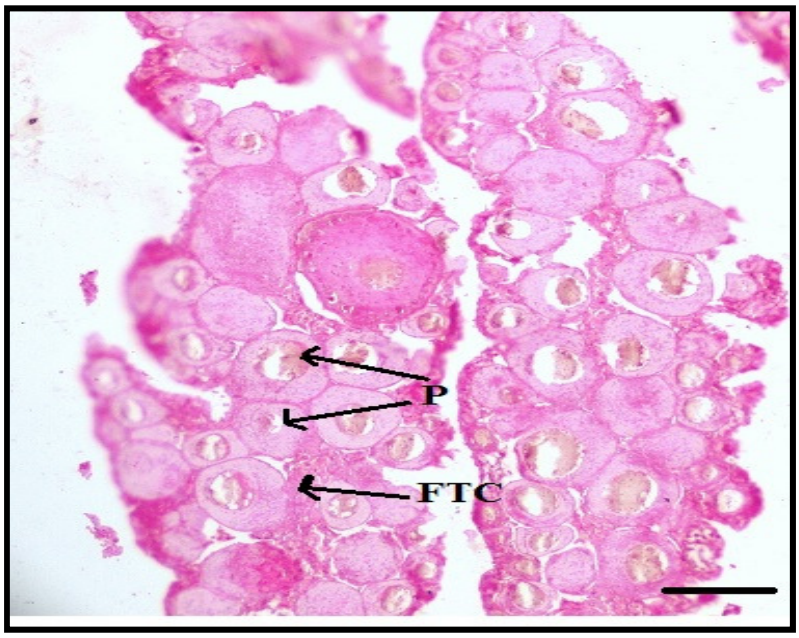


Figure 27. Ovary of *L. rohita* showed perinucleolar stage of oocyte with several oocytes yolk granules (YG) and follicular connective tissue (FTC). Bar scale = 20 µm.

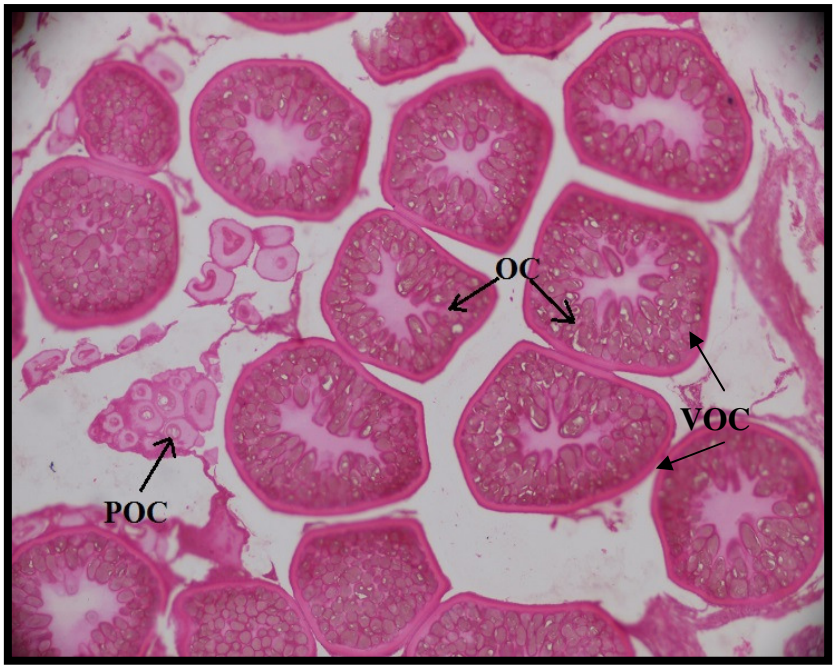


Figure 28. Structure showed ovary with perinucleolar stage of oocyte (POC), vitellogenic oocytes (VOC) filled with yolk granules (YG), follicular monolayer simple with squamous lining. Bar scale = 40 μm.

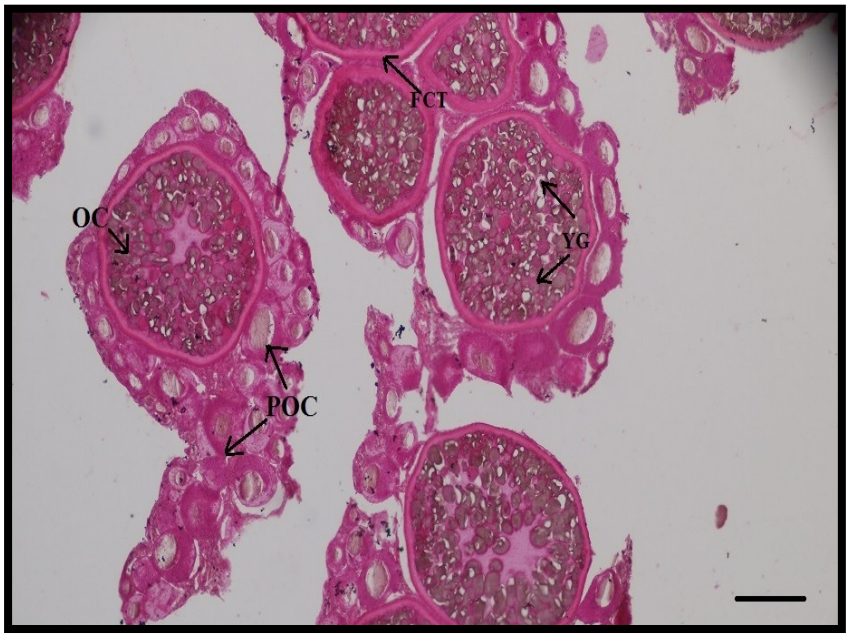


Figure 29. Ovary of *L. rohita* showed ripe stage of oocyte and follicular trilayer consists of thick acidophilic *zona radiata* (ZR), cuboidal follicular cell layer and stratified squamous thecal cell layer. Oil droplets (OD) were also appeared. Bar scale = 40 μm.

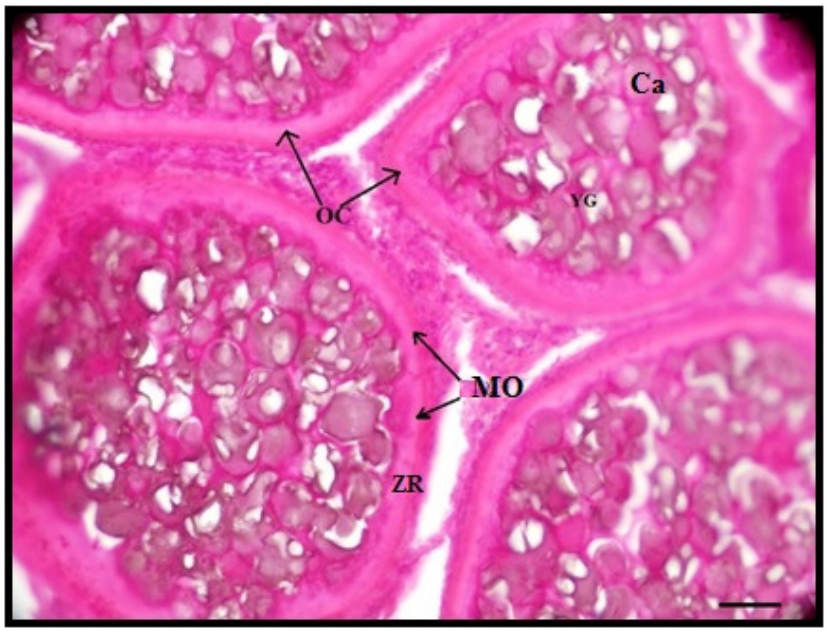


Figure 30. Ovary of *L. rohita* showed vitellogenic oocyte (V) with ripe mature oocyte (MO). The oocytes increased in size, cortical alveoli (Ca) were progressively displaced towards periphery. Bar scale = 40 µm.

Photomicrograph of developmental stage of testis during experimental work throughout all stages of control group of *Labeo rohita* (H&E stain)

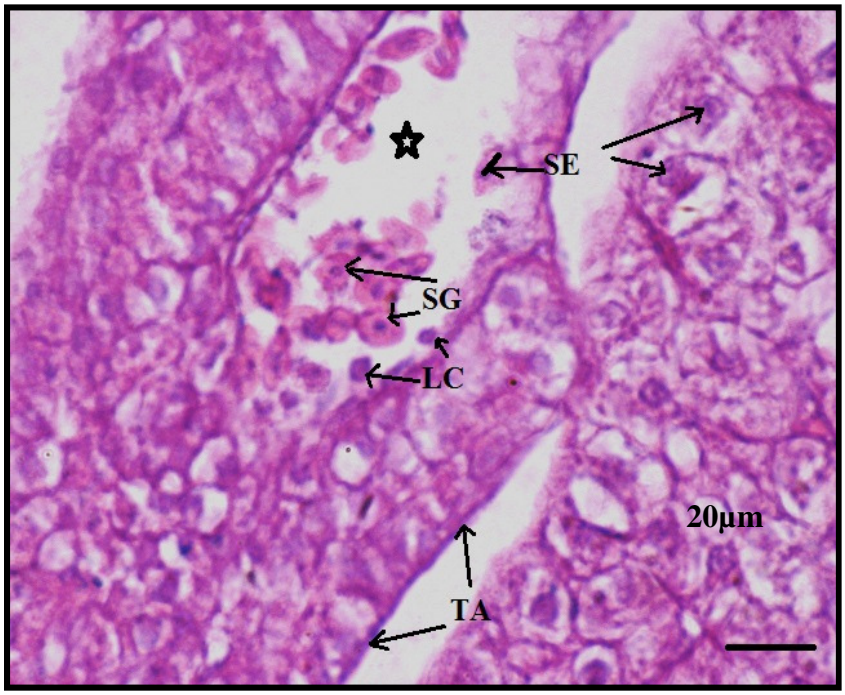


Figure 31. Structure of testes of *L. rohita* showed tunica albugena (TA), primary spermatogonial cell (SG), leyding cell (LC), sertoli cell (SE), astric indicate some lobules were devoid of germ cells. Bar scale = 20µm.

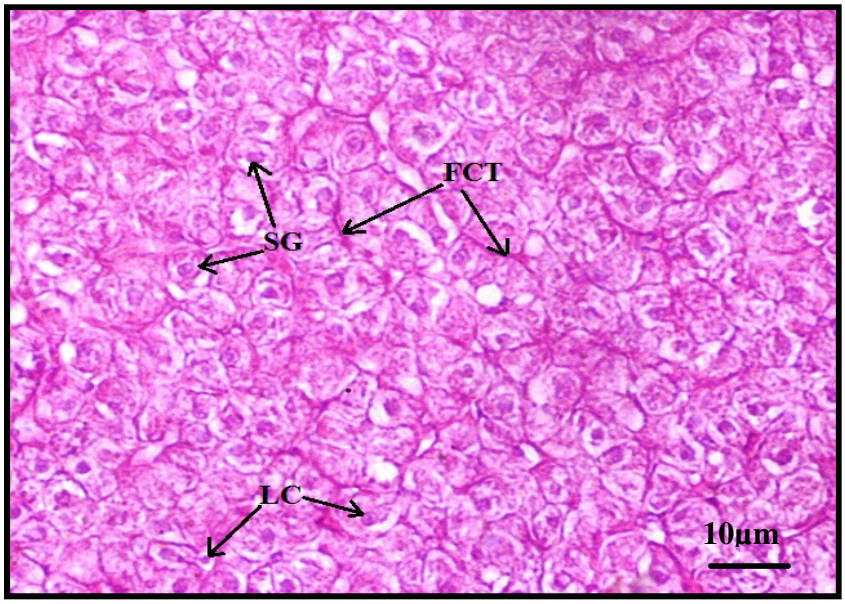


Figure 32. Structure of testes of *L. rohita* showed Spermatogonia cell (Primary and secondary) Fibrous connective tissue (FCT), Leyding cell (LC) Bar scale = 20µm.

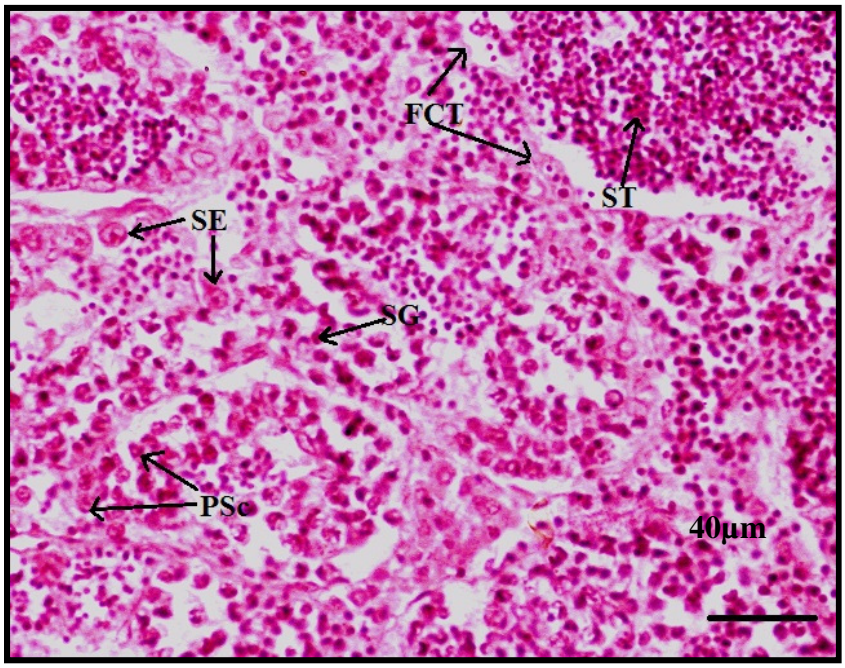


Figure 33. Structure of testes of *L. rohita* showed spermatogonia (SG), covered with fibrous connective tissue (FCT), Sertoli cells (ST) spermatids (ST), and lumen of lobule appeared. Bar scale = 40µm.

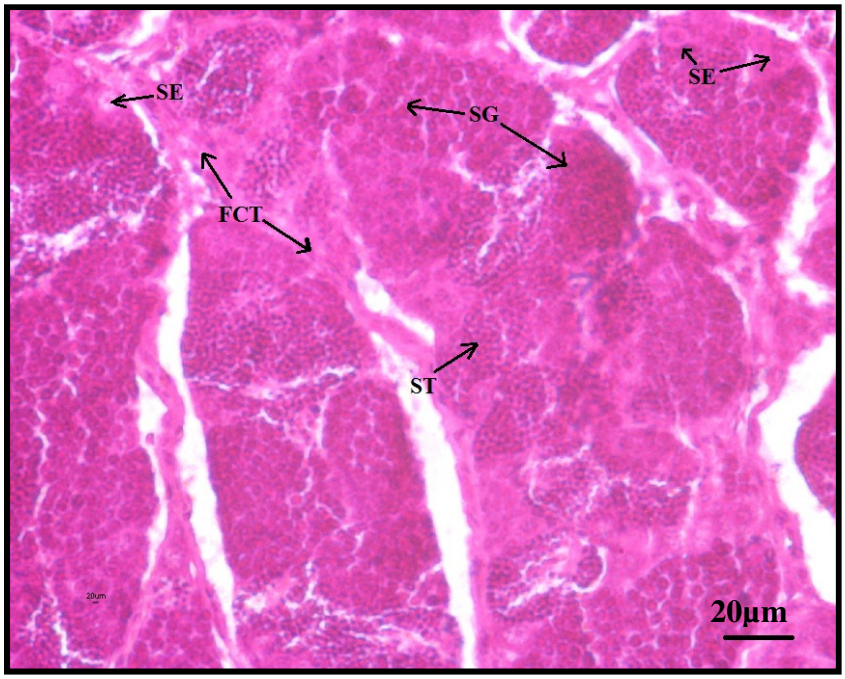


Figure 34. Testes of *L. rohita* showed matured stage with spermatocytes (SC), spermatogonia (SG), Sertoli cell (SE) fibrous connective tissue (FCT), Bar scale = 20µm**.**

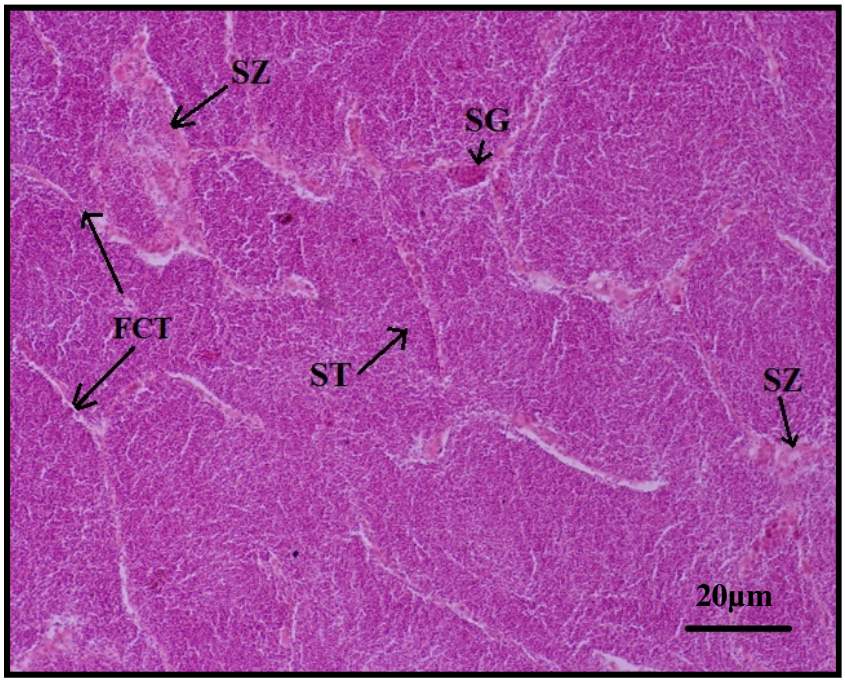


Figure 35. Testes of *L. rohita* at matured stage showed cysts form of spermatocytes (SC), spermatogonia (SG), fibrous connective tissue (FCT), and (SZ) Spermatozoa Bar scale = 20µm**.**

Photomicrographs of Phthalic acid ester (DMP. DBP, DEHP) treated ovary of *L. rohita* (H&E stain).

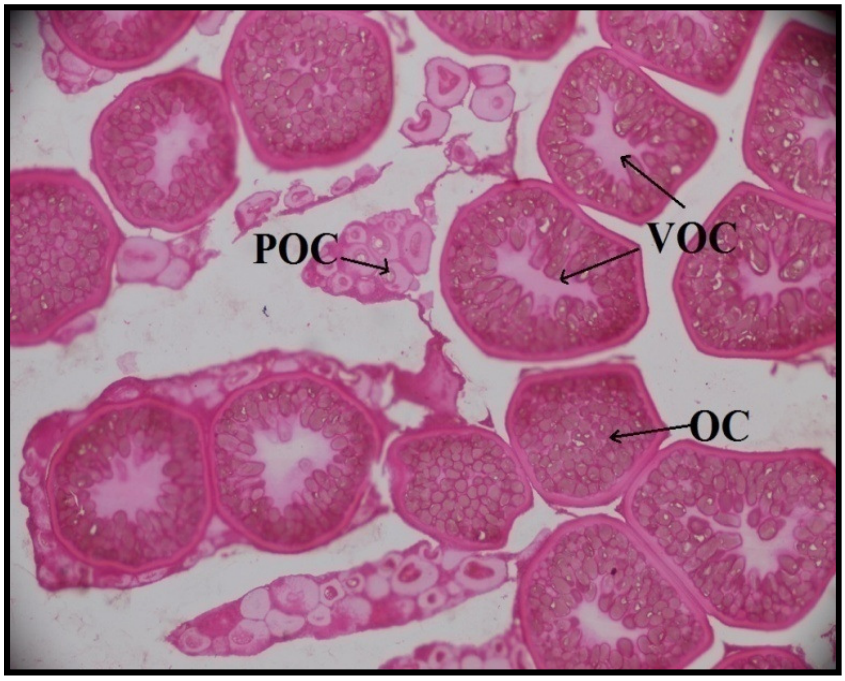


Figure 36. DMP treated ovary of *L. rohita* shows perinucleolar stage of oocyte (POC), Vitellogenic oocytes (VOC) filled with yolk granules (YG) Follicular monolayer having simple squamous lining. Bar scale = 40 µm

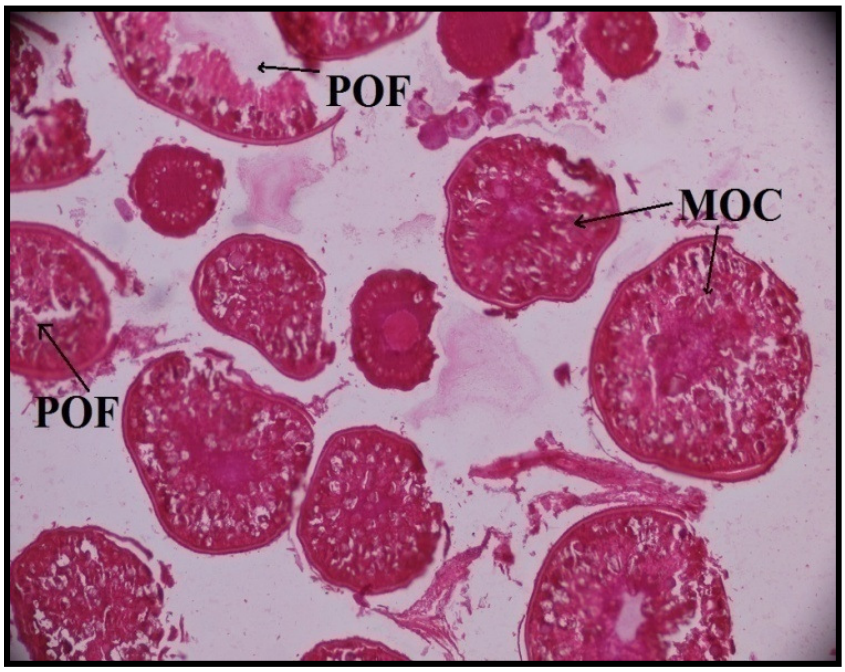


Figure 37. Structure of DBP treated ovary showed post ovulatory follicles (POF) and maturing oocytes (MOC), ova of oocytes were going to decrease and disappearance of the thecal layer of the ripe oocyte is noticed. Bar scale = 20 µm.

Photomicrograph of testes of *L.rohita* treated with phthalic acid ester (DMP, DBP, DEHP) (H&E stain).

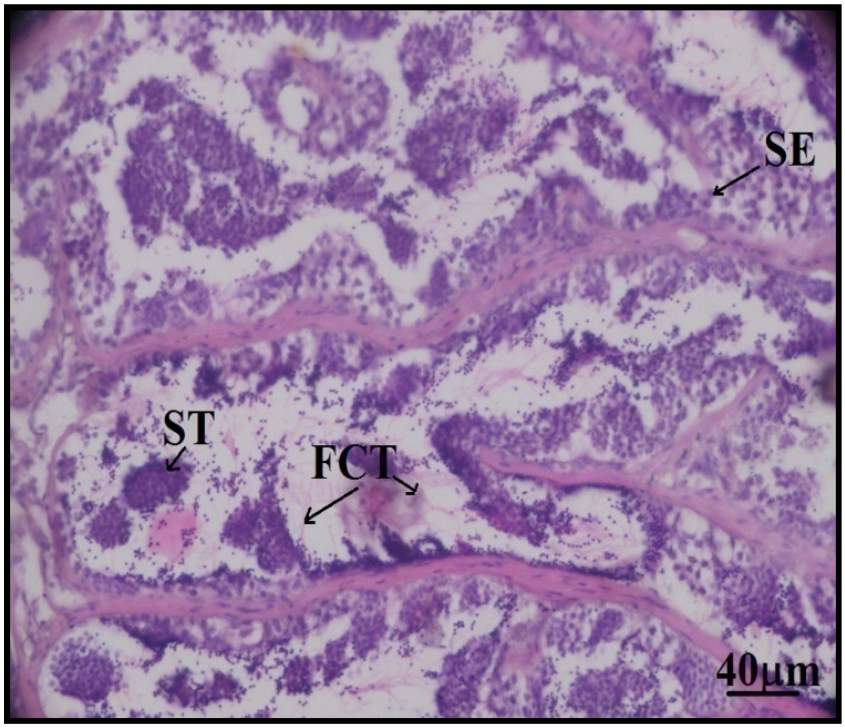


Figure 38. DBP treated testes of *L. rohita* showed bunch of spermatocytes (ST), spermatids (ST) and leyding cells (LD) and degenerated fibrous connective tissue (FCT) and lobules, arrow shows low proportion of spermatozoa. Bar scale = 40µm.

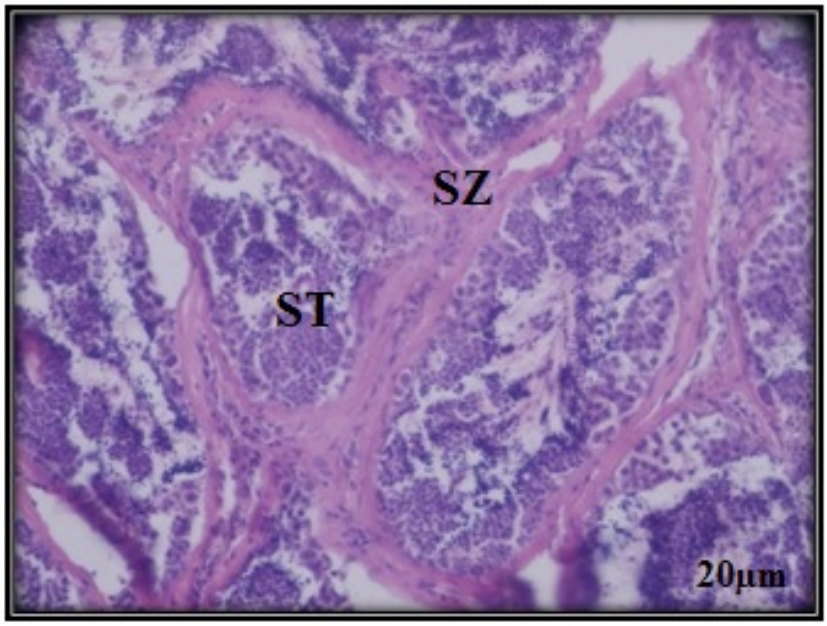


Figure 39. DEHP treated testes of *L. rohita* showed bunch of spermatocytes (ST), and spermatozoa. Lower proportion of spermatozoa. Bar scale = 20µm.

Photomicrograph of Intersex gonad of *L rohita* treated with phthalic acid ester (H&E stain).

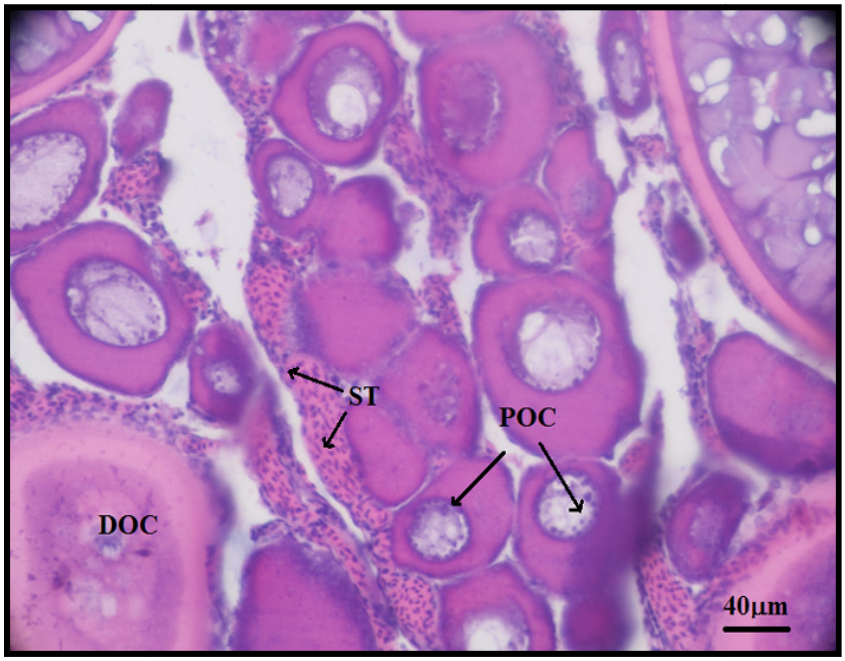


Figure 40. Structure showed mature spermatids with developing ST (Spermatocytes) and developing oocyte (OC) and there are several degenerated oocytes (DOC) H&E X- bar 40 µm.

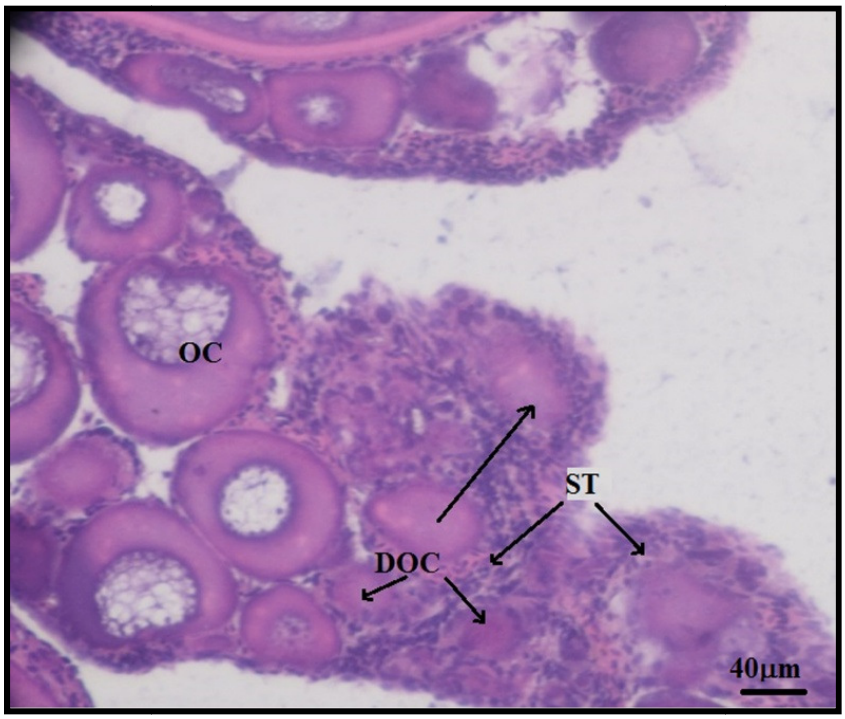


Figure 41. Histological section of ovary of *L. rohita* 0.5mg L^{-1} DEHP treated showing developing primary oocyte (OC), degenerated oocytes (DOC) and developing spermatids (ST). Bar scale = 40 µm.

Photomicrograph of *L. rohita* ovaries showing developmental structure and ovarian interstitial tissues of control group of fish *L rohita* from HCH experiment (H&E stain).

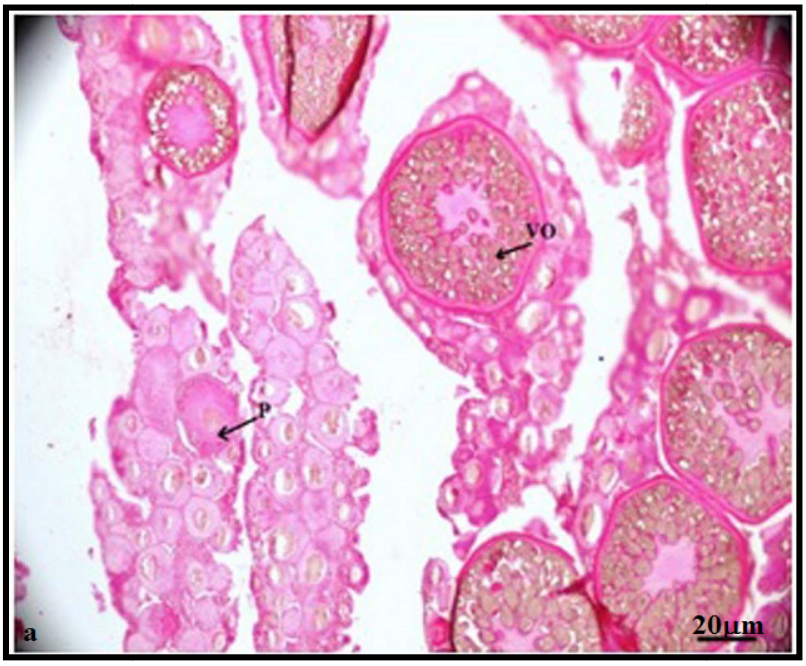


Figure 42. Structure showed ovary of *L. rohita* with vitellogenic oocyte (VO), cortical alveolar oocyte (CA), chromatin nucleolar oocyte I surrounded by Zona radiate (ZR) and Blood capillary (B) Bar scale = 20 µm.

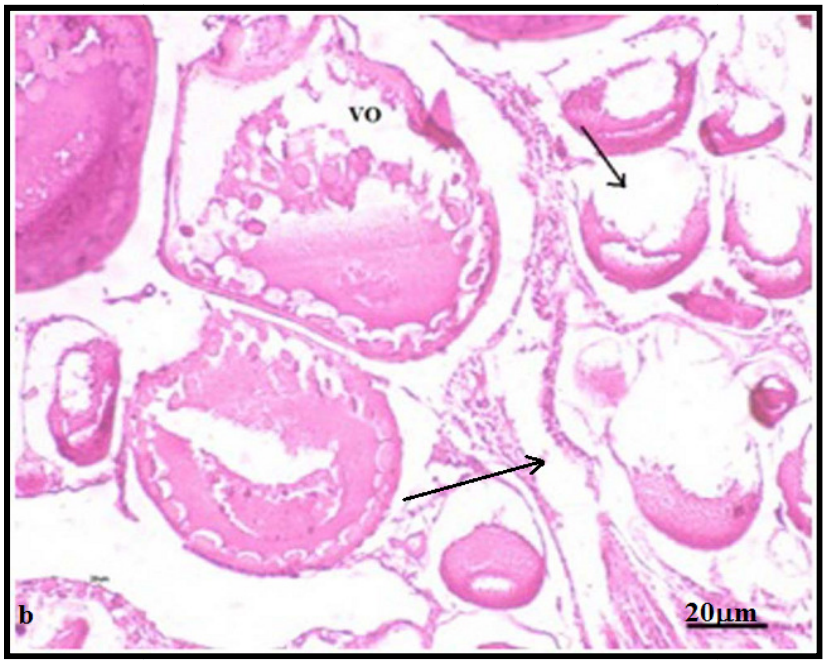


Figure 43. Gonads of HCH treated *L. rohita* showed developing oocytes, arrow showed degenerated follicular connective tissue (FTC), ovules goes to atresia Bar scale = 20µm.

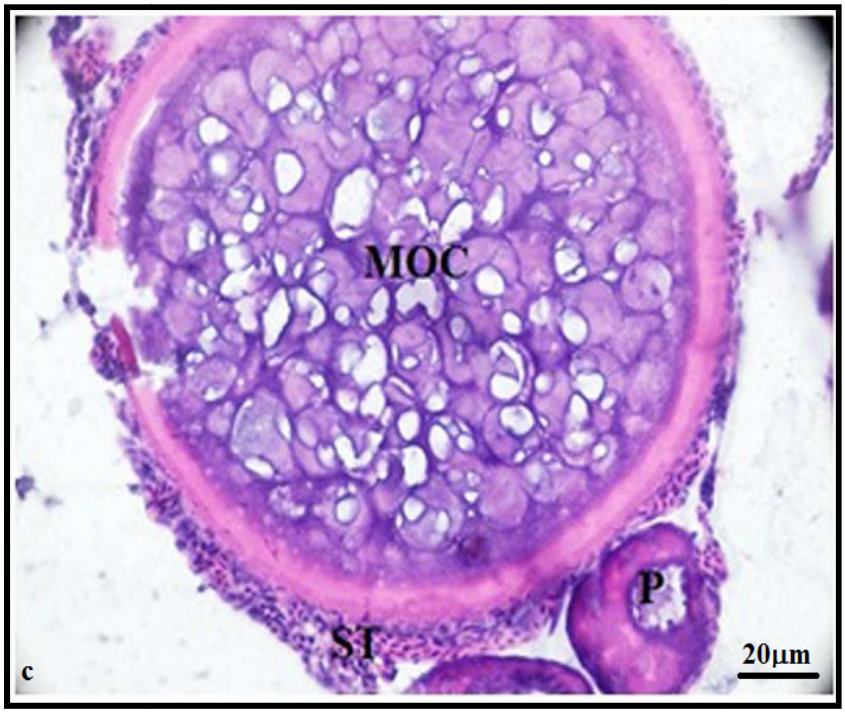


Figure 44. Gonads of HCH treated *L. rohita* showed intersex condition number of spermatids (ST) stage surrounded the mature oocytes (MOC). Bar scale = 20µm.

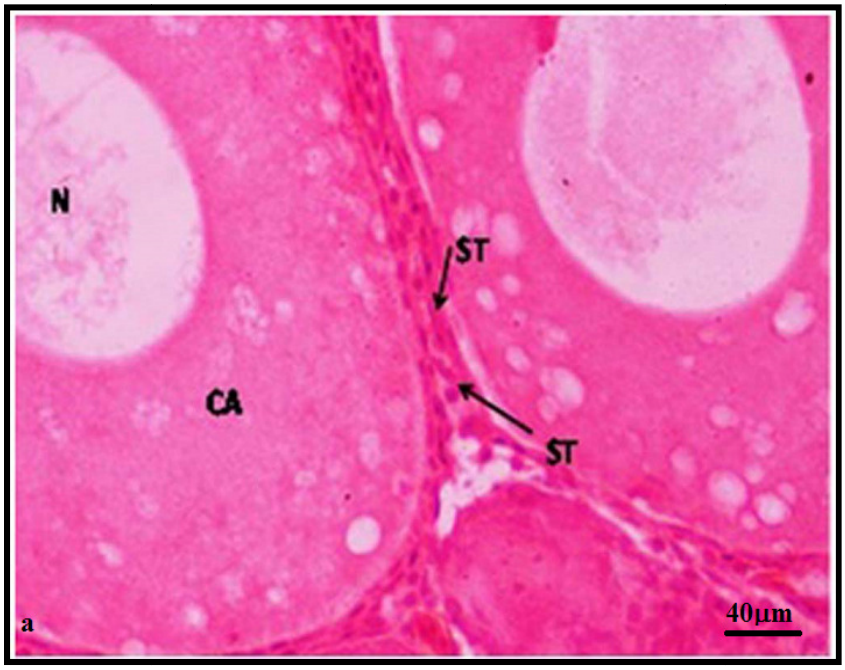


Figure 45. High dose 0.5mg/L HCH treated structure showed intersex gonad spermatids (ST) surrounded the cortical alveolar oocytes (CA) Bar scale =40µm.

Discussion

Effect of Phthalic Acid ester (DMP) on *L. rohita*

Effect of DMP on Gonado somatic index (GSI):

The exposure concentrations used in this experimental group were not expected to cause significant effects on GSI and growth. In our result we found that different dose of DMP (0.2mg/L, 0.3mg/L, 0.5mg/L) did not significantly affect (P>0.05) the weight of *L. rohita*. The results of this experimental group showed that there was no significant difference (P>0.05) in GSI. This finding aligns with other studies where exposure of phthalic acid ester has been reported (Corradetti *et al.* 2013). Similar effects were not observed in male carp collected from waste water, municipal and industrial effluents that have a lower GSI and a higher incidence of gonadal abnormalities (Stansley and Washuta, 2007). Kim *et al.* (2002) reported that zebra fish *Danio ranio* exposed to phthalic acid ester (0.2 and 20 mg/L) for three weeks had significantly higher gonado somatic index in males as compared with the unexposed fish. Similarly, the reduced GSI in male walleyes from the sewage treatment plant has also been reported (Folmar *et al.,* 2001; Barse *et al.,* 2007; Zanotelli *et al.* (2010) further reported that Phthalic acid ester exposed for 90 days had decreasesd body length and weight.

Effect of DMP on biochemical Parameters:

Certain phthalates emerge to be weakly estrogenic as reported by Jobling *et al.* (1995) who investigated the estrogenicity of a variety of phthalate esters including di (2-ethyhexyl) phthalate (DEHP), butyl benzyl phthalate (BBP), and di-n nbutylphthalate (DBP) in an assay to assess the ability of the compounds to inhibit binding of E_2 to the ER in rainbow trout liver. DEHP has been reported to show the lowest affinity for trout ER while DBP has been reported to show higher affinity. Phthalates are the most

ubiquitous man made contaminants in the environment, and that thousands of tons of plastics containing phthalates are disposed annually in landfill sites, enabling these compounds to migrate into surface and ground waters. In our findings we found out that different doses of DMP (0.2mg/L, 0.3mg/L, 0.5mg/L) did not significantly affect (P >0.05) the biochemical parameter in *L. rohita*. We found that there were no significant difference in serum testosterone level, estradiol level, vitellogenin and aromatase level in DMP exposed *L. rohita.* The effect of flutamide on juvenile rainbow trout in a concentration of 25 mg/L has not been reported to show significant affect on E2 level in serum and the authors concluded that anti-androgens do not add effect on estrogens due to different modes of action but these chemicals cause additive inhibiting/stimulating effect on the gonad development (Bhatia, 2014). However, a contradictory reoprt was published by Oishi and Hiraga (1980) showing significant decrease in serum testosterone levels after DMP administered diet at 2% (approximately 2000 mg/kg) for 7 days. Tyler *et al.* (1999) exposed fathead minnows to estradiol-17b in the water (nominal concentrations of 25 ng/L, 50 ng/L, and 100 ng/L) from 24 h post fertilization to 30 d post hatch showing induced Vitellogenin (VTG) synthesis in a dose-dependent manner and also shorter periods of exposure to 100 ng/L estradiol-17b (24 h post fertilization to 10 or 20 d post hatch) similarly showed induced vitellogenic response. Similar result obtained King *et al.* (2008) and Korte *et al.* (2000)

Effect of DMP on RNA/DNA Ratio and gonadal development:

In our findings, we found out that different dose of DMP (0.2mg/L, 0.3mg/L, 0.5mg/L,) did not significantly reduce (P >0.05) the RNA/DNA ratio in the gonads of *L. rohita.* We found that there is no malformation in histology of DMP exposed *L. rohita* gonads. Similar results were reported by Grey *et al.* (2000) where no

malformation in gonadal development and sexual differentiation was shown in DMP exposed rat.

Effect of Phthalic Acid ester (DBP) on *L. rohita*

Effect of DBP on Gonado somatic index (GSI):

Based on several reports in the literature, investigation of potential effects of DBP exposure on GSI, and gonad histology seemed warranted. In our findings, we found that different doses of DBP (0.2mg/L, 0.3mg/L, 0.5mg/L) not significantly affect ($P>0.05$) the weight of *L. Rohita* and no significant difference ($P<0.05$) was observed in GSI (Mandiki *et al.*, 2005). A related finding has been reported by Villeneuve *et al.* (2002), when they exposed 4-Nonylphenol (NP) to common carp. A number of studies on fish have been reported that phthalate may or may not induce effect on GSI and growth of fish after EDCs exposure. But in EDCs exposed *Fathead minnows* tended to have noticeably lower GSI than control, reduced ovarian growth and altered oocyte development and decreased fecundity in females (Baatrup *et al.,* 2001; Brion *et al.,* 2004; Makyen *et al.,* 2000; Mandiki *et al.,* 2005; Rey, 2006). Similar results have been observed by Rebecca *et al.* (2007) when they exposed Fundulus *heteroclitus* to endocrine disrupting chemicals (EDS) at nominal concentrations of 0–100 ng/L for 21 days resulting in higher GSI compared to control fish.

Effect of DBP on biochemical Parameters:

From the present finding it was observed that different doses of DBP (0.2mg/L, 0.3mg/L, 0.5mg/L) had significantly ($P>0.05$) affected the reproductive hormone level. DBP treated fish was analyzed and find out significant ($P<0.5$) reduction in testosterone level and increased estradiol level in serum. Serum aromatase and vitellogenin slightly increased. Similar findings was obtained by Jobling *et al.* (2009),

in this study adult male three-spined sticklebacks (Gasterosteus aculetaus) were exposed to DBP concentration 0, 15, and 35 μg DBP/L for 22 d and analyzed for changes in nesting behavior, androgen concentrations, spiggin concentrations, and steroidogenic gene expression. Plasma testosterone concentrations were significantly privileged in males compared to control. Similarly sexually mature mummichog were alienated by sex and exposed to the estrogen 17-ethynylestradiol (EE2) at nominal concentrations of 0–100 ng/L for 21 days, half of the fish were sampled on Day 21. At 100 ng/L, male fish had induction of VTG increased GSI, decreased testosterone production and decreased circulating 11-ketotestosterone (11-KT). Similarly Zebrafish exposed to flutamide concentration 250mg/kg body weight for 2 week decrease in testosterone level of Asian catfish *Clarias batrachus* (Bottero *et al.,* 2005). Similarly Shilling and William (2000) reported decrease in E2, VTG and P450 content. Female fish had decreased circulating estradiol (E2) and testosterone (T) at 100 ng/L (Rebecca *et al.,* 2007). These studies concluded anti androgenic nature of phthalic acid ester. Christiansen *et al.* (1998) injected immature rainbow trout with 500 mg/kg of either BBP or DBP a pre exposure blood sample was taken on Day 0 followed by a blood sample and sacrifice on Day 9. BBP increased vitellogenin, while the DBP did not increase the vitellogenin concentration above the detection limit. It is interesting to note here that Christiansen *et al.* (1998) did not detect an increase in vitellogenin production in hepatocytes exposed to DBP, however this same compound showed a fairly strong affinity for rainbow trout liver ER (Jobling *et al.,* 1995), possibly signifying an antagonistic potential for DBP. Earlier study reported that the impact of 17-β estradiol to stimulate vitellogenin (VTG) on *Xenopus laevis* (Barnhoorn *et al.,* 2004). Likewise in mammalian study Exposure of DBP/Kg/Body Weight/day on rat showed a significant reduction in weight and circulating

testosterone level (Mitchell *et al.,* 2012) Similarly foetal rat testes exposed 10µM Methyl hexyl phthalate (MEHP) resulted in reduction of testosterone, androstenedione and 5αdihydrotestosterone level (Chauvigne *et al.,* 2011)

Increased vitellogenin (Vtg) levels have been measured in male carp downstream from sewage treatment plants (Stansley and Washuta, 2007). This was followed by many reports that vitellogenin hormone level is a specific marker for assessing the effect of endocrine disrupting chemicals (Cheek *et al*., 2004; Folmar *et al*., 2000; Jobling *et al*., 1999). Moreover reproductive hormone level has been prominent technique to observe the effect of phthalic acid ester on fish reproductively. Divergent result obtained by Labadie and Budzinski (2006), decrease of androgens observed in juvenile turbot and adult common carp (Mandich *et al.,* 2007). In fathead minnows, prochloraz reduces plasma androgen concentrations, suggesting inhibition of cytochrome P450-dependent ezymes (Ankley *et al.,* 2005). But similar result obtained Tributyltin (Cooke, 2002) and androgens such as methyltestosterone (Hornung *et al.,* 2004) are known to hamper aromatase activity in fathead minnow. Similarly several previous studies supported our result that induction of VTG protein after phthalate exposure (Barse *et al.,* 2007; Bhatia *et al.,* 2014).

Effect of DBP on RNA/DNA Ratio of *L. rohita*:

The group of fish treated with DBP showed significant decrease of RNA/DNA ratio in high dose of the experimental period, but in median and low dose there was no significant ($P>0.05$) changes in RNA/DNA ratio in DBP exposed *L. rohita.* A related findings has been described by An *et al.* (2013) they investigated organotin effect and the biological responses of wild veined rapa whelk (*Rapana venosa*) from river and they found that RNA/DNA ratio was significantly lower in females ($p<0.05$), and a

slight increase in DNA damage was also observed in females and imposex individuals as compared to males.

Effect of DBP on gonadal development and structure of *L. rohita*:

Histological analysis of the gonads performed, confirmed that the gonads transformed to intersex. Intersex population was the intermediate form of ovary and testes. Therefore by the observation from the present study the resemblance seen in number of studies reported the induction of testicular oocytes appearance and a significant increase in the frequency of intersex individuals after EDCs exposure (Hayes *et al.,* 2002; Kloas *et al.,* 2009; Mackenzie *et al.,* 2003; Mosconi *et al.,* 2002; Qin *et al.,* 2003). Recently the effect of exposure to 2g/Kg DnBP on sexual differentiation in European pikeperch (*Sander lucioperca*) testicular development was deferred with increase frequency of intersex condition (Jarmolowicz *et al.,* 2013). Exposure to exogenous hormones during larval period can induce complete sex reversal or abnormal gonadal intersex, suggesting that gonadal differentiation in fishes is at risk for disruption by EDCs (Hogan *et al.,* 2008; Khul *et al.,* 2005: Mackenzie *et al.,* 2003; Petterson and Berg, 2007). This study showed significant decrease in spermatozoa in male testes (Figure 14a) exposed to high dose of DBP a relate result observed Lahnsteiner *et al.* (2005) that adverse effects of BPA on sperm quality in brown trout (Salmo trutta f. fario) shows delay in spermiation and decrease in sperm density, motility and velocity in males exposed to BPA at low dose. The present study of histological structure showed that many spermatids surrounded by the ovarian cavity in all group of intersex population (Figure 15a). Developing ovary showed perinucleolar oocytes (POC) Spermatocytes (ST) imposex condition in DBP exposed fish group. Similar findings was mentioned by Stansley and Washuta (2007) that male carp downstream from sewage treatment plants displayed intersex gonads in which

oocytes are present in the testicular tissue. Captivatingly, by histopathological analysis it is apparent that due to endocrine disrupting compound ovatestis developed in medaka (Gronen *et al*., 1999), carp (Jobling *et al.,* 2009). Also, fish exposed to estrogen increased number of atretic follicles in the ovaries and decreased number of vitellogenic follicles (Ven der Ven *et al*., 2003). Following this several other researchers observed intersex (such as testicular oocytes) common in fish on exposure of EDCs (VanAerle *et al.,* 2001; Blazer *et al.,* 2007; Kavanagh *et al.,* 2004; Krisfalusi and Nagler, 2000; Nolan *et al.,* 2001). The gonads affected by EDCs exposure confirmed a higher incidence of oocyte atresia was found in the fish gonadal tissue (Jobling *et al*., 2002; Rasmussen *et al*., 2005). The histological examination showed that the majority of the fish sampled from the contaminated sites had degenerated testes in which the lobular arrangements were disrupted and spermatogenesis was impaired. Similar study was done by Jarmolowiczl *et al.* (2013) and the impact of di-n-butyl phthalate on the development of the reproductive system of European pikeperch (*Sander lucioperca*) at some stage in the sex differentiation period (age 61–96 days post hatch) was observed. A total of 240 fish were divided into 6 groups (40 fish per tank), treatments consisted of a control group (0 g di-n-butyl phthalate·kg-1 feed) and five groups with concentration 0.125, 0.25, 0.5, 1, and 2 g di-nbutyl phthalate·kg-1 feed, respectively. Histological changes of the sex ratio, fish gonads, survival and growth of fish were evaluated. Di-n-butyl phthalate seriously disturbed sex differentiation process of pikeperch. Concluding these findings, histopathological analyses revealed that the administration of 2 g di-n-butyl phthalate·kg-1 significantly affected the sex ratio. The feminization process (intersex gonads) at concentrations of 1 g and 2 g di-n-butyl phthalate·kg-1 were observed. The effect of Di-n-butyl phthalate, an endocrine disrupting compound on the African clawed frog Xenopus

laevis, was studied by Lee and Veerramachaneni (2005) the study revealed that in all DBP treated groups, seminiferous tubule diameter and the average number of germ cell nests per tubule were lesser and the number of no germ in tubules was significantly high. The number of secondary spermatogonial cell nests was significantly reduced. Several lesions occurred in testes including denudation of germ cells, vacuolization of Sertoli cell cytoplasm, thickening of lamina propria of seminiferous tubules and focal lymphocytic infiltration. EDCs exposure was known to reduce fertility and lead to population collapse (Jobling *et al.,* 2002b; Kidd *et al.,* 2007; Nash *et al.,* 2004). Phthalic acid ester inhibits the progression of spermatogenisis by arrest of meiosis oestrogenic chemicals similarly disrupt spermatogenisis in fish and reduced the production of spermatozoa (Sohoni *et al.,* 2001). Similar findings observed in Exposure to 9–70µg/L prochloraz for 2 weeks induced an inhibition of the process of spermatogenesis in rainbow trout (*Onchorynchus mykiss*). In another study Le Gac *et al.* (2001) reported a significant reduction in testicular growth in male rain-bow trout following a 3-week exposure to 30µg/L Octonyl phenol (OP).

Effect of Phthalic acid ester (DEHP) on *L. rohita*

Effect of DEHP on Gonado somatic index (GSI) of *L. rohita*:

In our findings, we found that different doses of DEHP (0.2mg/L, 0.3mg/L, 0.5mg/L) do not significantly affect ($P>0.05$) the weight of *L. rohita*. The results of this study shows that there is no significant difference ($P<0.05$) in GSI at low dose but in high dose there was significant decrease in GSI of DBP exposed *L. rohita*. Similar findings were observed on Medaka (*Oryzias latipes*) adults exposed to DEHP 10, 50, and 100µg/L for 5 days where no significant effects could be observed on GSI (Kim *et al.,* 2002). Eggs exposed to 0.01, 0.1, 1.0, 10.0 µg/L DEHP brought about significantly

reduced female body weight-and gonadosomatic index (Chikae *et al.,* 2004a). Similar results are reported by Zanotelli *et al.* (2010) delineating insignificant difference (P>0.05) in GSI compared to control when Japanese medaka, (*Oryzias latipes*) embryo/larva/newly fertilised eggs were exposed to 0, 0.01, 0.1, 1.0 and 10.0 µg/L bis (2-ethylhexyl) phthalate (DEHP) until hatching time. No dose dependent effects were seen except for decreased body weights in male fish where successful hatching, sex ratio, normal GSI, no mortality and body weight was observed. In another study no alternations in GSI of fish exposed to BPA (0.6–11 or 0.2–20 µg/L), VZ (100–800 µg/L) and DEHP (1–100 µg/L) was found (Hatef *et al.,* 2012a, b, c, d). Elzeinova *et al.* (2008) also observed decrease GSI in mice due to the exposure of EDCs. In contrast, the study conducted by Hatef *et al.* (2012b, c) confirmed higher potency of E2 to altered GSI. Similar results are reported by other authors when exposed fish in the adult stage, had higher mortality rates and male body weights were lower for those individuals hatched from eggs exposed to DEHP, but no effects were seen in female body weights or the gonadosomatic index (GSI) of either sex (Chikae *et al.,* 2004a). Further study showed reduction in female body weights at 0.1, 1.0, and 10.0 µg/L and the GSI was reduced in males at 0.01 – 10 µg/L (Chikae *et al.,* 2004b). In another study in female medaka under chronic exposure conditions of 10 – 50 µg/L for 3 months there was high variability in GSI (Kim *et al.,* 2002). Diverse results have been documented by many authors after exposure of estrogenic and anti-androgenic contaminants in the laboratory conditions and also at contaminated sites (Ankley *et al.,* 2001; Bjorkblom *et al.,* 2009; Panter *et al.,* 2002b; Sepulveda *et al.,* 2002). Related findings was reported by Rey (2006) showed that the EDCs exposed fish *Fatherad minnows* tended to have noticeably lower GSI than control. In another study authors observed reduction in GSI of fish (VanAerle *et al.,* 2001; Makynen *et al.,*

2000). Comparative study was done by Vito *et al.* (2010) dose-dependent DEHP inhibited growth. The EDCs exposed fish fathead minnows tended to have noticeably lower GSI than control, and there was reduced ovarian growth, altered oocyte development and decreased fecundity in females (Brion *et al.,* 2004; Mandiki *et al.,* 2005; Rey, 2006). GSI of male walleye from the STP effluent channel in two field seasons were not significantly different (P>0.05) when compared to the pooled sample of fish from the effluent channel to fish from the reference site (Mandiki *et al.,* 2005). Analogous results was reported by Villeneuve *et al.* (2002) exposed 4-Nonylphenol (NP) to common carp and there was no significant difference (P>0.05) in GSI compared to control.

Effect of DEHP on biochemical Parameters:

In the present study, we find out that different doses of DEHP (0.2mg/L, 0.3mg/L, 0.5mg/L) significantly (P>0.05) affected the reproductive hormone level. DEHP treated fish was analyzed and found significant (P<0.5) reduction in testosterone level and increased estradiol level (P<0.05) in serum and also significantly increased serum aromatase and vitellogenin level. Similarly Mimeault *et al.* (2006) also reported significant decreases in the levels of plasma testosterone in goldfish after 14 days exposure to 1.5mg/L gemfibrozil. Related findings observed by Hatef *et al.* (2011) reported disruption of male reproductive physiology in goldfish exposed to environmentally relevant concentrations of BPA via alternations of androgens T and 11-KT. Our results in agreement with a considerable amount of evidence from the literature showing that phthalic acid ester particularly DEHP induced the reproductive hormone level. Alike Velasco-Santamaria *et al.* (2011) studied exposure of Bezafibrate to male zebrafish at a concentration of 70mg/g for 21 days and showed significant decreased in plasma 11- Keto-Testosterone level. Chang *et al.* (2011)

measured plasma T and E2 levels were significantly decreased in females at 100µg/L butachlor and a significantly elevated level of VTG in males was seen in 30 days of exposure. but dissimilar result revaled by Kuiper *et al.* (2008) they reported for polybrominated diphenyl ethers (PBDEs) which produced a significant dose-dependent increase in circulating T3 and T4 levels in zebrafish. Recent result was obtained by Xu *et al.,* 2013 that DEHP groups concentration were 0.1, 1.0, 10.0, 100.0 micromol/L investigate the effects of DEHP in testosterone synthesis and the related genes expression in the fetal testis of male mouse by organ culture *in vitro* compared to the control group, the levels of testosterone synthesis in 0.1, 1.0, 10.0 micromole/L groups were increased, but decreased in 100.0 micromole/ L group which were exposed in DEHP for 48 hours and 72 hours. However, effects of 30-day dietary (pre-pubertal) exposure to different doses (0 (control), 1, 10, 50, 200 and 400 mg/kg bodyweight/day) of di (n-butyl) phthalate (DBP) on plasma testosterone level the non-significant changes observed for plasma testosterone levels and histology (Bello *et al.,* 2014). Similar findings in the African catfish (*Clarias gariepinus*) treatments with androgens inhibited C17–20 lyase activity in the testis (Cavaco *et al.,* 2001; Schulz and Miura, 2002), and MT depressed the in vitro production of T in the testis of the mummichog (Sharpe *et al.,* 2004).

Vitellogenin level mainly in immature females and males is considered a useful biomarker of exposure to estrogenic compounds in aquatic environments (Blaise *et al.,* 2003; Gagne *et al.,* 2002; Marin and Matozzo, 2004; Vandenbergh and Janssen, 2001). Exposure of E2 mimics increase in the number of estrogen receptor as well as the induced the synthesis of Vitellogenin hormone (Andersen *et al.,* 2003; Bowman *et al.,* 2002) leading to an increase in oestrogen production that consequently induces VTG accumulation in the liver and bloodstream (Folmar *et al.,* 2001; Hayes *et al.,*

2006; Jobling and Tyler, 2006; Korte *et al.,* 2000; Nadzialek *et al.,* 2011; Wingfield and Mukai, 2009). For instance, the phospholipoglycoprotein vitellogenin, which is an egg yolk precursor in oviparous organism (including most fishes), is usually only present in minute or undetectable quantities in males but exposure to EEDCs leads to the induction of hepatic VTG gene and protein expression in male fish (Sumpter and Jobling, 1995; Van der Ven *et al.,* 2007). An induction of VTG production was noted in adult male sticklebacks in response to EE2 exposure at nominal concentration of 20 ng/l in adult zebrafish at nominal concentrations of 1.6 ng/l (Fenske *et al.,* 2001), and fathead minnow at nominal concentrations of 5 ng/l EE2 (Panter *et al.,* 2002). Ye *et al.* (2014) studied on Marine medaka larvae (*Oryzias melastigma*) were exposed to either DEHP (0.1 and 0.5 mg/L) or MEHP (0.1 and 0.5 mg/L) nominal values for 6 months DMSO was used as carrier solvent (0.1%) the effects on reproduction, sex steroid hormones, liver vitellogenin (VTG), gonad histology and the expression of genes involved in the hypothalamic-pituitary-gonad (HPG) axis were investigated. A significant increase in plasma 17β-estradiol (E2) along with a significant decrease in testosterone (T)/E2 ratios was observed in males, Increased concentrations of T and E2 were observed in females, The liver VTG level was significantly increased after DEHP and MEHP exposure in males. Similar findings supported by many authors that exposure of EDCs many estrogenic and androgenic chemicals inclined the Serum Vitellogenin hormone level in fish. (Ankley and Johnson, 2004; Desforges *et al.,* 2010; Folmar *et al.,* 2001; Jin *et al.,* 2008; King and Hassell, 2008; Liu *et al.,* 2009; Martyniuk *et al.,* 2007; Menuet *et al.,* 2004; Mills and Chichester, 2005; Nadzialek *et al.,* 2011).

There are several authors who worked on the expression level of CYP 19 gene after exposed endocrine disrupting compound and found the CYP 19 mRNA level tend to

increase (Kalliveretaki *et al.,* 2006; Kazeto *et al.,* 2004; Montserrat *et al.,* 2004). They may induce an increase in the expression and activity of P450 aromatase. Wang *et al.* (2013) was studied on adult Chinese rare minnow (*Gobiocypris rarus*) were exposed to DEHP concentration 0 µg/L, 3.6µg/L, 12.8 µg/L, 39.4 µg/L, and 117.6 µg/L for 21-days. Exposure of DEHP resulted in significant higher concentration of testosterone (T) and lower concentrations of estradiol (E2), similar result obtained that in male increases of T and E2 levels were consistent through up-regulation of Cyp17 and Cyp19a in the gonads. Moreover, the T/E2 ratio was significantly increased in females but reduced in males; significant increase in the levels of hepatic vitellogenin (VTG) gene transcription was observed in both females and males caused a significant decrease of E2 and an increased T/E2 ratio in females but a significant increase of E2 and decreased T/E2 ratio in males. Ye *et al.* (2014) observed a significant increase in plasma 17β- estradiol (E2) along with a significant diminish in testosterone (T)/E2 ratio (to less than 1/3 of control ratio) in male *O. melastigma* (LOEC 0.1 mg/L). A significant decrease in testosterone (T)/E2 ratios was also observed in males. Increased concentrations of T and E2 were observed in females. Han *et al.* (2009) studied the effect of DEHP concentration 5.5 to 20.5mg/L on sex hormone level in common carp (*Cyprinus carpio*) observed induction in Vitellogenin but reduction in Vitellogenin also seen in several studies (Carnevali *et al.,* 2010; Caunter *et al.,* 2004; Kim *et al.,* 2002; Wang *et al.,* 2013; Ye *et al.,* 2014) Caunter *et al.* (2004) observed increased Vtg concentrations in F2 female Fathead minnow exposed to 5 µg/l DEHP in water and 500 mg/kg in food for three generations. Also male fish from the same study had increased Vtg concentrations although not significant using conservative statistics. Vtg was analyzed semi-quantitatively in Japanese medaka (*Oryziaz latipes*) by Kim *et al.* (2002) after a chronic exposure from

1 or 2 days post-hatch (dph) until the age of 3 months to the nominal concentrations of 1, 10 or 50 µg DEHP/L. Carnevali *et al.* (2010) reported increase of plasma Vitellogenin levels in female zebrafish (*Danio rerio*) exposed to DEHP concentration 0.02, 0.2, 2, 20 and 40 mg/l for three weeks. Aromatase enzyme is the potential target of environmental contamination (Kallivretaki *et al.,* 2006). It is likely that EDCs affect reproduction either by disrupting the synthesis or degradation of endogenous hormones or by directly activating steroid hormone receptor-mediated gene expression (Larkin *et al.,* 2003). Comparable result obtained by Ankley *et al.* (2002) accumulation of sperm in testes has been caused by aromatase inhibitors in fathead minnow (*Pimephales promelas*), which may be referred to increased levels of androgen steroids in plasma Kinnberg *et al.* (2003) in guppies (*Poecilia reticulata*). In male Japanese medaka, brain aromatase activity augmented due to 10 day exposure to E2 (Melo and Ramsdell, 2001) Similarly high ovarian and brain aromatase activity has been reported in female mosquitofish exposed to paper mill effluents (Orlando *et al.,* 2002) Nevertheless Short time exposure to high concentrations of MT (µg/L range) decreased the aromatase activity in male Japanese medaka (Melo *et al.,* 1999) and fathead minnow (Hornung *et al.,* 2004).

Effect of DEHP on RNA/DNA Ratio of *L. rohita*

The group of fish treated with DEHP showed significant decrease of RNA/DNA ratio in high dose of the experimental period but in median and low dose there was no significant (P>0.05) changes in RNA/DNA ratio in DEHP exposed *L. rohita.* Similarly three juvenile fish species: the red drum (*Sciaenops ocellatus*), the orange-spotted grouper (*Epinephelus coioides*) and the marine medaka (*Oryzia melastigma*) were subjected to long-term (i) food deprivation, (ii) exposure to benzo[a]pyrene, (iii) hypoxia, respectively. No significant change in RNA: DNA ratio was detected for

both fed and unfed groups throughout the experimental period (Cheung *et al.,* 2007). The RNA:DNA ratio was most sensitive, and decreased significantly (by 50 to 86%) following exposure to 4.5 and 3.5 mg O2 l–1 low oxygen level for 1 week this study disclosed negative impact on growth due to decrease in RNA/DNA ratio (Zhou *et al.,* 2001). Similar study done on common carp, *Cyprinus carpio* under hypoxia and found that the RNA: DNA ratios were correlated with reduced growth. The common carp were exposed to 0.11–1.1 g of the pentabromo-substituted diphenyl ether (BDE-47) mg−1. By contrast, both DNA levels and RNA: DNA ratios were not affected (Gardestrom *et al.,* 2006). RNA/DNA might be a more sensitive parameter for growth development (Gorokhova, 2005; Gorokhova and Kyle, 2002; Rosa and Nunes, 2003). Ibiam and Grant (2005) found that RNA: DNA measurements more sensitive then acute toxicity in nematodes. In addition, they found it to be equally or more sensitive growth-related toxicity. Also, Yang *et al.* (2002) showed that the RNA: DNA ratio was equally sensitive as an endpoint as the growth in the micro algae Skeletonema costatum. Muscle RNA/DNA ratio were shown and compared with the control, intestine RNA/DNA ratio was significantly decreased ($P<0.05$) with a decreasing rate at 48.01%. Li *et al.* (2010) reported in fish, energy metabolism is allied to various environmental stress factors including toxicant exposure. Therefore, RNA to DNA ratio could be used as a possible biomarker for evaluating the environmental pollutants, but not be confirmed. Some studies supported the application of RNA/DNA ratio to evaluate the effects of toxicants on fish because they found a depressed RNA/ DNA ratio in fishes exposed to metal and organic contaminants (James and Sampath 1999; Kim and Kang 2004).

Raksheskar (2012) studied on the impact of sublethal concentration of endocrine disrupting compound (Cypermethrin) exposed to fresh water fish. The RNA/DNA

ratio and DNA, RNA were estimated in gill of freshwater fish *Channa striata*. The sublethal concentration of Cypermethrin (0.00078 µl/lit) for 24, 48, 72 and 96 hours of different time intervals. The concentration of Cypermethrin demonstrated reduce level of DNA as 6.79, 15.77, 15.66, 15.36 in experimental and 15.20, 14.11, 12.16, 10.16 respectively for experimental RNA at different exposure period. Whereas the RNA/DNA ratio changed 2.23, 2.48, 2.27, 1.97 at different time interval respectively as compares to control group. Moreover, Tripathi *et al.* (2000) reported that inhibitory effects of fenvalarate on DNA, RNA and protein content show extreme toxicity of pyrethoidal compound on the main biochemical machinery of the fresh water catfish, *Clarias batrachus*. Triphati *et al.* (2001) was investigated the effect of sublethal concentration of two different kinds of pesticides Fenvalerate (synthetic pyrethroid) and Monocrotophos (organophosphate) for 24, 48, 72 and 96 hrs were observed on DNA and RNA contents in particular tissues like gills, liver, kidney and muscle of freshwater fish *Punctius arenatus* (Day) The decrease of DNA and RNA content was found to be significant statistically at $P < 0.01$ or $P < 0.001$. The level of DNA and RNA was found to be reduced in the gills, liver, kidney and muscle of *Punctius arenatus* (Day) due to period of exposure and different concentration of two different pesticides. Pesticide toxicity indicates alteration in nucleic acid synthesis (Nutan *et al.,* 2010). Tripathi *et al.* (2003) also reported that fish exposed to Dimethoate (organophosphate) exhibited a decrease in nucleic acid (DNA and RNA) content. Similar study done by Triphati *et al.* (2002) the effects of sublethal concentration of Fenvalerate on DNA, RNA, RNA/DNA ratio and protein contents were estimated in gill and kidney tissues of *Clarias batrachus*. Fenvalerate reduced the DNA content in gills, whereas it does not produce any significant effect on DNA in kidney.

1.4 Effect of DEHP on gonadal development and structure of *L. rohita*:

In DEHP treatment group histological observation indicated clear cut difference from the control group. Histological analysis of the gonads confirmed that the gonads visually identified as intersex, Intersex population was the intermediate form of ovary and testes. Histological structure showed many spermatids surrounded by the ovarian cavity in all group of intersex population. Developing ovary shows perinucleolar oocytes (POC) Spermatocytes (ST) imposex condition in DEHP exposed fish group. Similar result have been shown in salmon (*Salmo salar*), exposure to 1500 mg DEHP kg in the food for a for four months during early life resulted in a small incidence of intersex (Norman *et al.,* 2007). Endocrine disrupting compound stimulates the production of VTG and eggshell Zr protein by the liver of female fish (Arukwe and Goksoyr, 2003) there are so many workers reported that in lower vertebrates estrogen directly influence a number of development and reproductive events such as hepatic vitellogenisis, egg member protein synthesis, oocyte growth germ cell development, gonadal sex determination (Li *et al.,* 2008; Nori, 2004; Pablo *et al.,* 2005). Salmon (*Salmo salar*) fry exposure of DEHP concentration 16.5 mg/kg body weight result Intersex fish (Norman *et al.,* 2007).

However, a series of chemical have been reported to have potential to directly or indirectly disrupt the hormonal level of fish by interrupting with the regulation of CYP 19 gene expression either in vivo and in vitro (Kalliveretaki *et al*., 2006). The abnormal sexual development such as compromised reproductive function and characteristic may be due to the induction of aromatase by EDCs (Larkin *et al*., 2003). Similarly abnormal level of circulating steroid hormones have been reported in fish exposed to pulp and paper mill effluents (Guillette *et al*., 1994). Estradiol (17β) also stimulate the aromatase activity in Black porgy and increase estradiol and vitellogenin hormone level in serum by the exposure of estrogen 17β Estradiol (Chang and Lin,

1998). The exposures of ortho-para dichlorodiphenyltrichloroethane (op- DDT) induced the sex reversal in Medaka and also find the higher brain aromatase activity (Kuhl and Brouwer, 2006). Ovatestis abnormality by EDCs exposure have been reported in medaka (Gronen *et al*., 1999) and Carp (Gimeno *et al*., 1994, 1997) Arukwe (2001) reported related study with the exposure of high concentration of synthetic environmental estrogen reproductive and developmental problem like feminization of male, masculinization of female and lower fertility. These abnormalities Bruna *et al.* (2013) was evaluated the effects of environmentally relevant concentrations of DEHP 0.2 and 20 mg/L on the reproductive physiology of adult male zebrafish (*Danio rerio*) DEHP impaired reproduction in zebrafish by inducing a mitotic arrest during spermatogenesis, rising of DNA fragmentation in sperm cells and markedly reducing embryo production it was up to 90%. In conclusion, moderately shortterm exposure to environmentally relevant concentrations of DEHP is able to alter spermatogenesis and affect reproduction in zebrafish.

There are several studies supported our result like feminized male, increased plasma estrogen levels, Decreased testes size and related spermatogenesis in male and increased aromatase level (Baatrup and Jung 2001; Bayley *et al.,* 2002; Jensen *et al.,* 2004; Kinnberg and Toft 2003; Powlowski *et al*., 2004) Correspondingly due to endocrine exposure reduced ovarian growth and altered oocyte development and decreased fecundity in females, presence of oocytes and ovarian duct in testes of male fish was showed (Brion *et al.,* 2004; Jensen *et al.,* 2004; Kiparissis *et al.,* 2003; Makyen *et al.,* 2000; Mandiki *et al.,* 2005; Nash *et al.,* 2004; Pawlowski *et al.,* 2004). Treating fish with exogenous sex steroid hormones during early developmental stages may cause sex-inversion or sex reversal in spite of the genotypic sex (Tzchori *et al.,*

2004). Similar observation by Norman *et al.* (2007) in Salmon (Salmo salar) fry treated with DEHP dose of 16.5 mg/kg intersex fish fishes were found. Dutta *et al.* (2006) conducted a histopathological study on the effect of endosulfan on testes of bluegill fish *Lepomis macrochirus* after 24, 48, 72 and 96 hours there were signs of connective tissue splintering, breakage of primary spermatocyte wall and its separation from the seminiferous tubules, damage and migration of primary spermatogonia into the lumen and damage to connective tissue and seminiferous tubules was observed. The histological data support the hypothesis that phthalic acid ester inhibits the progression of spermatogenesis potentially by causing an arrest of meiosis.

Therefore by the observation from the present study the resemblance showed by Chikae *et al.,* 2004a, reported that Japanese medaka were exposed to 0.01, 0.1, 1.0, and 10.0 μg/L DEHP until hatching resulted in delayed hatching time. Another study by Chikae *et al.,* 2004a adult stage, mortality rates exposed to DEHP, but no effects were seen in female body weights or the gonadosomatic index (GSI) of either sex. A later study were also observed GSI was reduced in males at 0.01 – 10 μg/L in rat female body (Chikae *et al.,* 2004b). Kim *et al.,* 2002 reported the sublethal effect of DEHP in female medaka under chronic exposure conditions of 10 – 50 μg/L for 3 months showed reduced GSI. Akinbemi *et al*. (2004; 2001) investigated that the DEHP concentration 5 or 25 mg/L can alter testicular function and decrease the level of Testosterone in blood serum.

Similar study reported the effects of Di-(2-ethylhexyl)-phthalate (DEHP) in female reproductive system of zebrafish there was a significant decrease in ovulation and embryo production was observed. Treatment with EE2 or the 2 mg/l DEHP dose led to a significant increase in the number of vitellogenic oocytes. This increase was

associated with a significant diminish in pre-vitellogenic oocytes observed in the same experimental groups; EE2 and 2 mg/l DEHP shifted pre-vitellogenic oocytes towards vitellogenic induction. Interestingly, no post-vitellogenic oocytes were found in the EE2, 20 or 40 mg/l DEHP exposed females in all treated groups the GSI (gonad-somatic index) became higher. A significant increase in vtg levels in the plasma of treated females was observed with the highest induction found with 40 mg/l DEHP, clearly showing the estrogenic activity of DEHP studied by Oliana *et al.* (2010).

Histopathology has proven to be a useful biomarker to evaluate the risk to aquatic organisms by exposure to both natural and anthropogenic EDCs that may interfere with reproduction and development (Bateman *et al.,* 2004). Mild intersex characteristics were recently shown to be able to compete with normal males and contribute to the next generation in a competitive breeding scenario (Matthiessen *et al.,* 2008; Singh *et al.,* 2013) and fish kills (Ripley *et al.,* 2008). Tetreault *et al.* (2011) reported alterations in gonad development compared to that of fish from reference sites during the examined of gonad sections from downstream of STP discharges. The abnormal sexual development such as compromised reproductive function and characteristic may be due to the induction of aromatase by EDCs chemical (Larkin *et al.,* 2003; Singh, 2012; Singh *et al.,* 2014). Zutshi and Murthy (2001, 2003) reported ultrastructural changes in the testes of the fish *Glossogobius giuris* induced by the pesticide fenthion. Uren-Webster *et al.* (2010) investigated the effects of di (2-ethylhexyl) phthalate (DEHP) on the reproductive health of male zebrafish (*Danio rerio*). Males were treated with 0.5, 50 and 5000 mg DEHP kg−1 (body weight) for a period of 10 days via intraperitoneal injection, a significant increase in the hepatosomatic index and levels of hepatic vitellogenin transcript were observed

following exposure to 5000 mg DEHP kg−1. Exposure to 5000 mg DEHP kg−1 also resulted in a reduction in fertilisation success of oocytes spawned by untreated females caused alterations in the proportion of germ cells at specific stages of spermatogenesis in the testis, including a reduction in the proportion of spermatozoa and an increase in the proportion of spermatocytes. Our study concluded degeneration of vitellogenic oocyte in developing ovary although we do not measure separate level of VTG concentration in male and female fish but degenerating oocytes (Figure 15b). Similar findings by Bhatia *et al.* (2013) supported my findings. Similarly Murray rainbow fish exposed to DnBP concentration 125-1000µg/L observed significant reduction in the proportion of spermatozoa (Bhatia *et al.,* 2013).

Effect of HCH on *L. rohita*

Effect of HCH on Gonado somatic index (GSI) of *L. rohita*:

The results of this study delineated that exposure of **γ-**HCH to *L. rohita* reduced the gonado-somatic index in all experimental group. Similar study EDCs exposed fish feather minnow has been reported to have noticeably lower GSI than control (Rey, 2006). GSI decreases have been reported in adult rainbow trout and carp exposed to estrogens either in water or via their food (Gimeno *et al.,* 1998; Jobling *et al.,* 1996; Komen *et al.,* 1989). Results of this study along with previous literature showed that GSI can be suitably considered for detection of estrogen mimics (Bjorkblom *et al.,* 2009; Jobling *et al.,* 1996). Related findings have been documented by many authors showing that exposure of estrogenic and anti-androgenic contaminants in the laboratory and in fish inhabiting contaminated sites with reduced GSI (Ankley *et al.,* 2001; Patino *et al.,* 2003; Sepulveda *et al.,* 2002; Sun *et al.,* 2009; Zha *et al.,* 2008).

Effect of HCH on biochemical Parameters of *L. rohita*:

In this study, we found decreased serum 17-β estradiol (E2) after HCH treatment. Similar results have been shown by revealing an antiestrogenic potency of HCH that influences sexual development in amphibians (Levy *et al.,* 2004). In the same way exposure to pp-DDE decreased estradiol in largemouth bass (*Micropterus salmoides*) females, but increased 11-ketotestosterone Dieldrin on the other hand decreased estradiol, 11-ketotestosterone in both sexes, altered the expression of vitellogenin and genes involved in hormone synthesis and metabolism which considerably lowers plasma hormone levels (Garcia-Reyero *et al.,* 2006). Other reports suggested decreased testosterone level in mature male common carp exposed to 1000mg/L BPA (Mandich *et al.,* 2007), in juvenile turbot *Psetta maxima* exposed to 59mg/ L BPA (Labadie and Budzinski, 2006) and in larvae of brown trout (*Salmo trutta*) exposed to 50mg/L BPA (Bjerregaard *et al.,* 2008). Another related study reported by Iwamatsu *et al.* (2006a), showed that aromatizable testosterone in high concentrations may induce a significant increase in E2 content in embryos of medaka *Oryzias latipes* and can cause paradoxical sex reversal. Similar observations were made in zebrafish (Orn *et al.,* 2003). In the African catfish (*Clarias gariepinus*) in vivo treatments with androgens inhibited testis development (Cavaco *et al.,* 2001; Schulz and Miura, 2002).

Hormonal regulation between estrogen and androgen is necessary in sexual differentiation of teleost fish and it depends upon activity of some steroid synthesis including enzyme particularly cytochrome P450 aromatase complex. This enzyme directly participate in the regulation of sexual differentiation because aromatase inhibitor treated female fish have various degree of masculinization *Oncorhynchus mykiss* (Guiguen *et al.,* 1999), zebra fish *Danio ranio* (Uchida *et al.,* 2004)

Orinochronis niloticus (Singh *et al.,* 2011; Kwon *et al.,* 2000a) common carp (Singh *et al.,* 2013). In this study serum aromatase value did not significantly (P>0.05) change in HCH treated fish while Celius *et al.* (1999) investigated the production of vitellogenin and zona radiata (eggshell) proteins in primary hepatocytes of atlantic salmon exposed to a variety of suspected EDCs including o, p'-DDT and lindane (γ–HCH) cells treated with 1, 5, and 10 μM DDT and lindane induced vitellogenin and zona radiata proteins in an approximate dose-dependent manner.

Effect of HCH on RNA/DNA Ratio

The results of this study revealed that fish exposed to **γ-**HCH decreased RNA/ DNA ratio. Similar observation has been reported where inhibitory effects of fenvalarate on DNA, RNA and protein content has been shown to have tremendous toxicity of pyrethoidal compound on the main biochemical machinery of the fresh water catfish, *Clarias batrachus* (Tripathi *et al.,* 2001). In another analogues study significant decline in the nucleic acid content has been reported in *Channa punctatus* subjected to dimethoate an organophosphate (Tripathy *et al .,* 2003). Our results showed that effect of high dose of HCH affected the DNA, RNA and RNA/DNA ratio so very lower concentration may affect fishes suggesting that the poor growth of experimental fish was due to imbalance in RNA/DNA ratio. The percentage reduction of nucleic acid level was statistically significant in tissues of the fish (Rathod and Kshirsagar, 2010; Raksheskar, 2012). Further, it is reported that fish exposed to Dimethoate (organophosphate) exhibited a decrease in nucleic acid (DNA and RNA) content (Anderson *et al.,* 1994; Tripathi, 2003). Thus hypothesis of this study cleared that the RNA/DNA ratio can be considered as early warning signals for detection of effects any EDCs at the species level.

Effect of HCH on gonadal development and structure of *L. rohita*:

In HCH treatment group histological observation indicated clear cut difference from the control group. Histological structure of testis showed different stage of spermatogenesis. In controlled condition cross section of testis showed ducts, Sertoli cells (SE), tunica albuginea (TA) and it revealed the presence of spermatogonia (SG) spermatids (ST) and spermatozoa (SZ). In this study HCH exposed fish showed disorganization of tubular elements semeniferous tubules degenerated when treated with 0.5mg/L HCH. HCH treated gonad showed developing oocyte (OC) after 60 days treatment of 0.5mg/L and a clear mature oocyte was observed in developing testes having number of spermatocytes surrounding it. In controlled condition, immature ovary showed the perinuclear stage and vitellogenic stage. In perinuclear stage the number of nucleoli increased and arranged along the inner side of the nuclear membrane. In vitellogenic stage, oocyte size was found to be increased. The nucleus was convoluted and follicular layer were well developed. In HCH treated fish, ovular cell were not clear and no perinuclear condition was observed and yolk vesicles were surrounded by the nucleus of the ovule. There are many other similar reports suggesting E2 content in embryos of medaka *Oryzias latipes* and can cause paradoxical sex reversal (Iwamatsu *et al.*, 2006a). Similar observations were made in zebrafish (Orn *et al.,* 2003). These considerations led us to suppose that exposure to organochlorene could increase the possibility of a persistent intersex condition. The absence of intersex individuals in control groups may indicate that the observed effect in HCH-exposed groups would be a demasculinization/feminization phenomenon or a delay of intersex individuals in turning into males or females. A recent study investigated the effect of exposure of EDCs on sexual differentiation in European pikerch (*Sander lucioperca*) testicular development was delayed with increased

frequency of intersex condition (Jarmolowicz *et al.,* 2013). Synchronized conclusion observed by many authors that abnormal gonadal development, such as delayed maturation, high levels of atresia or intersexuality may also be detected histologically. Such parameters are frequently investigated in fish from contaminated environments or those exposed to anthropogenic chemicals (Brion *et al.,* 2004; Mattson *et al.,* 2001)

Mechanism of Action of these chemicals:

The abnormalities and sub lethal changes caused by endocrine disrupting chemicals are reported in several aquatic animals (Bayley *et al.,* 2002; Harris *et al.,* 2001; Kinnberg *et al.,* 2000; Kinnberg and Toft 2003; Kloas, 2002; Lavado *et al.,* 2004; Loomis *et al.,* 2000; Maclatchy *et al.,* 2003; Makynen *et al.,* 2000; Mandiki *et al.,* 2005; Schwaiger *et al.,* 2002). It has been shown that Endocrine disruptoring chemicals (EDCs) can affect endocrine systems through various ways. It may be receptor-mediated or receptor-independent pathways mimicking the action of estrogens or androgens. While acting though receptor, these compounds have affinity to bind with receptor and activate the gene cascade thereby affecting the reproductive physiology either by disrupting the synthesis or degradation of endogenous hormones or by directly activating steroid hormone receptor-mediated gene activation pathways (Caserta *et al.,* 2008; Patisaul *et al.,* 2009). Exposure of estradiol (E2) results in the synthesis of specific proteins that activate several genes that encode proteins like estrogen receptor (ER), Vitellogenin (Vtg), the egg yolk precursor proteins, and choriogenins required for making the egg membrane (Arukwe *et al.,* 2001; Bowman *et al.,* 2000; 2002 Celius *et al.,* 2000; Folmar *et al.,* 2000; Funkenstein *et al.,* 2000; Hemmer *et al.,* 2001; Lattier *et al.,* 2001) and affecting the expression of Vtg. (Matozzo *et al.,* 2008). These compounds also have got agonist activity to bind aromatase receptor (Brion *et al.,* 2012; Hinfray *et al.,* 2008; Kallivretaki *et al.,* 2006).

It is likely that EDCs will have their own specific gene expression profiles because they may bind with low affinity to more than one steroid receptor resulting in a complex gene activation pattern (Kallivretaki *et al.,* 2006). There is clear cut evidence that these compounds have both agonist and antagonist activity i.e. either binds with ER receptor or AR receptor (Paech *et al.,* 1997). However, several questions are raised for the fate of the gene expression as EDCs can bind to both the ER and AR. Scientific investigations are going on to unravel the dilemma and to understand the mechanisms that might be involved with different kind of EDCs. Estrogenic compounds are increasingly being recognized in environment which has potential to induce feminization of wild fish because it blocks androgen action (Filby *et al.,* 2006). Feminization by anti androgen and estrogen may in part be attributed to increased expression of CYP 19 genes which is responsible for the production of E_2 by catalyzing its conversion from testosterone. It involves conversion of ▲4 3-one a ring of the androgens to the corresponding phenolic ring characteristic of estrogen. Many endocrine chemicals have this phenolic ring so these are easily recognized by receptor and thus cause disruption. (Craig *et al.,* 2011) Modulation of aromatase CYP19 expression and function can dramatically modify the rate of estrogen production, disturbing the local and systemic levels of estrogens. Two *cyp19* genes are present in most teleosts, *cyp19a* and *cyp19b*, primarily expressed in the ovary and brain, respectively both aromatase CYP19 isoforms are concerned in the sexual differentiation and regulation of the reproductive cycle (Paech *et al.,* 1997). Alteration of aromatase CYP19 expression and/or activity by up regulation or down regulation, may cause possibility for several EDC classes to affect *cyp19* expression on the transcriptional level (Kalliveretaki *et al.,* 2006; Kazeto *et al.,* 2004; Montserrat *et al.,* 2004).

Conclusion

In the last decade, research has focused on potential interactions of chemicals with various hormone receptors, with particular emphasis on the thyroid hormone and estrogen receptor, and more recently the androgen receptor. Numerous chemicals have been reported to be agonists or antagonists for the estrogen receptor in various *in vitro* systems. However, receptor affinityare usually very low relative to endogenous hormones such as 17b-estradiol and testosterone. There may exist several mechanisms of interference of such pseod-receptors with the endocrine system. The endocrine disruptor is typically defined as ‘‘an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse effects in an intact organism or its progeny or (sub) populations’’ (IPCS, 2002). Consequently, key enzymes involved in steroid hormone synthesis and metabolism are being considered as important targets for endocrine disrupting chemicals particularly, the cytochrome P450 (CYP) enzymes which are responsible for the highly specific reactions in the steroid biosynthetic pathway.

While with the above discussion it is clear that exposure to EDCs can have adverse effects on reproductive physiology and behaviour in fish. It is important to recognise the prevalence of such compounds in the environment and to find out their potential adverse affect. Further, efforts to understand the mechanisms underlying EDC effects, particularly those seen in environment in low doses. However, such compounds have no clear mechanism of action and these may cause the epigenetic potential effects to destroy the next generation population. It is now considered consider that improvements in detection techniques of EDCs are essential, together with effective monitoring of discharges and pollutants in aquatic bodies. Studies on the immunological effects of endocrine disruptors is however, recommended. A further

long-term study of populations exposed to EDCs is also recommended to assess the effects on age and the structure of population as well as on altering population size.

Summarizing the weight of evidence DEHP induces vitellogenin protein And estradiol in most of the tested fish species, indicating an estrogenic mode of action. Overall DEHP acts as a weak estrogen and/or anti-androgen changing the sex ratio of fish in one study, inducing ovo-testis in another, decreasing reproductive output in combination with Vtg induction in a third study as well as decreasing male reproductive output in a fourth study. Further available studies support this conclusion and other studies suggest that DEHP may also have thyroid effects in fish and/ or amphibian species. Well similar result obtained with DBP which is the isomer of the phthalic acid ester so we can conclude that Phthalic acid ester is a week estrogenic chemical viewing above result, But result point out a clear cut difference between Organochlorine (HCH) and plastic originated compound like phthalic acid ester that both have different mode of mechanism and both have different adverse effect on aquatic animal. Results revealed that that Due to the imbalance in the reproductive hormone the reproductive development should be arrested. It could be clear cut concluded that these compounds directly affect the endocrinology of animals.

Gonadosomatic index also a vital parameter to see the direct effect of these chemical on the physiological development. RNA plays significant role in protein synthesis hence depletion in RNA contents also results in depletion in protein level. Hence, there is decrease in RNA level thus reducing protein synthesis. Thus, from the present investigation, it can be accomplished that the marked decrease in the DNA and RNA content upon exposure to pesticides may be due to decrease in protein synthesis, impairment of nucleic acid metabolism, the degradation of cells, resulting in the reduction in the DNA and RNA content.

In the present investigation, it can be concluded that under exposure to pesticides the DNA and RNA content decreased in the tissues of the fish, *L. rohita* leading to a decrease in protein synthesis and cellular degradation. The present data happens to constitute the first report on pesticide causing depletion in the nucleic acid level amongst the freshwater fishes. Further it could also be concluded that these intersex fishes are not able to reproduce because of lack of the sexual differentiation. These are sterile. It should be noted, however the present dataset was restricted to relatively limited information and results but more study need to be conducted to fully elucidate the pathway involve in the disruption of gonaldal function in fish.

Summary

Sexual reproduction in vertebrates is mediated by steroid hormones and numerous endocrine disrupting chemicals (EDCs) discharged into the environment have been shown to interfere with hormone signaling via several mechanisms. In fish, sex determination and differentiation are particularly susceptible to endocrine disruption and exposures to various synthetic and natural endocrine disrupting chemicals have been associated with significant effects on reproductive development and function in wild fish populations. Endocrine disrupting chemicals (EDCs) are chemicals that can have an effect on the endocrine system of animals including aquatic animals (fishes, molluscs, and amphibian), birds and humans. Several EDCs have been detected in water, air, and soil environments. Estrogenic EDCs (e-EDCs) are able to induce an estrogen-like response in organisms The most common e-EDCs listed in literature are Estrone (E1), 17β-Estradiol (E2), Ethinylestradiol (EE2), Estriol (E3), Bisphenol A (BPA), Nonylphenol (NP), Nonylphenol ethoxylates (NPnEO), and Octylphenol, Phthalic acid ester (DMP, DBP, DEHP). In view of the above mentioned facts, the thesis work was conduced focusing on the effects of EDCs on fish reproduction endocrinology and hormone biosynthesis. In particular, we have examined the effect of EDCc on an Indian major carp *Labeo rohita*. Degradation of these compounds leads to a lowering of solubility and increased estrogenicity. EDCs can act at cellular level; induce endocrine disruption via a number of routes that involve steroid receptor binding (agonists), blocking steroid receptor binding (antagonists), or by disrupting the biosynthesis and or metabolism of steroids (Sharpe and Irvine, 2004). Paper mill extracts and municipal wastewater are polluted with a huge number of organic substances containing both natural and xenobiotics compounds (Gehring *et al.,* 2002; Ying *et al.,* 2002).These chemicals mimic endogenous hormone action or inhibit their activity modulating the endocrine system (Guiletter, 2000). Significant concentrations

of EDCs have been detected in sediments and wastewater by several authors in Uttar Pradesh, India. Therefore, monitoring of EDCs in aquatic animals are necessary. On account of above facts,, this study was undertaken to elucidate the impacts of EDCs (phthalic acid ester and organochlorine) under controlled laboratory studies. The experimental animal *L. rohita*, weighing 100g-150g were exposed to phthalic acid ester (DMP, DBP, and DEHP) and γ-HCH in different doses of 0.2mg/L, 0.3mgL, 0.5mg/L. Phthalic acid ester was dissolved in 1% DMSO and γ-HCH was dissolved in 1% Acetone and diluted the stock solution for the required concentration in aquaria water where the fihes were treated for 90 days with renewal of water every third day. This study investigated the effect of these endocrine disrupting chemicals on the gonads (GSI), histopathological changes of gonads, plasma concentrations of Vitellogenin (VTG), 17β-estradiol (E2), Testosterone and Aromatase, and also RNA/DNA ratio.

Methodology used:

Labeo rohita (rohu) was collected from private fish ponds, Lucknow. Identified taxonomically and maintained in laboratory condition. Fishes were fed with healthy diet daily commercially available palette diet (Tyio Pvt. Ltd.) daily. Length and weight of each fish was measured before and after the experiment and the collected data gave the information about growth of fish under controlled and treatment condition. Weight of fish was taken by digital electronic balance and length was measured by a calliper scale. Gonado somatic index (GSI) was calculated according to Singh *et al.* (2013). Estradiol-17β, testosterone and Vitellogenin levels in the serum was measured using kit provided by Enzo -Life Sciences, India, Serum Aromatase was determined by kit (Uscn Life Science Inc). The microscopic anatomy of the cellular changes in gonadal tissues was done using histological techniques

described by Coolidge and Howard (1979). RNA/DNA ratio was measured by using spectrophotometric method by Schinder (1957).

Results:

Effect of Phthalic acid ester (DMP, DBP and DEHP) on *L. rohita*

GSI value in control group ranged from 0.18±0.03-0.32±0.01 and in all DMP treated groups showed insignificant changes compared to control group. GSI in all treatment group ranged 0.19±0.01 - 0.316 ±0.015. Further, there was no change observed in GSI of fish exposed to both DBP and DEHP. In DBP and DEHP exposed groups the testosterone value of decreased significantly ($P<0.05$) and it was 961pg/ml and 463.5 pg/ml respectively in high dose of 0.5mg/L but in control it was 1523.3pg/ml. Serum estradiol and vitellogenin level increased significantly ($P<0.05$) in both groups of treatment DBP and DEHP. Estradiol level range from 438pg/ml to 529pg/ml in DBP and DEHP at different experimental doses and the vitellogenin slightly increased which was highly significant in DEHP treated group where it was 1701 pg/ml but in control group it was 1018pg/ml. Serum Aromatase value was insignificant in DBP treated group but in DEHP treated group it significantly ($P<0.05$) increased compared to control, where it was 79.74 pg/ml and in DEHP treated group it was 213.3pg/ml. The group of fish treated with DBP showed significant decrease of RNA/DNA ratio in high dose of the experimental period after 90 days. RNA/DNA ratio value in control group ranged from 2.2±0.01-2.12±0.09 and in DBP treated group it was not significantly ($P>0.05$) affected in 0.2mg/L, 0.3mg/L and 0.5mg/L after 30 and 60 days. The group of fish treated with DEHP showed also significant decrease of RNA/DNA ratio in high dose 0.5mg/L where it was 1.95±0.05. RNA/DNA ratio also significantly reduced ($P<0.01$)) after 90 days but in low dose and after 60 and 90 days there was insignificant difference between control and DEHP treated group. In both

the groups histological structure of gonad showed intersex condition where many spermatids were surrounded by the ovarian cavity in all group of intersex population. Developing ovary showed perinucleolar oocytes (POC), spermatocytes (ST) delineating imposex condition. There were a lower proportion of spermatozoa in the testes of males exposed with 0.5mg/L DEHP compared to control group. Conversely, there was significantly less count of spermatocytes in the testes of fish exposed with 0.2mg, and 0.3mg/L DBP compared to control. At the high concentration of DEHP, the microphotograph showed degenerating oocytes possibly due to endocrine disruption.

Effect of Organochlorine γ-HCH on *L. rohita*

The experimental fishes were found unhealthy throughout the experimental period. γ-HCH exposed fish had significantly ($P<0.5$) decreased GSI compared to control group. GSI value in control group was 0.17±0.01, 0.26±0.01and 0.35±0.01 after 30, 60 and 90 respectively. However, it reduced to 0.16±0.01, 0.26±0.01 and 0.29±0.01 after 30, 60 and 90 days respectively. HCH treated fish was analysed and found that serum testosterone level significantly ($P<0.5$) increased compared to control group but serum estradiol and vitellogenin level decreased significantly. Serum aromatase level was not significantly ($P>0.05$) different from control thought the experiment period in control group where it was 79.5±4.5, 89.5±2.3 and 87.6±4.5 pg/ml after 30, 60 and 90 days. The fish treated with HCH showed a significant decrease of RNA/DNA ratio in all group of treatments. RNA/DNA ratio value in control group ranged from 2.6±0.09-2.74±0.05 and in HCH treated group it significantly reduced to 1.05±0.09. Gonads of HCH treated *L. rohita* showed intersex condition where developing oocyte, degenerated follicular connective tissue (FTC), atresia, number of spermatids (ST) surrounding the mature oocytes (MOC) were observed.

This study sensitized and created awareness among people to EDCs and their harmful effects on aquatic animals including fish. This study reported effect of endocrine disruption chemicals in *L. rohita*. The level of serum steroid (vitellogenin and testosterone) used as a successful biomarker to check endocrine disruption in fish and aquatic animals. Estrogens and estrogen mimic endocrine disrupting chemicals and are probably responsible for elevated incidence of intersex characteristics among fish and other animals, particularly those exposed during growth and developmental phases of life. Exposure of effluent on fish included development of gonadal abnormalities, changes in sexually dimorphic characteristics, and sex reversal. Many of these effects are mediated through the estrogen mimicking actions of the compounds released into the environment. In the present investigation, it can be concluded that under exposure of endocrine disrupting chemicals affect the reproductive physiology of *L. rohita.* EDCs exposed fishes were intersex (sterile) in reproductive condition. suggesting that future monitoring should considered important to see if there is an increase in the masculinization or feminization percentage and to determine the limit of dosage of EDCs that can be permeable. The use of house hold waste material should be discarded carefully (not through running waterof natural aquatic bodies) to minimize the effects of EDCs.

References

Aditya, A.K. and Chattopadhyaya, S. (2000). Accumulation of methyl parathion in the muscle and gonad of *Labeo rohita*, Journal of Environmental Biology. 21(1): 55–57.

Akatsuka, N., Komatsuzaki, E., Ishikawa, A., Suzuki, I., Yamane, N., Miyata, S. (2005). Expression of the gonadal p450 aromatase gene of Xenopus and characterization of the 5-flanking region of the aromatase gene, Journal of Steroid Biochemistry & Molecular Biology. 96: 45–50.

Akingbemi, B.T., Ge, R., Klinefelter, G.R., Zirkin, B.R. and Hardy, M.P. (2004). Phthalateinduced Leydig cell hyperplasia is associated with multiple endocrine disturbances, Proceedings of the National Academy of Sciences USA. 101: 775-80.

Akingbemi, B.T., Youker, R.T., Sottas, C.M., Ge, R., Katz, E., Klinefelter, G.R., Zirkin, B.R. and Hardy, M.P. (2001). Modulation of rat leydig cell steroidogenic function by di (2- ethylhexyl) phthalate, Biology of Reproduction. 65: 1252-9.

An, Li-hui, Yanqiang, Z., Shuang-shuang, S., Yue, L., Zi-cheng, Li, Hao, C., Xing-ru, Z., Kun, L., Junmin, G., Bing-hui, Z. (2013). Imposex effects on the veined rapa whelk (Rapana venosa) in Bohai Bay, China, Ecotoxicology. 22 (3): 538–547.

Andersen, L., Bjerregaard, P. and Korsgaard, B. (2003). Vitellogenin induction and brain aromatase activity in adult male and female zebrafish exposed to endocrine disrupters, Fish Physiology Biochemistry. **28:** 319–321.

Anderson, S, et al. (1994) Environmental Health and Perspective. 101: 3–8.

Ankley, G. T., Kuehl, D. W., Kahl, M. D., Jensen, K. M., Linnum, A., Leino, R. L., and Villeneuve, D. A. (2005). Reproductive and developmental toxicity and bioconcentration of perfluorooctanesulfonate (PFOS) in a partial-life cycle test with the fathead minnow (Pimephales promelas). General and Comparative Endocrinology.

Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J., Makynen, E.A. (2001). Description and evaluation of a short-term reproduction test with the fathead minnow (Pimephales promelas), Environmental Toxicology and Chemistry. 20:1276-1290.

Ankley, G.T., Jensen, K.M., Makynen, E.A., Kahl, M.D., Korte, J.J., Hornung, M.W., Henry, T.R., Denny, J.S., Leino, R.L., Wilson, V.S., Cardon, M.C., Hartig, P.C., Gray, L.E. (2003). Effects of the androgenic growth promoter 17-

trenbolone on fecundity and reproductive endocrinology of the fathead minnow, Environmental Toxicology and Chemistry. 22: 1350–1360.

Ankley, G.T., Johnson, R.D. (2004). Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. Inter Lab Anim Res J. 45:467–481.

Arditsoglou, A. and Voutsa, D. (2008). Determination of phenolic and steroid endocrine disrupting compounds in environmental matrices, Environmental Science Pollution Research. 15 (3): 228-236.

Arukwe, A., Celius, T., Walther, B.T. and Goksoyr, A. (2000). Effects of xenoestrogen treatment on zona radiata protein and vitellogenin expression in Atlantic salmon (Salmo salar), Aquatic Toxicology. 49:159–170.

Arukwe, A., Goksøyr, A. (2003). Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption, Comparative Hepatology. 6; 2(1).

Arukwe, A., Kullman, S.W., Hinton, D.E. (2001). Differential biomarker gene and protein expressions in nonylphenol and estradiol-17β treated juvenile rainbow trout (Oncorhynchus mykiss), Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology. 129:1–10.

Baatrup, E., Junge, M. (2001). Antiandrogenic pesticides disrupt sexual characteristics in the adult male guppy (*Poecilia reticulata*), Environmental Health Perspectives. 109: 1063–1070.

Babler, S.J.M. (1970). The occurrence of a penis-like outgrowth behind the right tentacle in spent female of Nucella lapillus (L.), Proceedings of the Malacological Society of London. 39: 231–233.

Barlas, N., Selmanoglu, G., Songur, S., Kockaya, A.E. and Erdemli, E. (2002). Biochemical and histopathological effects of carbendazim to rat male reproduction, Pesticides. 17: 59 – 71.

Barnhoorn, I.E.J., Bornman, M.S., Pieterse, G.M. and VanVuren, J.H.J. (2004). Histological evidence of intersex in feral sharptooth catfish (Clarias gariepinus) from an estrogen-polluted water source in Gauteng, South Africa, Environmental Toxicology. 19 (6): 603-608.

Barse, A.V., Chakrabarti, T., Ghosh, T.K., Pal, A.K., Jadhao, S.B. (2007). Endocrine disruption and metabolic changes following exposure of *Cyprinus carpio* to diethyl phthalate, Pesticide Biochemistry and Physiology. 88(1):36–42.

Bateman K.S, Stentiford G.D, Feist S.W. (2004). A ranking system for the evaluation of intersex condition in European Flounder (Platichthys flesus), Environmental Toxicology and Chemistry. 23:2831–2836. doi:10.1897/03-541.1.

Batty, J., Lim, R. (1999). Morphological and reproductive characteristics of male mosquito fish (Gambusia affinis holbrooki) inhabiting sewage-contaminated waters in New South Wales, Australia, Archives of Environmental Contamination and Toxicology. 36 (3): 301-307.

Bayley, M., Junge, M., Baatrup, E. (2002). Exposure of juvenile guppies to three anti androgens causes demasculinization and a reduced sperm count in adult males, Aquatic Toxicology. 56:227-239.

Belloa, U.M., Mary-Catherine, M., Herman, B., Groenewalda, T.A., Airea, A.A. (2014). The effects on steroidogenesis and histopathology of adult male Japanesequails (Coturnix coturnix japonica) testis following pre-pubertal exposureto di(n-butyl) phthalate (DBP), Comparative Biochemistry and Physiology, Part C. 166: 24–33.

Bhatia, H., Kumar, A., Chapman, J.C. (2015). Long-term exposures to di-n-butyl phthalate inhibit body growth and impair gonad development in juvenile Murray rainbowfish (Melanotaenia fluviatilis), Applied Toxicology 35(7): 806–816.

Bjerregaard, L.B., Korsgaard, B., Bjerregaard, P. (2006). Intersex in wild roach (Rutilus rutilus) from Danish sewage effluentreceiving streams, Ecotoxicology and Environmental Safety. 64:321–328.

Bjerregaard, L.B., Lindholst, C., Korsgaard, B., Bjerregaard, P. (2008). Sex hormone concentrations and gonad histology in brown trout (Salmo trutta) exposed to 17beta-estradiol and bisphenol A, Ecotoxicology. 17: 252-263.

Bjorkblom, C., Högfors, E., Salste, L., Bergelin, E., Olsson, P.E., Katsiadaki, I. and Wiklund, T. (2009). Estrogenic and androgenic effects of municipal wastewater effluent on reproductive endpoint biomarkers in three-spined stickleback (Gasterousteus aculeatus), Environmental Toxicology and Chemistry. 28:1063-1071.

Blaise, C., Gagne, F., Salazar, M., Salazar, S., Trottier, S., Hansen, P.D. (2003). Experimentally induced feminisation of freshwater mussels after long-term exposure to a municipal effluent, Fresenius, Bulletin of Environmental Contamination and Toxicology. 12: 865–70.

Blazer,V.S., Iwanowicz, L.R., Iwanowicz, D.D., Smith D.R., Young, J.A., Hedrick, J., Foster, S.W. and Reeser, S.J. (2007). Intersex (Testicular Oocytes) in Smallmouth Bass Micropterus dolomieu From the Potomac River and Selected Nearby Drainages, Journal of Aquatic Animal Health. 19:242-253.

Bogdan, G., Carmen, G., Stelian, D., Anca, B., Simona, P. (2011). Heavy Metals Acting as Endocrine Disrupters, Animal Science and Biotechnologies. 44(2): 89-93

Bottero, S., Cevasco, A., Monteverde, M., Pedemonte, F., Bertolotti, R., Viganò, L., Mandich, A., Massari, A. (2005). In vivo and in vitro exposures of carp and carp tissues to graded concentrations of endocrine disrupting chemicals, Proceedings of the National Academy of Sciences. 1040: 234–238.

Bowman, C.J., Kroll, K.J., Gross, T.G., Denslow, N.D. (2002). Estradiol-induced gene expression in largemouth bass (Micropterus salmoides), Molecular and Cellular Endocrinology.196: 67–77.

Bowman, C.J., Kroll, K.J., Hemmer, M.J., Folmar, L.C., Denslow, N.D. (2000). Estrogen induced vitellogenin mRNA and protein in sheepshead minnow (Cyprinodon variegatus), General of Comparative Endocrinology. 120: 300–313.

Brion, F., Le Page, Y., Piccini, B., Cardoso, O., Tong, S.K., Chung, B.C. (2012). Screening estrogenic activities of chemicals or mixtures in vivo using transgenic (cyp19a1b-GFP) zebrafish embryos, PLOS ONE 7: e36069.

Brion, F., Tyler, C.R., Palazzi, X., Laillet, B., Porcher, J.M., Garric, J., Flammarion, P. (2004). Impacts of 17_-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*), Aquatic Toxicology. 68: 193–217.

Cai, Y., Jiang, G. and Liu, J. (2003). Solid-phase extraction of several phthalate esters from environmental water samples on column packed with polytetrafluoroethylene turning, Analytical Science. 19: 1491-1494.

Calamari, D., Tremolada, P, Di Guardo, A. and Vighi, M. (1994). Environmental Science and Technology. 28:429-434.

Canadian Arctic Contaminants Assessment Report (CACAR) 1997. Jensen, J., Adare, K., Shearer, R. (eds.). Department of Indian Affairs and Northern Development, Ottawa

Carnevali, C., Galassi, M.D., Bonasoro, S., Patruno, F., Thorndyke, M.C. (2001). Regeneration and endocrine disrupters in crinoid echinoderms: arm

regeneration in Antedon mediterranea after experimental PCB exposure, Journal of Experimental Biology. 204: 835-842.

Carnevali, O., Tosti, L., Speciale, C., Peng, C., Zhu, Y., Maradonna, F. (2010). DEHP Impairs Zebrafish Reproduction by Affecting Critical Factors in Oogenesis, PLOS One 5(4): 1-10.

Caserta, D., Maranghi, L., Mantovani, A., Marci, R., Maranghi, F., Moscarini, M. (2008). Impact of endocrine disruptor chemicals in gynaecology, Human Reproduction Update.14(1): 59-72.

Caunter, J.E., Williams, T.D. and Shillabeer, N. (2004). Di-2-Ethylhexylphtalate: Multi-generation study with the fathead minnow (Pimephales promelas), Study No AJ0172, Performed by Brixham environmental laboratory Astra Zeneca UK Ltd, sponsored by European Council for Plasticisers and Intermediates.

Cavaco, J.E., van Baal, J., van Dijk, W., Hassing, G.A., Goos, H.J., Schulz, R.W. (2001a). Steroid hormones stimulate gonadotrophs in juvenile male African catfish (Clarias gariepinus), Biol. Reprod. 64: 1358–1365.

Celius, T., Haugen, T.B., Grotmol, T. and Walther, B.T. (1999). A sensitive zonagenetic assay for rapid in vitro assessment of estrogenic potency of xenobiotics and mycotoxins, Environmental Health Perspectives. 107: 63-68.

Celius, T., Matthews, J.B., Giesy, J.P., Zacharewski, T.R. (2000). Quantification of rainbow trout (Oncorhynchus mykiss) zona radiata and vitellogenin mRNA levels using real-time PCR after in vivo treatment with estradiol-17β or α-zearalenol, The Journal of Steroid Biochemistry and Molecular Biology. 75:109–119.

Cespedes, R., Petrovic, M., Raldua, D., Saura, U., Pina, B., Lacorte, S. (2004). Integrated procedure for determination of endocrine-disrupting activity in surface waters and sediments by use of the biological technique recombinant yeast assay and chemical analysis by LC-ESI-MS, Analytical and Bioanalytical Chemistry. 378: 697–708.

Chang, C.F. and Lin, B.Y. (1998). Estradiol-17β stimulates aromatase activity and reversible sex change in protandrous black porgy, Acanthopagrus schlegeli, Journal of Experimental Zoology. 280(2): 165–173.

Chauvigne, F., Plummer, S., Lesne, L., Cravedi J-P, Dejucq-Rainsford, N., Fostier, A. (2011). Mono-(2-ethylhexyl) Phthalate Directly Alters the Expression of Leydig Cell Genes and CYP17 Lyase Activity in Cultured Rat Fetal Testis, PLoS ONE 6(11): e27172.

Cheek, A.O., King, V.W., Burse, J.R., Borton, D.L., Sullivan, C.V. (2004). Bluegill (Lepomis macrochirus) vitellogenin: purification and enzyme-linked immunosorbent assay for detection of endocrine disruption by papermill effluent, Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 137(3): 249–260.

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I.L. (2008). Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish, General of Comperative Endocrinology. 155: 31–62

Cheunga, C.H.Y., Chailleb, P.M., Randallc, D.J., Grayd, J.S.,. Au, D.W.T (2007). The use of scale increment as a means of indicating fishgrowth and growth impairment, Aquaculture. 266: 102–111.

Chikae, M., Hatano, Y., Ikeda, R., Morita, Y., Hasan, H. and Tamiya, E. (2004a). Effects of bis(2- ethylhexyl) phthalate and benzo[a]pyrene on the embryos of Japanese medaka (Oryzias latipes), Environmental Toxicology and Pharmacology. 16: 141-145.

Chikae, M., Ikeda, R., Hatano, Y., Hasan, Q., Morita, Y., Tamiya, E. (2004b). Effects of bis(2-ethylhexyl) phthalate, γ-hexachlorocyclohexane, and 17β-estradiol on the fry stage of medaka (Oryzias latipes), Environmental Toxicology and Pharmacology. 18: 9-12.

Christiansen, L.B., Pedersen, K.L., Korsgaard, B., Bjerregaard, P. (1998). Estrogenicity of xenobiotics in rainbow trout (Oncorhynchus mykiss) using in vivo synthesis of vitellogenin as a biomarker, Marine Environmental Research. 46: 137–140.

Connon, R.E., Geist, J., Werner, I. (2012). Effect-Based Tools for Monitoring and Predicting the Ecotoxicological Effects of Chemicals in the Aquatic Environment, Sensors (Basel, Switzerland) 12(9): 12741-12771 doi: 10.3390/s120912741.

Cooke, G.M. (2002). Effect of organotins on human aromatase activity in vitro. Toxicology Letter. 126: 121–130.

Corradetti, B., Stronati, A., Tosti, L., Manicardi, G., Carnevali, O., Bizzaro, D. (2013) Bis-(2-ethylexhyl) phthalate impairs spermatogenesis in zebrafish (Danio rerio), Reproductive Biology. 13(3):195-202

Cortazar, E., Bartolome, L., Delgado, A., Etxebarria, N., Fernandez, L.A., Usobiaga, A. and Zuloaga, O. (2005). Optimisation of microwave-assisted extraction for the determination of nonylphenols and phthalate esters in sediment samples and

comparison with pressurised solvent extraction, Analytica Chimica Acta. 534: 247-254.

Corton, J. C., and Lapinskas, P. J. (2005). Peroxisome proliferator-activated receptors: Mediators of phthalate ester-induced effects in the male reproductive tract? Toxicol. Sci. 83, 4–17.

Crago, J., Klaper, R. (2012). A mixture of an environmentally realistic concentration of a phthalate and herbicide reduces testosterone in male fathead minnow (Pimephales promelas) through a novel mechanism of action, Aquatic Toxicology. 110– 111: 74– 83.

Craig, Z.R, Wang, W, Flaws, J.A. (2011). Endocrine-disrupting chemicals in ovarian function: effects on steroidogenesis, metabolism and nuclear receptor signalling, Reproduction. 142:633–646.

Crain, D. A., Eriksen, M., Iguchi, T., Jobling, S., Laufer, H., LeBlanc, G. A. & Guillette Jr, L. J. 2007 An ecological assessment of bisphenol-A: evidence from comparative biology, Reproductive Toxicology. 24L: 225 –239.

Das, K.B. and Mukherjee, C.S. (2000). A Histopathological study of carp (*Labeo rohita*) exposed to hexachlorocyclohexane, Veterinarski Arhiv. 70(4): 169-180.

Denslow, N., Sepulveda, M. (2008). Ecotoxicological effects of endocrine disrupting compounds on fish reproduction. In: Babin, P.J, Cerda, J., Lubzens, E. (Eds.),The Fish Oocyte-From Basic Studies to Biotechnological Applications, Springer, Dordrecht, the Netherlands,pp. 255-322.

Desforges, J.P.W., Peachey, B.D.L., Sanderson, P.M., White, P.A., Jules, M. and Blais, J.L. (2010). Plasma vitellogenin in male teleost fish from 43 rivers worldwide is correlated with upstream human population size, Environmental Pollution. 158:3279-3284.

Devanathan, G., Subramanian, A., Someya, M., Sudaryanto, A., Isobe, T., Takahashi, S., Chakraborty, P., Tanabe, S. (2009). Persistent organochlorines in human breast milk from major metropolitan cities in India. Environomental Pollution. 157: 148-154.

Devlin, R.H. and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences, Aquaculture. 208:191-364.

Dethlefesen, V., Von Westernhagen, H. and Cameron, P. (1996). Malformation in North Sea pelagic fish embryos during the period 1984-1995, ICES Journal of Marine Science. 53: 1024-1035.

Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C. (2009). Endocrine-Disrupting Chemicals: An Endocrine Society Scientific Statement, Endocrine Reviews. 30(4): 293-342.

Dutta, H.M., Misquitta, D., Khan, S. (2006). The Effects of Endosulfan on the Testes of Bluegill Fish, Lepomis macrochirus: A Histopathological Study, Archives of Environmental Contamination and Toxicology. 51(1): 149–156.

Elzeinova, F., Novakova, V., Buckiova, D., Kubatova, A., Peknicova, J. (2008). Effect of low dose of vinclozolin on reproductive tract development and sperm parameters in CD1 outbred mice, Reproductive Toxicology. 26: 231–238.

Falconer, I.R., Chapman, H.F., Moore, M.R. and Ranmuthuga, G. (2006). Endocrine-disrupting compounds: A review of their challenge to sustainable and safe water supply and water reuse, Environmental Toxicology. 21(2): 181–191. DOI: 10.1002/tox.20172.

FAO (2003). The State of Food Insecurity in the World, Rome, Italy.

Fenske, M., van Aerle, R., Brack, S., Tyler, C.R., Segner, H. (2001). Development and validation of a homologous zebrafish vitellogenin ELISA and its application for studies on estrogenic chemicals, Comperative Biochemistry and Physiology C. 129: 217–232.

Ferraz, N., Carnevia, D., Nande, G., Rossotti, M., Miguez, M.N., Last, J.A. and Gonzalez-sapienza, G. (2007). Specific immunoassays for endocrine disruptor monitoring using recombinant antigens covered by degenerated primer PCR., Analytical and Bioanalytical Chemistry. 389: 2195-2202.

Filby, A.L., Thorpe, K.L., Maack, G., Tyler, C.R. (2007). Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish, Aquatic Toxicology. 81(2): 219-31.

Filby, A.L., Thorpe, K.L., Tyler, C.R. (2006). Multiple molecular effect pathways of an environmental oestrogen in fish, Journal of Molecular Endocrinology. 37: 121–134.

Folmar, L.C., Gardner, G.R., Schreibman, M.P., Magliulo-Cepriano, L., Mills, L.J., Zaroogian, G., Gutjahr-Gobell, R., Haebler, R., Horowitz, D.B., Denslow, N.D. (2001). Vitellogenin-induced pathology in male summer flounder (Paralichthys dentatus), Aquatic Toxicology. 51: 431–441.

Folmar, L.C., Hemmer, M., Hemmer, R., Bowman, C., Kroll, K., Denslow, N.D. (2000). Comparative estrogenicity of estradiol, ethinyl estradiol and

diethylstilbestrol in an in vivo, male sheepshead minnow (Cyprinodon variegatus), vitellogenin bioassay, Aquatic Toxicology. 49:77–88.

Fossi, M.C., Casini, S., Marsili, L., Neri, G., Mori, G., Ancora, S. (2002). Biomarkers for endocrine disruption in three species of Mediterranean large pelagic fish, Marine Environmental Research. 54: 667 671.

Foster, P. M. (2006). Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. Int. J. Androl. 29, 140–147; discussion 181–185.

Fromme, H., Gruber, L., Schlummer, M., Wolz, G., Bohmer, S., Angerer, J., Mayer, R., Liebl, B., Bolte, G. (2007b). Intake of phthalates and di(2-ethylhexyl) adipate: Results of the Integrated Exposure Assessment Survey based on duplicate diet samples and biomonitoring data, Environmental International . 33: 1012-1020.

Fry, D.M., Toone, C.K. (1981). DDT-induced feminization of gull embryos, 213(4510) 922-924. doi: 10.1126/science.7256288.

Funkenstein, B., Bowman, C., Denslow, N.D., Cardinali, M., Carnevali, O. (2000). Contrasting effects of estrogen on transthyretin and vitellogenin expression in males of the marine fish Sparus aurata, Molecular and Cellular Endocrinology. 167:33–41.

Gagne, F., Blaise, C., Aoyama, I., Luo, R., Gagnon, C., Couillard, Y., Campbell, P., Salazar, M. (2002). Biomarker study of a municipal effluent dispersion plume in two species of freshwater mussels, Environmental Toxicology. 17: 149–159.

Garcia-Reyero, N., Barber, D.S., Gross, T.S., Johnson, K.G., Sepulveda, M.S., Szabo, N.J., Denslow, N.D. (2006). Dietary exposure of largemouth bass to OCPs changes expression of genes important for reproduction. Aquatic Toxicology. 78: 358–369.

Gardestrom, J., Gorokhova, E., Gilek, M., Grahn, M., Holmborn, B.E. (2006). Biomarkers of copepod feeding status Bengtsson, and M. Breitholtz. A multilevel approach to predict toxicity in copepod populations: Assessment of growth, genetics, and population structure, Aquatic Toxicology. 79: 41–48.

Gehring, M., Weltin, D., Tennhardt, L., Vogel, D., Bilitewski, B. (2002). Elimination of bisphenol A during wastewater treatment: detailed mass balances derived from laboratory experiments, Proceedings of the AWWA Endocrine Disruptors and the Water Industry Symposium, April 18 – 20, Cincinnati, Ohio, USA.

Georgescu, B., Georgescu, C., Dărăban, S., Bouaru, A., Pascalau, S. (2011) Heavy Metals Acting as Endocrine Disrupters , Animal Science and Biotechnologies. 44 (2).

Gimeno, S., Komen, H., Venderbosch, P.W.M., Bowmer, T. (1997). Disruption of sexual differentiation in genetic male common carp (Cyprinus carpio) exposed to an alkylphenol during different life stages, Environmental Science Technology. 31:2884–2890.

Gorokhova, E. (2003). Relationships between nucleic acid levels and egg production rates in Acartia bifilosa: implications for growth assessment of copepods in the northern Baltic proper, Marine Ecology Progress Series. 262:163-172.

Gray LE Jr., Ostby J, Furr J, Price M, Veeramachaneni DN, Parks L. (2000). Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicological Science. 58: 350-365.

Gronen, S., Denslow, N., Manning, S., Barnes, S., Barnes, D., Brouwer, M. (1999). Serum vitellogenin levels and reproductive impairment of male Japanese medaka (Oryzias latipes) exposed to 4-tert-octylphenol, Environmental Health Perspectives. 107: 385–390.

Guiguen, Y., Baroiller, J.F., Ricordel, M.J., Iseki, K., Mcmeel, O.M., Martin, S.A., Fostier, A. (1999). Involvement of estrogens in the process of sex differentiation in two fish species: The rainbow trout (Oncorhynchus mykiss) and a tilapia (Oreochromis niloticus), Molecular Reproduction and Development. 54:154–162.

Guiletter, L.J. (2000). Contaminant-induced endocrine disruption in wildlife. Growth hormone and IGF, Research. 10: 45–50.

Guillette, L.J., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.F., Woodward, A.R. (1994). Developmental abnormalities of the gonad and abnormal sex-hormone concentrations in juvenile alligators from contaminated and control lakes in Florida, Environmental Health Perspectives. 102(8): 680-688.

Gunnarsson, L., Kristiansson, E., Forlin, L., Nerman, O. and Larsson, D.G. (2007). Sensitive and robust gene expression changes in fish exposed to estrogen – a microarray approach, BMC Genomics. 8: 149.

Gunwant, P. (2014). Gadekar studies on the seasonal histomorphological changes in the ovary of Indian major carp, *Labeo rohita* (ham.), The Bioscan. 9(3): 1037-1042.

Halm, S., Pounds, N., Maddix, S., Rand-Weaver, M., Sumpter, J.P., Hutchinson, T.H., Tyler, C.R. (2002). Exposure to exogenous 17β-oestradiol disrupts P450aromB mRNA

expression in the brain and gonad of adult fathead minnows (Pimephales promelas). Aquatic Toxicology 60: 285-299.

Han, S.W., Lee, H., Han, S.Y., Lim, D.S., Jung, K.K., Kwack, S.J., Kim, K.B., Lee, B.M. (2009). An exposure assessment of di-(2-ethylhexyl) phthalate (DEHP) and di-n-butyl phthalate (DBP) in human semen, Journal of Toxicology and Environmental Health, Part A. 72:1463–1463.

Hansen, P.D., Dizer, H., Hock, B., Marx, A., Sherry, J., McMaster, M., Blaise, C. (1998). Vitellogenin- biomarker for endocrine disruptor, Trends in Analytical Chemistry. 17: 448-451.

Harries, J.E., Runnalls, T., Hill, E., Harris, C.A., Maddix, S., Sumpter, J.P., Tyler, C.R. (2000). Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (Pimephales promelas), Environmental Science & Technology. 34: 3003-3011

Harris, C.A., Santos, E.M., Janbakhsh, A., Pottinger, T.G., Tyler, C.R. and Sumpter, J.P. (2001). Nonylphenol affects gonadotropin levels in the pituitary gland and plasma of female rainbow trout, Environmental Science and Technology. 35: 2909-2916.

Harris, R.M., Kirk, C.J., Waring, R.H. (2005). Non-genomic effects of endocrine disruptors: inhibition of estrogen sulfotransferase by phgenols and chlorinated phenols, Molecular and Cellular Endocrinology. 244: 72–74.

Hashimoto, S., Bessho, H., Hara, A., Nakamura, M., Iguchi, T. and Fujita, K. (2000). Elevated serum vitellogenin levels and gonadal abnormalities in wild male flounder (Pleuronectes yokohamae) from Tokyo Bay, Japan, Marine Environmental Research. 49: 37 53.

Hassanin, A., Kuwahara, S., Tsukamoto, Y., Ogawa, K., Hiramatsu, K., Sasaki, F. (2002). Gonadosomatic index and testis morphology of common carp (Cyprinus carpio) in rivers contaminated with estrogenic chemicals, Journal of Veterinary Medical Science. 64: 921–926.

Hatef, A., Alavi, S.M.H., Abdulfatah, A., Fontaine, P., Rodina, M., Linhart, O. (2012a). Adverse eff ects of Bisphenol A on reproductive physiology in male goldfi sh at environmentally relevant concentrations, Ecotoxicology and Environmental Safety. 76: 56–62.

Hatef, A., Alavi, S.M.H., Milla, S., Butts, I.A.E., Rodina, M., Carnevali, O., Fontaine, P., Linhart, O. (2012b). Di-(2- ethylhexyl)-phthalate impairs sperm quality in goldfi sh associated with disruption in androgenesis. (submitted)

Hatef, A., Alavi, S.M.H., Milla, S., Křišťan, J., Golshan, M., Fontaine, P., Linhart, O. (2012c). Anti-androgen vinclozolin impairs sperm quality and steroidogenesis in goldfish, Aquatic Toxicology. 122–123: 181–187.

Hatef, A., Zare, A., Alavi, S.M.H., Habibi, H.R., Linhart, O. (2012d). Modulations in androgen and estrogen mediating genes and testicular response in male goldfish exposed to bisphenol A, Environmental Toxicology and Chemistry. 10.1002/etc.1919.

Haubruge, E., Petit, F., Gage, M.J. (2000). Reduced sperm counts in guppies (Poecilia reticulata) following exposure to low levels of tributylin and bisphenol A, Proceedings of the Royal Society B: Biological Sciences. 267: 2333-2337.

Hayes, T.B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., Lee, M., Mai, V.P., Marjuoa, Y., Parker, J. and Tsui, M. (2006). Pesticide mixtures, endocrine disruption, and amphibians: are we underestimating the impacts?, Environmental Health Perspectives. 114: 40-50.

Hayes, T.B., Collins, A., Lee, M., Mendoza, M., Noriega, N., Stuart, A.A. (2002). Hermaphroditic demasculinized frogs after exposure to the herbicide atrazine at slow ecologically relevant doses, Proc Natl Acad Sci U S A. 99(8):5476-80.

Hecker, M., Tyler, C.R., Hoffmann, M., Maddix, S., Karbe, L. (2002). Plasma biomarkers in fish provide evidence for endocrine modulation in the Elbe River, Germany, Environmental Science & Technology. 36:2311–2321.

Hemmer, M.J., Hemmer, B.L., Bowman, C.J., Kroll, K.J, Folmar, L.C., Marcovich, D. (2001). Effects of p-nonylphenol, methoxychlor, and endosulfan on vitellogenin induction and expression in sheepshead minnow (Cyprinodon variegatus), Environmental Toxicology and Chemistry. 20: 336–343.

Hilscherova, K., Jones, P.D., Gracia, T., Newsted, J.L., Zhang, X.W., Sanderson, J.T., Yu, R.M.K., Wu, R.S.S., Giesy, J.P. (2004). Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR, Toxicological Sciences. 81: 78–89.

Hinfray, N., Cheshenko, K., Ait-Aissa, S., Palluel, O., Porcher, J., Pakdel, F., Eggen, R.I.L., Kah, O. and Brion, F. (2008). Brain and gonadal aromatases as molecular and biochemical targets of endocrine disrupters in a model species, the zebrafish (Danio rerio), Cybium. 32(2): 34-36.

Hjelmborg, P.S., Ghisari, M. and Bonefeld-jorgensen, E.C. (2006). SPE-HPLC purification of endocrine disrupting compounds from human serum for assessment of xenoestrogenic activity. Analytical and Bioanalytical Chemistry. 385: 875-887.

Hogan, N.S., Duarte, P., Wade, M.G., Lean, D.R.S., Trudeau, V.L. (2008). Estrogenic exposure affects metamorphosis and alters sex ratios in the northern leopard frog (Rana pipiens): identifying critically vulnerable periods of development, General of Comparative Endocrinology. 156: 515-523.

Holbech, H., Kinnberg, K. Petersen, G.I., Jackson, P., Hylland, K., Norrgren, L. and Bjerregaard,P. (2006). Detection of endocrine disrupters: Evaluation of a Fish Sexual Development Test (FSDT), Comparative Biochemistry and Physiology C-Toxicology and Pharmacology. 144: 57-66.

Hornung, M.W., Jensen, K.M, Korte, J.J, Kahl, M.D, Durhan, E.J, Denny, J.S, Henry, T.R, Ankley, G.T. (2004). Mechanistic basis for estrogenic effects in fathead minnow (Pimephales promelas) following exposure to the androgen 17α - methyltestosterone: conversion of 17α -methyltestosterone to 17α –methylestradiol, Aquatic Toxicology . 66: 15–23.

Houtman, C.J. (2010). Emerging contaminants in surface water and their relevance for the production of drinking water in Europe, Journal of Integrative Environmental Sciences. 7(4): 271–295.

Huang, H., Leung, L.K. (2009). Bisphenol A down regulates CYP19 transcription in JEG-3 cells, Toxicology Letter. 189: 248–252.

Huang, P.C., Tien, C.J., Sun, Y.M., Hsieh, C.Y. and Lee, C.C. (2008). Occurrence of Phthalates in sediment and biota: Relationship to aquatic factors and the biota-sediment accumulation factor, Chemosphere .73 : 539-544.

Hwang, K.A., Park, S.H., Yi, B.R., Choi, K.C. (2011). Gene Alterations of Ovarian Cancer Cells Expressing Estrogen Receptors by Estrogen and Bisphenol A Using Microarray Analysis, Laboratory Animal Research. 27(2):99-107. doi:10.5625/lar.2011.27.2.99.

Ibiam, U. and Grant, A. (2005). RNA/DNA ratios as a sublethal endpoint for large-scale toxicity tests with the nematode Caenorhabditis elegans. Environmental Toxicology and Chemistry. 24(5):1155-9.

IPCS. (2002). Global assessment of the state-of-the-science of endocrine disruptors. Geneva, International Programme on Chemical Safety, World Health Organization and United Nations Environment Programme.

Iwamatsu, T., Kobayashi, H., Sagegami, R., Shuo, T. (2006a). Testosterone content of developing eggs and sex reversal in the medaka (Oryzias latipes). General of Comperative Endocrinology. 145: 67–74.

James, R., Sampath, K. (1999). Effect of zeolite on the reduction of cadmium toxicity in water and a freshwater fish Oreochromis mossambicus, Bulletin of Environmental Contamination and Toxicology. 62: 222–229.

Jarmołowicz, S., Demska-Zakes, K., Zakes ,Z. (2013). Impact of di-n-butyl phthalate on reproductive system development in European pikeperch (Sander lucioperca), Acta Veterinaria Brunensis.82: 197–201.

Jarosova, A. (2006). Phthalic acid esters (PAEs) in the food chain. Czech Journal of Food Sciences, 5: 223–231.

Jensen, J., Adare, K., Shearer, R. (1997). Canadian Arctic Contaminants Assessment Report (CACAR) (eds.), Department of Indian Affairs and Northern Development, Ottawa.

Jensen, K.M., Korte, J.J., Kahl, M.D., Pasha, M.S., Ankley, G.T. (2001). Aspects of basic reproductive biology and endocrinology in the fathead minnow (Pimephales promelas), Comparative Biochemistry and Physiology - Part C. 128: 127–141.

Jobling, S. and Tyler, C.R. (2006). Introduction: the ecological relevance of chemically induced endocrine disruption in wildlife, Environmental Health Perspectives. 114: 7–8.

Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawlowski, S., Baunbeck, T., Turner, A.P. and Tyler, C.R. (2004). Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent, Aquatic Toxicology. 66: 207-222.

Jobling, S., Coey , S. Whitmore, J.G., Kime, D.E., Van Look, K.J.W., McAllister, B.G., Beresford, N., Henshaw , A.C., Brighty , G., Tyler, C.R. and Sumpter, J.P. (2002b). Wild intersex roach (Rutilus rutilus) have reduced fertility, Biology of Reproduction. 67: 515-524.

Jobling, S., Coey, S., Whitmore, J.G., Kime, D.E., Van Look, K.J.W., McAllister, B.G. (2002). Wild intersex roach (Rutilus rutilus) have reduced fertility, Biology of Reproduction. 67: 515–524.

Jobling, S., Reynolds, T., White, R., Parker, M.G., Sumpter, J.P. (1995). A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic, Environmental Health Perspectives.103:582–587. doi:10.2307/3432434.

Jyothi, B. and Narayan, G. (1999). Certain Pesticide-induced carbohydrate metabolic disorders in the serum of freshwater fish Clarias batrachus (Linn.). Food and

Chemical Toxicology. 37: 417-421.

Kallivretaki, E., Eggen, R., Neuhauss, S., Alberti, M., Kausch, U., Segner, H. (2006). Aromatase in zebrafish: A potential target for endocrine disrupting chemicals, Marine Environmental Research. 62: 187 -190.

Kang, I.J., Yokota, H., Oshima, Y., Tsuruda, Y., Oe, T., Imada, N., Tadokoro, H., Honjo, T. (2002). Effects of Bisphenol A on the reproduction of Japanese medaka (Oryzias latipes), Environmental Toxicology and Chemistry. 21: 2394-2400.

Karels, A.A., Manning, S., Brouwer, T.H., Brouwer, M. (2003). Reproductive effects of estrogenic and antiestrogenic chemicals on sheepshead minnows (*Cyprinodon variegatus*), Environmental Toxicology and Chemistry. 22: 855-865.

Kaur, K. and Virk, S. (1983). Effect of BHC and Sumithion on ovarian recrudescence in common carp Cyphnus carpio, Indian Journal of Experimental Biology. 10: 182- 187.

Kaur, T. and Saxena, P.K. (2002). Impact of pollution on the ovarian maturation in certain fresh water teleosts dwelling in river Sutlaj : A morphological study, Pollution Research 21 (2): 227 – 233.

Kavanagh, R. J., Balch, G.C., Kiparissis, Y., Niimi, A.J., Sherry, J., Tinson, C. and Metcalfe, C.D. (2004). Endocrine disruption and altered gonadal development in white perch (Morone americana) from the lower Great Lakes region, Environmental Health Perspectives. 112: 898-902.

Kazeto, Y., Allen, R.P., Trant, J.M. (2004). Effects of endocrine disrupting chemicals on the expression of CYP19 genes in zebrafish (Danio rerio) juveniles, Aquatic Toxicology.69: 25–34

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M. and Flick, R.(2007). Collapse of a fish population following exposure to a synthetic estrogen, Proceedings of the National Academy of Science. 104: 8897-8901.

Kim, E.J., Kim, J.W. and Lee, S. K. (2002). Inhibition of oocyte development in Japanese medaka (Oryzias latipes) exposed to di-2-ethylhexyl phthalate, Environment International. 28: 359-65.

Kim, S.G., Kang, J.C. (2004). Effect of dietary copper exposure on accumulation, growth and haematological parameters of the juvenile rockfish Sebastes schlegeli, Marine Environmental Research. 58: 65–82.

King, S.C. and Hassell, K. (2008). Preliminary Assessment of an Indirect Elisa and Western Blot Techniques to Screen Vitellogenin in Barramundi as a Potential Biomarker of Exposure to EDCs in Australian Tropical Waters, Australasian, Journal of Ecotoxicology. 14: 109-116.

Kinnberg, K., Korsgaard, B., Bjerregaard, P. and Jespersen, A. (2000). Effects of nonylphenol and 17β-estradiol on vitellogenin synthesis and testis morphology in male platyfish Xiphophorus maculates, The Journal of Experimental Biology. 203: 171-181.

Kinnberg, K., Toft, G. (2003). Effects of estrogenic and antiandrogenic compounds on the testis structure of the adult guppy (*Poecilia reticulata*), Ecotoxicology Environmental Safety. 54: 16–24.

Kiparissis, Y., Metcalfe, T.L., Balch, G.C., Metcalfe, C.D. (2003). Effects of the antiandrogens, vinclozolin and cyproterone acetate on gonadal development in the Japanese medaka (*Oryzias latipes*), Aquatic Toxicology. 63: 391– 403.

Kirby, M.F., Allen, Y.T., Dyer, R.A., Feist, S.W., Katsiadaki, I., Matthiessen, P., Scott, A.P., Smith, A., Stentiford, G.D., Thain, J.E., Thomas, K.V., Tolhurst, L., Waldock, M.J. (2004). Surveys of plasma vitellogenin and intersex in male flounder (Platichthys flesus) as measures of endocrine disruption by estrogenic contamination in United Kingdom estuaries: Temporal trends, 1996 to 2001, Environmental Toxicology and Chemistry. 23: 748–758.

Kishida, M., McLellan, M., Miranda, J.A., Callard, G.V. (2001). Estrogen and xenoestrogens unregulated the brain aromatase isoform (P450aromB) and perturb markers of early development in zebrafish (*Danio rerio*), Comparative Biochemistry and Physiology - Part B. 129: 261–268.

Klaper, R., Rees, C.B., Drevnick, P., Weber, D., Sandheinrich, M., Carvan, M.J. (2006). Gene expression changes related to endocrine function and decline in reproduction in fathead minnow (Pimephales promelas) after dietary methyl mercury exposure, Environmental Health Perspectives. 114: 1337–1343.

Kloas, W. (2002). Amphibians model for the study of endocrine disruptors, International Review of Cytology. 216: 1-57.

Knorr, S., Braunbeck, T. (2002). Decline in reproductive success, sex reversal, and developmental alterations in Japanese medaka (Oryzias latipes) after continuous exposure to octylphenol, Ecotoxicology and Environmental Safety. 51(3): 187–196.

Korte, J.J., Kahl, M.D., Jensen, K.M., Pasha, M.S., Parks, L.G., LeBlanc, G.A., Ankley, G.T. (2000). Fathead minnow vitellogenin: complementary DNA sequence and messenger RNA and protein expression after 17β-estradiol treatment, Environmental Toxicology and Chemistry. 19: 972–981.

Krisfalusi, M., Nagler, J.J. (2000). Induction of gonadal intersex in genotypic male rainbow trout (Oncorhynchus mykiss) embryos following immersion in estradiol- 17beta, Molecular Reproduction and Development. 56: 495–501.

Kuhl, A.J., Brouwer, M. (2006). Antiestrogens inhibit xenoestrogen-induced brain aromatase activity but do not prevent xenoestrogen-induced feminization in Japanese medaka (Oryzias latipes), Environmental Health Perspectives. 114(4): 500-506.

Kuhl, A.J., Manning, S., Brouwer, M. (2005). Brain aromatase in Japanese medaka (Oryzias latipes): molecular characterization and role in xenoestrogen-induced sex reversal, The Journal of Steroid Biochemistry and Molecular Biology. 96: 67–77.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., Gustafsson, J.A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β, Endocrinology. 139: 4252–4263.

Kumar, V., Chakraborty, A., Viswanath, G., Roy, P. (2008) Androgenic endocrine disruptors in wastewater treatment plant effluents in India: Their influence on reproductive processes and systemic toxicity in male rats, Toxicology and Applied Pharmacology. 226(1):60-73.

Kwon, J.Y., McAndrew, B.J., Penman, D.J. (2001). Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia Oreochromis niloticus. Molecular Reproduction and Development. 59: 359–370.

Labadie, P., Budzinski, H. (2006). Alteration of steroid hormone profile in juvenile turbot (Psetta maxima) as a consequence of short-term exposure to 17alpha-ethynylestradiol, Chemosphere. 64 (8): 1274-86.

Lahnsteiner, F., Berger, B., Kletzl, M., Weismann, T. (2005). Effect of bisphenol A on maturation and quality of semen and eggs in the brown trout, Salmo trutta f. fario. Aquatic Toxicology. 75:213–224.

Larkin, P., Knoebl, I., Denslow, N.D. (2003). Differential gene expression analysis in fish exposed to endocrine disrupting compounds, Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.136: 149–161.

Latini, G., Verzotti, A., De Felice, C. (2004). Di-2-ethylhexylphthlate and endocrine disruption: a review, Current Drug Targets - Immune, Endocrine & Metabolic Disorders. 4: 37–40.

Lattier, D.L., Gordon, D.A., Burks, D.J., Toth, G.P. (2001). Vitellogenin gene transcription: a relative quantitative exposure indicator of environmental estrogens, Environmental Toxicology and Chemistry. 20: 1979–1985

Lavado, R., Thibuat, R., Raldua, D., Martin, R., Porte, C. (2004). First evidence of endocrine disruption in feral carp from the Ebro River, Toxicology and Applied Pharmacology. 196: 247-257.

Leanos-Castaneda, O., Van Der K.G. (2007). Functional characterization of estrogen receptor subtypes, ERα and ERβ,mediating vitellogenin production in the liver of rainbow trout, Toxicology and Applied Pharmacology. 224: 116–125.

Lee, C., Na, J.G., Lee, K.C., Park, K. (2002). Choriogenin mRNA induction in male medaka, Oryzias latipes as a biomarker of endocrine disruption, Aquatic Toxicology. 61(3–4):233–241.

Lee, S.K., Veeramachaneni, D.N. (2005). Subchronic exposure to low concentrations of di-n-butyl phthalate disrupts spermatogenesis in Xenopus laevis frogs, Toxicological Sciences. 84(2): 394-407.

Lee, Y.M., Seo, J.S., Kim, I.C., Yoon, Y.D., Lee, J.S. (2006). Endocrine disrupting chemicals (bisphenol A, 4-nonylphenol, 4-tert-octylphenol) modulate expression of two distinct cytochrome P450 aromatase genes differently in gender types of the hermaphroditic fish Rivulus marmoratus, Biochemical and Biophysical Research Communications. 345: 894–903.

Levy, G., Lutz, I., Kruger, A., Kloas, W. (2004). Bisphenol-A induces feminization in Xenopus laevis tadpoles, Environmental Research 94:102–111.

Loomis, A.K., Thomas, P. (2000). Effects of estrogens and xenoestrogens on androgen production by Atlantic croaker testes in vitro: evidence for a nongenomic action mediated by an estrogen membrane receptor, Biology of Reproduction. 62: 995–1004.

Luskova, V., Svoboda, M. and Kolarova, J. (2002). The effect of diazinon on blood plasma biochemistry in Carp (Cprinus carpio L.), Acta Veterinaria Brunensis. 71: 117-123.

Mackenzie, C.A., Berrill, M., Metcalfe, C., Pauli, B.D. (2003). Gonadal differentiation in frogs exposed to estrogenic and antiestrogenic compounds, Environmental Toxicology Chemistry. 22: 2466-2475.

MacLatchy, D.L., Courtenay, S.C., Rice, C.D., Van der Kraak, G.J. (2003). Development of a short-term reproductive endocrine bioassay using steroid hormone and vitellogenin end points in the estuarine mummichog (Fundulus heteroclitus), Environmental Toxicology and Chemistry. 22: 996–1008.

Magadalenal, N.N., Prabhu, R., Inbaraj, R.M. (2011). In vitro effect of endocrine disrupting chemical on the gonadal steroidogenesis in an Indian major carp, *Labeo rohita* .Indian Journal of Science and Technology. (9th ISRPF issue).

Makynen, E.A., Kahl, M.D., Jensen, K.M., Tietge, J.E., Wells, K.L., Van Der Kraak, G., and Ankley, G.T. (2000). Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (Pimephales promelas), Aquatic Toxicology. 48, 461–475.

Malik, A. 2006. Studies on the trace organics and major ion chemistry of the Gomati River. Ph.D. Thesis submitted to the Gurukul Kangri University, Hardwar, India.

Malik, A., Singh, K.P. and Ojha, P. (2007). Residues of Organochlorine Pesticides in Fish from the Gomti River, India, Springer Science Business Media, LLC.

Malik, A., Singh, K.P., Mohan, D., Patel, D.K. (2004). Distribution of polycyclic aromatic hydrocarbons in Gomti river system of India, Bulletin of Environmental Contamination Toxicology. 72:1211–1218.

Mandich, A., Bottero, S., Benfenati, E., Cevasco, A., Erratico, C., Maggioni, S., Massari, A., Pedemonte, F., Vigano,` L. (2007). In vivo exposure of carp to graded concentrations of bisphenol A, General and Comparative Endocrinology. 153:15-24.

Mandiki, N.M., Virginie, G., Koen, M., Dirk, E., Els, D.R., Tom, D.B., Steven, A.S., Declerck, L.D.M., Catherine, B., Edwige, V.H., Marie-Louise, S., Patrick, K. (2014). Effect of land use on pollution status and risk of fish endocrine disruption in small farmland ponds. Syaghalirwa, Hydrobiologia. 723: 103–120.

Mandiki, S.N.M., Babiak, I., Bopopi, J. M., Leprieur, F. and Kestemont, P. (2005). Effects of sex steroids and their inhibitors on endocrine parameters and gender growth differences in Eurasian perch (Perca fluviatilis) juveniles, Steroids. 70: 85-94.

Marciniak, A.T. and Witczak, A. (2010). Distribution of endocrine-disrupting pesticides in water and fish from the Oder River Poland, Acta Ichthyologica et piscatorial. 40(1): 1–9 doi:10.3750/aip2010.40.1.01.

Marin, M.G., Matozzo, V. (2004). Vitellogenin induction as a biomarker of exposure to estrogenic compounds in aquatic environments, Marine Pollution Bulletin. 48: 835–839.

Martyniuk, C.J., Gerrie, E.R., Popesku, J.T., Ekker, M., Trudeau, V.L. (2007). Microarray analysis in the zebrafish (Danio rerio) liver and telencephalon after exposure to low concentration of 17alpha-ethinylestradiol, Aquatic Toxicology 84:38–49.

Martyniuk, C.J., Spade, D.J., Blum, J.L., Kroll, K.J., Denslow, N.D. (2011). Methoxychlor affects multiple hormone signaling pathways in the largemouth bass (Micropterus salmoides) liver, Aquatic Toxicology. 101 483–492.

Mastin, J.P. (2005). Environmental cardiovascular disease, Cardiovascular Toxicology. 5: 91–94.

Matozzo, V., Gagne, F., Marin, M.G., Ricciardi, F. and Blaise, C. (2008). Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review, Environment International. 34: 531-545.

Matta, M.B., Linse, J., Cairncross, C., Francendese, L., Kocan, R.M. (2001). Reproductive and transgenerational effects of methylmercury or Aroclor 1268 on Fundulus heteroclitus, Environmental Toxicology and Chemistry. 20: 327–335.

Matthiessen, P. (2008). An assessment of endocrine disruption in mollusks and the potential for developing internationally standardized mollusk life cycle test guidelines, Environmental Assessment and Management. 4(3): 274–284.

Matthiessen, P., Allen, Y., Bamber, S., Craft, J., Hurst, M., Hutchinson, T., Feist, S., Katsiadaki, I., Kirby, M., Robinson, C. (2002). The impact of oestrogenic and androgenic contamination on marine organisms in the United Kingdom—Summary of the EDMAR programme Endocrine Disruption in the Marine, Environment Marine Environmental Research.54: 645–649.

Matthiessen, P., Gibbs, P.E. (1998) Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks, Environmental Toxicology and Chemistry. 17(1): 37-43.

Matthiessen, P., Sumpter, J.P. (1998). Effects of estrogenic substances in the aquatic environment. In: Fish Ecotoxicology (Braunbeck, T., Hinton, D.E., Streit, B., Eds.). Burkhauser Verlag, Basel, Switzerland, pp. 319-335.

Melo, M.M., Nascimento, E.F., Oliveira, N.F.J. (1999). Intoxicação de bovinos por aflatoxina B1 presente em polpa cítrica: relato de um surto, The Arquivo Brasileiro de Medicina Veterinária e Zootecnia.

Mitchell, R.T., Childs, A.J., Anderson, R.A., van den Driesche. S., Saunders, P.T., McKinnell, C., Wallace, W.H., Kelnar, C.J., Sharpe, R.M. (2012). Do phthalates affect steroidogenesis by the human fetal testis? Exposure of human fetal testis xenografts to di-n-butyl phthalate, The Journal of Clinical Endocrinology & Metabolism. 97: E341-E348.

Moens, L.N., van der Ven, K., Van, R.P., Del-Favero, J., De Coen, W.M. (2007). Gene expression analysis of estrogenic compounds in the liver of common carp (Cyprinus carpio) using a custom cDNA microarray, Journal of Biochemical and Molecular Toxicology. 21: 299–311.

Mohan, M.R. (2000) Malathion induced changes in the fresh water fish, *Glossocobtus giuris* (ham), Pollution research. 19 (1) 273-75.

Montserrat, N., Gonzalez, A., Mendez, E., Piferrer, F., Planas, J.V. (2004). Effects of follicle stimulating hormone on estradiol-17b production and P-450 aromatase (CYP19) activity and mRNA expression in brown trout vitellogenic ovarian follicles in vitro, General and Comparative Endocrinology. 137: 123-131.

Mosconi, G., Carnevali, O., Franzoni, M.F., Cottone, E., Lutz, I., Kloas, W., Yamamoto, K., Kikuyama, S., Polzonetti-Magni, A.M. (2002). Environmental estrogens and reproductive biology in amphibians. General of Comperative Endocrinology. 126: 124-129.

Murmur, S., Srivastava, V.K. (2011). Protective Action of an Anti-oxidant (Vitamin-C) Against Bisphenol-toxicity in Cirrhinus mrigala (Ham.), Turkish Journal of Fisheries and Aquatic Sciences. 11: 25-29.

Nadzialek, S., Depiereux, S., Mandiki, S.N.M. and Kestemont, P. (2011). In vivo biomarkers of estrogenicity: limitation and interpretation in wild environment, Archive Environmental Contamination. 60: 471-478.

Narayana, K., Prashanthi, N., Nayanatara, A., Kumar,H.H., Abhilash, K., Bairy, K.L. (2006). Neonatal methyl parathion exposure affects the growth andfunctions of the male reproductive system in the adult rat, Folia Morphologica (Warsz). 65(1):26-33.

Nash, J.P., Kime, D.E., Van der Ven, L.T., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R. (2004). Long-term exposure to environmental concentrations of the pharmaceutical ethinylestradiol causes reproductive failure in fish, Environmental Health Perspectives. 112: 1725–1733.

Nayak, A., Raha, R. and Das, A.K. (1995). Organochlorine pesticide residues in middle stream of the Ganges River, India, Bulletin of Environmental Contamination and Toxicology. 54: 68–75.

Nolan, M., Jobling, S., Brighty, G., Sumpter, J.P., Tyler, C.R. (2001). A histological description of intersexuality in the roach, Journal of Fish Biology. 58(1): 160–176.

Norman, A., Borjeson, H., David, F., Tienpont, B. and Norrgren, L. (2007). Studies of uptake, elimination and late effects in Atlantic salmon (Salmo salar) dietary exposed to di-2-ethylhexyl phthalate (DEHP) during early life, Archives of Environmental Contamination and Toxicology (AECT).

Oishi, S., Hiraga, K. (1980). Testicular atrophy induced by phthalic acid esters: effect on testosterone and zinc concentrations, Toxicology and Applied Pharmacology 53(1): 35–41.

Orlando, E.F., William, P.D., and Louis J. Guillette Jr. (2002). Aromatase Activity in the Ovary and Brain of the Eastern Mosquitofish (Gambusia holbrooki) Exposed to Paper Mill Effluent, Environmental Health Perspectives. 110: 429-433.

Orn, S., Holbech, H., Madsen, T.H., Norrgren, L., Petersen, G.I. (2003). Gonad development and vitellogenin production in zebrafish (Danio rerio) exposed to ethinylestradiol and methyltestosterone. Aquatic Toxicology. 65: 397-411.

Orton, F., Carr, J.A., Handy, R.D. (2006). Effects of nitrate and atrazine on larval development and sexual differentiation in the northern leopard frog Rana pipiens. Environmental Toxicology and Chemistry. 25:65–71.

Paech, K., Webb, P., Kuiper, G.G., Nilsson, S., Gustafsson, J.A., Kushner, P.J. (1997). Differential ligand activation of estrogen receptors ERa and ERb AP1 sites, Science. 277: 1508–1510.

Pan, G., Dutta, H. (2000). Diazinon induced changes in the serum proteins of largemouth bass, Micropterus salmoides, Bulletin of Environmental Contamination and Toxicology. 64(2): 287-93.

Panter, G.H., Hutchinson, T.H., Lange, R., Lye, C.M., Sumpter, J.P., Zerulla, M. (2002). Utility of a juvenile fathead minnow screening assay for detecting (anti-) estrogenic substances, Environmental Toxicology and Chemistry. 21: 319–326.

Panter, G.H., Hutchinson, T.H., Lange, R., Whale, G., Sumpter, J.P., van Aerle, R., Tyler, C.R. (2002b). Quantifying histological development in the gonads of sexually maturing fathead minnows (Pimephales promelas): Phase two. ECFIC-EMSG AQ005. Brixham: CEFI-EMSG Aquatic Research Programme.

Parrott, J.L., Wood, C.S., Boutot, P., Dunn, S. (2003). Changes in growth and secondary sex characteristics of fathead minnows exposed to bleached sulphite mill effluent, Environomental. Toxicology and Chemistry.22: 2908–29.

Patino, R., Goodbred, S.L., Draugelis-Dale, R., Barry, C.E., Foott, J.S., Wainscott, M.R., Gross,T.S. and Covay, K.J. (2003). Morphometric and histopathological parameters of gonadal development in adult common carp from contaminated and reference sites in Lake Mead, Nevada, Journal of Aquatic Animal Health. 15:55–68.

Patisaul, H.B. and Adewale, H.B. (2009). Long-Term Effects of Environmental Endocrine Disruptors on Reproductive Physiology and Behavior, Frontiers in Behavioral Neuroscience. 3:10.

Pawlowski, S., Van Aerle, R., Tyler, C.R., Braunbeck, T. (2004). Effects of 17_-ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay, Ecotoxicology Environmental Safety. 57: 330–345.

Petrovic, M., Sole, M., Lopez de Alda, M., Barcelo, D. (2002). Endocrine disrupters in sewage treatment plants, receiving river waters and sediments. Integration of chemical analysis and biological effect on feral carps, Environmental Toxicology and Chemistry. 21: 2146 – 2156.

Pettersson, I., Berg, C. (2007). Environmentally relevant concentrations of ethynylestradiol cause female-biased sex ratios in Xenopus tropicalis and Rana temporaria, Environmental Toxicology and Chemistry. 26(5):1005-1009.

Pickford, D.B., Hetheridge, M.J., Caunter, J.E., Hall, A.T., Hutchinson, T.H. (2003). Assessing chronic toxicity of bisphenol A to larvae of the African clawed frog (Xenopus laevis) in a flow-through exposure system, Chemosphere. 53(3): 223–235.

Pratap, B.S., Singh, V. (2008). Bioaccumulation of hexachlorocyclohexane, dichlorodiphenyltrichloroethane, and estradiol-17*b* in catfish and carp during the pre-monsoon season in India, Fish Physiology and Biochemistry 34: 25–36 doi: 10.1007/s10695-007-9142-9.

Propper, R.C. (2005). The study of endocrine disruption compounds past approaches and new directions, Integrative and Comparative Biology, 45: 194-200.

Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R., Sumpter, J.P. (1994). Estrogenic effects of effluents from sewage treatment works. Chemical Ecology. 8: 275-285.

Qin, C., Burghardt, R., Smith, R., Wormke, M., Stewart, J., Safe, S. (2003). Peroxisome proliferator-activated receptor gamma agonists induce proteasomedependent

degradation of cyclin D1 and estrogen receptor alpha in MCF-7 breast cancer cells, Cancer Research. 63: 958–964.

Rahman, M.F., Siddiqui, M.K. (2006). Hematological and clinical chemistry changes induced by subchronic dosing of a novel phosphorothionate (RPR-V) in Wistar male and female rats, Drug Chemistry Toxicology. 29(1): 95-110.

Raksheskar, G.A. (2012). Influenced of Cypermethrin on DNA, RNA and RNA/DNA ratio in gills of the freshwater fish Channa striata, Bioscience Discovery. 3(1): 17-19.

Rasmussen, T.H., Teh, S.J., Bjerregaard, P., Korsgaard, B. (2005). Anti-estrogen prevents xenoestrogen-induced testicular pathology of eelpout (*Zoarces viviparus*), Aquatic Toxicology. 72: 177-194.

Rathod, N.D., R.V. Kshirsagar. (2010).Quantification of nucleic acid from fresh water fish Punctius arenatus (Day) exposed to pesticides, International Journal of Advanced Biotechnology and Research. 11(1): 43-51.

Ray, E. J. 2006. Effect of p,p' DDE on reproduction and biomarkers of endocrine disruption in fatherad minnows (*pimephales promelas*). MSc. Thesis University of florida.

Rebecca, E.M., Courtenay, S.C., Cagampan, S., MacLatchy, D.L. (2007). Effects on reproductive potential and endocrine status in the mummichog (Fundulus heteroclitus) after exposure to 17alpha-ethynylestradiol in a short-term reproductive bioassay, Aquatic Toxicology. 85(2): 154-66.

Ripley, J.L, Iwanowicz, L.R, Blazer, V.S. and Foran, C.M. (2008). Utilization of protein profiles as indicators of environmental impairment of smallmouth bass (Micropterus dolomieu) from the Shenandoah River, VA, USA. Environmental Toxicology and Chemistry 27: 1756-1767.

Robinson, M.R, Escriva, H.G., Laudet, V. (2003). The nuclear receptor superfamily, Journal of Cell Science. 116(4):585–586.

Rosa, R., Nunes, A. (2003) Seasonal changes in nucleic acids, amino acids and protein content in juvenile Norway lobster (Nephrops norvegicus), Marine Biology. 143: 565–572.

Samir, K.K., Xie, B., Thompson, M.L., Sung, S.W., Ong, S.K. and Leeuvent, J.V. (2006). Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems, Environmental Science and Technology. 40: 6537–6546.

Sanghi, R., Pillai, M.K., Jayalekshmi, T.R., Nair, A. (2003). Organochlorine and organophosphorus pesticide residues in breast milk from Bhopal, Madhya Pradesh, India. Human Experimental Toxicology. 22:73-76.

Schinder, W.C. (1957). Determination of nucleic acid in tissue by pentose analysis: Methods in enzymology, (Eds:S.P. Colowick and N.O.kaplon), Academic press, New York. 111: 680-684.

Schoenfuss, H.L., Bartell, S.E., Bistodeau, T.B., Cediel, R.A., Grove, K.J., Zintek, Lee, K.E. and Barber, L.B. (2008). Impairment of the reproductive potential of male fathead minnows by environmentally relevant exposures to 4-nonylphenol, Aquatic Toxocology. 86: 91-98.

Schug, T.T., Janesick, A., Blumberg, B. and Heindel, J.J. (2011). Endocrine disrupting chemicals and disease susceptibility, The Journal of Steroid Biochemistry and Molecular Biology. 127: 204–215.

Schulz, R.W., Miura, T. (2002). Spermatogenesis and its endocrine regulation, Fish Physiology Biochemistry. 26: 43–56.

Schwaiger, J., Mallow, U., Ferling, H., Knoerr, S., Braunbeck, T., Kalbfus, W. (2002). How estrogenic is nonylphenol? A transgenerational study using rainbow trout (Oncorhynchus mykiss) as a test organism, Aquatic Toxicology. 59: 177–189.

Sepulveda, M.S., Johnson, W.E., Higman, J.C., Denslow, N.D., Schoeb, T.R. and Gross, T.S. (2002). An evaluation of biomarkers of reproductive function and potential contaminant effects in Florida largemouth bass (Micropterus salmoides floridanus) sampled from the St. Johns River, Science of the Total Environment. 289: 133-144.

Sharma, B.M. Bharat, G.K., Tayal, S., Nizzetto, L., Cupr P., Larssen, T. (2014). Environment and human exposure to persistent organic pollutants (POPs) in India: A systematic review of recent and historical data, Environment International. 66: 48–64.

Sharpe, R.M., Irvine, D.S. (2004). How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health?, BMJ□: British Medical Journal. 328(7437): 447-451.

Shilling, A.D., Williams, D.E. (2000). Determining relative estrogenicity by quantifying vitellogenin induction in rainbow trout liver slices, Toxicology and Applied Pharmacology. 164: 330–335.

Shioda, T., Wakabayashi, M. (2000) Effect of certain chemicals on the reproduction of medaka (Oryzias latipes), Chemosphere. 40: 239-243.

Singh, A.K. (2013). Introduction of modern endocrine techniques for the production of monosex population of fishes, General and Comparative Endocrinology. 181: 146–155.

Singh, A.K. and Singh, R. (2013). *In vivo* response of melatonin, gonadal activity and biochemical changes during aromatase inhibited sex reversal in common carp *Cyprinus carpio* (L), Animal Reproduction Science. 136: 317– 325.

Singh, A.K. and Srivastava, R. (2013). An overview of gonadal disruption in fishes inhabiting natural aquatic environments and future research need. Advances in Fish Research, Ed. U.C.Goswami Vol V, Narendra Publishing House, Delhi. 103-115.

Singh, A.K., Ansari, A., Singh, R. (2011). Possible impact of pharmaceutical wastes on aquatic environment. In: Water Pollution and Management, (Eds) D.S. Malik, Sunil Kumar and Umesh Bharti. Biotech Books, New Delhi. 371-386.

Singh, K.P., Malik, A., Sinha, S. (2007). Persistent organochlorine pesticide residues in soil and surface water of northern Indo- Gangetic alluvial plains, Environmental Monitoring and Assessment. 125: 147–155.

Singh, K.P., Singh, Malik, A., Mohan, D. and Takroo, R. (2005). Distribution of persistent organochlorine pesticide residues in Gomti River, India, Bulletin of Environmental Contamination and Toxicology. **74:** 146–154.

Singh, P.B. and Canario, A.V.M. (2004). Reproductive endocrine disruption in the freshwater catfish, Heteropneustes fossilis, in response to the pesticide c-hexachlorocyclohexane, Ecotoxicological and Environmental Safety. 58(1): 77–83.

Singh, P.B. and Singh, V. (2008). Pesticide bioaccumulation and plasma sex steroids in fishes during breeding phase from north India, Environmental Toxicology and Pharmacology. **25:** 342–350.

Singh, S., Singh, P.B. and Singh, V. (2007). Exposure and recovery response of isomers of HCH, metabolites of DDT and estradiol-17β in the female catfish, *Heteropneustes fossilis*, Environmental Toxicology and Pharmacology. **24:** 245–251.

Singh, S., Singh, P.B. and Singh, V. (2008). Bioaccumulation of hexachlorocyclohexane, dichlorodiphenyltrichloroethane, and estradiol-17β in

catfish and carp during pre-monsoon season in India, Fish Physiology and Biochemistry. **34:** 25–36.

Snyder, E.M., Snyder, S.A., Kelly, K.L., Gross, T.S., Villeneuve, D.L., Fitzgerald, S.D., Villalobos, S.A., Giesy, J.P. (2004). Reproductive responses of common carp (Cyprinus carpio) exposed in cages to influent of the Las Vegas Wash in Lake Mead, Nevada, from late winter to early spring, Environmental Science and Technology. 38: 6385-6395.

Sohoni, P., Tyler, C.R., Hurd, K., Caunter, J., Hetheridge, M., Williams, T., Woods, C., Evans, M., Toy, R., Gargas, M., Sumpter, J.P. (2001). Reproductive effects of longterm exposure to bisphenol A in the fathead minnow (Pimephales promelas), Environmental Science and Technology. 35: 2917- 2925.

Srivastava, A**.,** Sharma, P.V., Tripathi, R**.**, Kumar, R**.,** Patel, D**.**K., Mathur, P.K. (2010). Occurrence of phthalic acid esters in Gomti River Sediment, India, Environmental Monitoring and Assessment, 169: 397–406.

Stansley, W., Washuta, E.J. (2007). Initial Survey of Plasma Vitellogenin and Gonadal Development in Male Carp (Cyprinus carpio) From Three Locations in New Jersey, USA, Bulletin of Environmental Contamination and Toxicology. 78:181–185.

Sumpter, J.P. and Jobling, S. (1995). Vitellogenesis as a Biomarker for Estrogenic Contamination of the Aquatic Environment, Environmental Health Perspectives 103(7):173-178.

Suneetha, K., Kumar, K.G. and Veeraiah, K. (2010). Changes in protein subunits induced by endosulfan and fenvalerate in fresh water fish Labeo rohita through SDS-PAGE, Journal of Environmental Biology. 31(5): 759-763.

Swan, S. H. (2008). Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. Environmental Research. 108: 177–184.

Takeuchia, S., Iidab, M., Kobayashia, S., Jina, K., Matsudac, T., Kojimaa, H. (2005). Differential effects of phthalate esters on transcriptional activities via human estrogen receptors α and β, and androgen receptor, Toxicology. 210(2–3): 223–233.

Tan, S., Mahaffey, K., Evidence for mercurty as an endocrine disrupter: an overview of the literature, 2003, http://www.scribd.com/doc/1790574.

Tanaka, J.N., Grizzle, J.M. (2002). Effects of nonylphenol on the gonadal differentiation of the hermaphroditic fish, *Rivulus marmoratus*, Aquatic Toxicology. 57(3): 117-25.

Tetreault, G.R., Bennett, C.J., Shires, K., Knight, B., Servos, M.R. and McMaster, M.E. (2011). Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges, Aquatic Toxicology. 104: 278-290.

Thibaut, R., Porte, C., 2004. Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. J. Steroid Biochem. Mol. Biol. 92, 485–494.

Thomas, K.V., Balaam, J., Hurst, M., Nedyalkova, Z., Mekenyan, O. (2004). Potency and characterization of estrogen-receptor agonists in United Kingdom estuarine sediments. Environmental Science and Technology. 23: 471-479.

Tijani, J.O., Fatoba, O.O., Petrik, L.F. (2013). A review of pharmaceuticals and endocrine-disrupting compounds: sources, effects, removal, and detections, Water, Air and Soil Pollution, 224: 1770-1798.

Tripathi, G. (1992). Relative toxicity of aldrin, fenvalerate, captan and diazinon to the freshwater food fish, *Clarias batrachus*, Biomedical Environmental Science, 5: 33-38.

Tripathi, G., Harsh, S., Verma, P. (2001). Fenvalerate induced macromolecular changes in the catfish, *Clarias batrachus*, Environmental Biology. 23: 143-146.

Tripathi, P.K. (2003). Asian Fisheries Science Issue. 16: 349–359.

Tripathi, P.K., Srivastava, V.K., Singh, A. (2003). Toxic effect of dimethoate (organophosphate) on metabolism and enzyme system of fresh water teleost fish *Channa punctatus*, Asian Fisheries Science. 16: 349-359.

Tyler, C.R., Aerle, R.V., Hutchinson, T.H., Maddix, S. and Trip, H. (1999). An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin, Environmental Toxicology and Chemistry. 18(2): 337–347.

Tzchori, I., Degani, G., Hurvitz, A., Moava, B. (2004). Cloning and developmental expression of the cytochrome P450 aromatase gene (CYP19) in the European eel (Anguilla anguilla), General and Comparative Endocrinology. 138: 271–280.

U.S. EPA (2002). A short term test method for assessing the reproductive toxicity of endocrine disrupting chemicals using the fathead minnow (Pimephales promelas). ubiquitous water pollutant, 4-nonylphenol, Gen. Comp. Endocrinol. 164: 91–99 U.S. Environmental Protection Agency, Washington, DC. EPA/ 600/R-01/067.

Uchida, D., Yamashita, M., Kitano, T., Iguchi, T. (2004). An aromatase inhibitor or high water temperature induce oocyte apoptosis and depletion of P450 aromatase activity in the gonads of genetic female zebrafish during sexreversal, Comparative Biochemistry and Physiology – Part A: Molecular & Integrative Physiology. 137: 11-20.

Uren-Webster, T.M., Lewis, C., Filby, A.L., Paull, G.C., Santos, E.M. (2010). Mechanisms of toxicity of di (2-ethylhexyl) phthalate on the reproductive health of male zebrafish, Aquatic Toxicology 99: 360–369.

Van Aerle, R., Nolan, T.M., Jobling, S, Christiansen, L.B., Sumpter, J.P., Tyler, C.R. (2001). Sexual disruption in a second species of wild cyprinid fish (the Gudgeon, Gobio gobio) in United Kingdom freshwaters, Environmental Toxicology and Chemistry. 20(12): 2841–2847.

Van den Belt, K., Wester, P.W., van der Ven, L.T., Verheyen, R., Witters, H. (2002). Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): time dependency and reversibility, Environmental Toxicology and Chemistry. 21: 767-775.

Van den Berg, H. (2009). Global status of DDT and its alternatives for use in vector control to prevent disease, Environmental Health Perspective. 117: 1656–1663.

Van der Ven, L.T., Van den Brandhof, E.J., Vos, J.H., Wester, P.W. (2007). Effects of estrogen agonist 17-estradiol and antagonist tamoxifen in a partial life-cycle assay with zebrafish (*Danio rerio*), Environmental Toxicology and Chemistry. 26: 92–99.

Van der Ven, L.T., Wester, P.W., Vos, J.G. (2003). Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (*Danio rerio*), Environmental Toxicology and Chemistry. 22: 908-913.

Vandenberg, L.N., Hauser, R., Marcus, M., Olea, N., Welshons, W.V. (2007). Human exposure to bisphenol A (BPA), Reproductive Toxicology. 24: 139–177.

Vandenbergh, G.F., Janssen, C.R. (2001). Vitellogenesis as a biomarker of endocrinedisruption in the freshwater amphipod Hyalella azteca In: Goos HJT, Rastogi, R.K., Vaudry, H., Pierantoni, R., editors. Perspective in Comparative

Endocrinology: Unity and Diversity. Bologna (Italy): Monduzzi Editore;2001. p. 261–8.

Verma, R. and Singh, A.K. (2013). Endocrine disruptor in aquatic bodies: Issues and challenges, Journal of Ecology Environment and Biology, 1(1): 17-21.

Verma, Y. and Rana, S.V.S. (2009). Endocrinal toxicity of industrial solvents A Mini Review, Indian Journal of Experimental Biology. 47: 537-549.

Verslycke, T., De Wasch, K., De Brabander, H.F., Janssen, C.R. (2002). Testosterone metabolism in the estuarine mysid Neomysis integer (Crustacea; Mysidacea): identification of testosterone metabolites and endogenous vertebrate-type steroids, General and Comparative Endocrinology. 126: 190-199.

Versonnen, B.J., Goemans, G., Belpaire, C. and Janssen, C.R. (2004). Vitellogenin content in European eel (Anguilla anguilla) in Flanders, Belgium, Environmental Pollution. 128(3): 363-371.

Vethaak, A.D., Lahr, J., Schrap, S.M., Belfroid, A.C., Rijs, G.B.J., Gerritsen, A., Boer, J.D., Bulder, A.S., Grinwis, G.C.M., Kuiper, R.V., Legler, J., Murk, T.A.J., Peijnenburg, W., Verhaar, H.J.M. and Voogt, P.D. (2005). An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands, Chemosphere. 59: 511-524.

Vigano, L. , Arillo, A., Bottero, S., Massari, A., Mandich, A. (2001). First observation of intersex cyprinids in the Po River (Italy). Science of Total Environment. 269: 189–194.

Villeneuve, D.L., Villalobos, S.A., Keitha, T.L., Snydera, E.M., Fitzgerald, S.D., Giesy J.P. (2002). Effects of waterborne exposure to 4-nonylphenol on plasma sex steroid and vitellogenin concentrations in sexually mature male carp (Cyprinus carpio), Chemosphere. 47(1): 15–28.

Vos, J.G., Dybing, E., Greim, H.A., Ladefoged, O., Lambre, C., Tarazona, J.V., Brandt, I. and Vethaak, A.D. (2000). Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation, Critical Reviews in Toxicology. 30(1): 71–133.

Wang, X., Yang, Y., Zhang, L., Ma, Y., Han, J. (2013). Endocrine disruption by di-(2-ethylhexyl) - phthalate in Chinese rare minnow Gobiocypris rarus), Environmental Toxicology and Chemistry. 32(8): 1846–1854.

Wingfield, J.C. and Mukai, M. (2009). Endocrine disruption in the context of life cycles: perception and transduction of environmental cues, General Comparative Endocrinology. 163: 92-96.

Xu, H., Huang, J., Li, M., Gao, Z.B., Zhu, Y.F., Li, Y. (2013). The effects of di-(2-ethylhexyl) phthalate (DEHP) in testosterone synthesis and its molecular mechanisms in the fetal testis of male mouse by organ culture in vitro, Sichuan Da Xue Xue Bao Yi Xue Ban. 44(4): 511-6.

Yang, O., Kim, H.L., Weon, J.I., Seo, Y.R. (2015). Endocrine-disrupting Chemicals: Review of Toxicological Mechanisms Using Molecular Pathway Analysis, Journal of Cancer Prevention. 20(1): 12-24.

Yang, S., Wu, R.S.S. and Kong, R.Y.C. (2002). Physiological and cytological responses of the marine diatom Skeletonnema costatum to 2,4-dichlorophenol, Aquatic Toxicology 60, 33–41.

Ye, T., Kang, M., Huang, Q., Fang, C., Chen, Y., Shen, H., Dong, S. (2014). Exposure to DEHP and MEHP from hatching to adulthood causes reproductive dysfunction and endocrine disruption in marine medaka (Oryzias melastigma), Aquatic Toxicology. 146: 115– 126.

Ying, G., Kookana, R.S., Ru, Y. (2002). Occurrence and fate of hormone steroids in the environment, Environment International. 28: 545–551.

Zanotelli, V.R.T., Neuhaussb, S.C.F. and Ehrengruber, M.U. (2010). Long-term exposure to bis(2-ethylhexyl)phthalate (DEHP) inhibits growth of guppy fish (Poecilia reticulata), Journal of Applied Toxicology. 30: 29–33.

Zerulla, M., Lange, R., Steger-Hartmann, T., Panter, G., Hutchin-son, T., Dietrich, D.R. (2002). Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (Pimephales promelas), Toxicology Letter. 131: 51-63.

Zha, J., Sun, L., Spear, P.A., Wang, Z. (2008). Comparison of ethinylestradiol and nonylphenol effects on reproduction of Chinese rare minnows (Gobiocypris rarus). Ecotoxicology and environmental safety. 71: 390–399.

Zhang, Y. and Zhou, J.L. (2008). Occurrence and removal of endocrine disrupting chemicals in wastewater treatment, Chemosphere.73: 848–853.

Zhanga, X., Hecker, M., Parka, J., Tompsett, A.R., Newstedb, J., Nakayamaf, K., Jonesb, P.D., Auh, D., Kongh, R., Wuh, R.S.S., Giesya, J.P. (2008). Real-time PCR array to study effects of chemicals on the Hypothalamic–Pituitary–Gonadal axis of the Japanese medaka, Aquatic Toxicology. 88: 173–182

Zhou, B.S., Wu, R.S.S., Randall, D.J. Lam, P.K.S. (2001). Bioenergetics and RNA /DNA ratios in the common carp (Cyprinus carpio) under hypoxia, Journal of Comparative Physiology B. 171: 49-57.

Zillioux, E.J., JohnsonI, C., Kiparissis, Y., Metcalfe, C.D., Wheat, J.V., Ward, S.G. (2001). The sheepshead minnow as an in vivo model for endocrine disruption in marine teleosts: a partial life-cycle test with 17 alphaethynylestradiol, Environmental Toxicology and Chemistry. 20: 1968–1978.

Zutshi, B. (2003). Effect of fenthion on the testis of freshwater fish G.giuris (histochemical and biochemical), Pollution Research. 22(2): 231-236.

Zutshi, B. and Murthy, P.S. (2001). Ultrastructural changes in testis of gobid fish Glossogobius giuris (Ham) induced by fenthion, Indian Journal of Experimental Biology. 39: 170 – 173.

Proc.Zool.Soc.India. 14 (1) : 79 - 90 : (2015)
ISSN 0972-6683 : INDEXED AND ABSTRACTED

14

ENDOCRINE-DISRUPTING COMPOUNDS IN RIVER GOMTI: SOURCES, DETECTIONS AND EFFECTS (A REVIEW)

RITA VERMA[1], A.K SINGH[2], KAMAL JAISWAL[1] AND SUMAN MISHRA[1]

[1]Babasaheb Bhimrao Ambedkar Central University, Lucknow, 226025

[2]Directorate of Coldwater Fisheries Research, Bhimtal (Nanital), 263136

Received - 22.01.2015 **Accepted - 20.04.2015**

ABSTRACT

Many EDCs and PPCPs in water have raised substantial concern among the public and pollution control board, however, very little is known about the fate of these compounds during drinking and wastewater treatment and consequently permitted limit remains undecided. A brief overview of some methods available for the assessment of endocrine disruption activity in environmental samples are provided discussing various aspects to understand the impacts of such compounds at the physiological as well as gene level. The relevance of endocrine-disrupting compounds (EDCs) as potential contaminants in River Gomti has been reviewed in this paper. The sources of EDCs and primary pathway to reach in the water bodies are also discussed. The adverse effect by these compounds on the physiology of different aquatic animals has been described. Several approaches for measuring expression of gene transcripts are discussed, including directed approaches, such as blotting techniques and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) as well as micro arrays. Each of these methods provides important information about the molecular mechanisms that result from exposure to EDCs.

Key words: *EDCs, Gomti River, PPCPs. RT PCR, Gene Array,*

INTRODUCTION

Endocrine disrupting compounds (EDCs) was first reported in the year 1991 during the Wingspread Conference Centre in Wisconsin and are recognised as synthetic or natural compounds that could mimic or block the natural hormones in the endocrine systems of animals. Endocrine disruption in aquatic animals is well documented and studied during the past few decades. It is also documented that how these chemicals disturb the endocrine regulation in fish. (Jensen et al., 2001; Kinnberg and Toft, 2003; Kazeto et al., 2004). Today, EDCs are ubiquitous in our environment and are available in air, water, soil (Zhang et al., 2008; Schug, et al., 2011.) They are detected in food products also due to packaging of plasticizers. They are also occurring naturally in plants (phytoestrogens, EE2, Estrone), present in household products (Detergent, personal care products), pesticides (Lindane (α-HCH, β-HCH, γ-HCH), DDT, endosulphan, atrazine), plastics (bisphenol A, phthalates like DMP(Di-methyl phthalate, DBP (Di-butyl phthalate) (Srivastava et al., 2010), pharmaceutical wastes (birth control pills, industrial waste chemicals (PCBs, dioxin by-products of incineration, paper pulp mill and fuel combustion, and metals i.e. cadmium, lead, mercury (Propper et al., 2005; Verma and Rana, 2009). A detailed information on different categories of EDCs, their primary pathways have been compiled and presented (Table1).

Level of some Endocrine disruptor compounds in River Gomti

The Gomti River, flowing through eight districts in Uttar Pradesh, India. It travels about 25,000 km^2 before it merges with the Ganga River. Due to so many small tributaries This River carrying the wastewater and industrial effluents from different towns and industrial units. The river is the major source of drinking water for the Lucknow City, the State capital of Uttar Pradesh with a population of about 204.2 million. Due to

the human intervention and agriculture runoff The River, receives the untreated wastewater and effluents from Lucknow, Jagdishpur, Sultanpur and Jaunpur directly in its course through more than 40 wastewater drains (Malik et al., 2007).

This river is contaminated with so many pesticides and organic chemicals like polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides OCPs ((Nayak et al., 1995; Singh et al., 2005; Malik et al., 2007; Singh and Singh, 2008; Srivastava, 2010; Singh et al., 2005; Singh et al., 2007) and plasticizers like phthalic acid ester (PAEs) (Srivastava et al., 2010). The concentrations compounds such as DMP, DEP, DBP, DEHP, and DOP were found as 10.54, 4.57, 10.41, 31.61, and 5.16 µg/kg, respectively (Srivastava et al., 2010). The level of bisphenol-A, HCH has also been found to be 3-5µg/L and 4-158µg/L in drainage near river Gomti. (Verma et al., 2013). Fish muscle has also been shown to be contaminated with photochemical ozone creation potential (POCPs (Organochlorine pesticide (OCPs) and the reported value ranged between 2.58 and 22.56 ng g^{-1} (Malik et al., 2004; Singh et al., 2005a). Similarly presence of aldrin, dieldrin and OCPs have also been reported in water and bed-sediments of the Gomti river (Malik, 2006) and in the soil, surface water and groundwater of the adjoining areas (Singh et al., 2005b, 2007).

Mechanism of Action :

Endocrine disruptor compounds (EDCs) can affect endocrine systems through various ways. It may be receptor-mediated or receptor-independent pathways mimicking the action of estrogens or androgens. While acting though receptor, these compounds have affinity to bind with receptor and activate the gene cascade thereby affecting the reproductive physiology either by disrupting the synthesis or degradation of endogenous hormones or by directly activating steroid hormone receptor-mediated gene activation pathways. Exposure of estradiol (E2) results in the synthesis of specific proteins that activate several genes that encode proteins like estrogen receptor (ER), Vitellogenin (Vtg), the egg yolk precursor proteins, and choriogenins required for making the egg membrane (Bowman et al., 2000; 2002 Celius et al., 2000; Folmar et al., 2000; Funkenstein et al., 2000; Arukwe et al., 2001; Hemmer et al., 2001; Lattier et al., 2001; Lee et al., 2002), and affecting the expression of Vtg. (Hansen et al., 1998; Matozzo et al., 2008). These compounds also have got agonist activity to bind aromatase receptor (Kallivretaki et al., 2006; Hinfray et al., 2008; Brion et al., 2012). It is likely that EDCs will have their own specific gene expression profiles because they may bind with low affinity to more than one steroid receptor resulting in a complex gene activation pattern (Kallivretaki et al., 2006). There is clear cut evidence that these compounds have both agonist and antagonist activity i.e. either binds with ER receptor or AR receptor (Paech et al., 1997). However, several questions have been raised for the fate of the gene expression as EDCs can bind to both the ER and AR. Several researches are going on day by day to unravel the dilemma and to understand the mechanisms that might be involved.

Impact of EDCs on aquatic Health :

Many environmental chemicals exhibit estrogenic or androgenic activity some of these occur naturally in plants, others are manmade by-products that are present in agricultural and industrial chemicals. Endocrine disrupting compounds may interact with steroid hormones and their receptors, or other hormones and transcription factors in the biochemical pathway of hormonal activity. Endocrine disruption in animals have been documented by several researchers and now becoming an emerging topic to differentiate how these chemicals disturb the endocrine regulation in general and in fish particular (Harris et al., 2001; Kinnberg and Toft, 2003). Researchers first alerted to aquatic contamination by endocrine-disrupting compounds through observation of a variety of reproductive changes in different species of fish, mollusks and amphibians sampled from contaminated river, drainage, downstream of sewage treatment plant (Howell et al., 1980; Bortone and Davis, 1994; Jobling et al., 1998, 2004; Batty and Lim, 1999; Folmar et al., 2001; Gagne et al., 2002). The mechanism of action of these chemicals abnormalities and sub lethal changes caused by these compounds are also reported in several aquatic animals (Guilleette et al., 1994; Candia et al., 2001; Kloas 2002; Lavado et al., 2004 Kinnberg et al., 2000; Shioda and Wakabayashi 2000;

Kinnberg and Toft, 2003; Bayley et al., 2002; Harris et al., 2001; Schwaiger et al., 2002; Loomis et al., 2000; Maclatchy et al., 2003; Makynen et al., 2000; Mandiki et al., 2005).

The EDCs exposed Fish Featherhead minnows tended to have noticeably lower GSI than control and the plasma estradiol level was reported lower than control (Rey, 2006). EDCs exposure was further reported to reduce to ovarian growth and alter oocyte development and there was decreased fecundity in females (Makyen et al., 2000; Baatrup et al., 2001; Brion et al., 2004; Mandiki et al., 2005). The presence of oocyte and ovarian duct was found in testes of male fish (Kiparissis et al., 2003; Kuhl et al., 2005) observing xenoestrogen type sex reversal.

Aromatase enzyme has been reported as the potential biomarker of environmental contamination (Kallivretaki et al., 2006). Aromatase (CYP 19) the enzyme that convert androgens to estrogens has been the subject of studies into the mechanism by which chemicals interfere with sex steroids hormone (Singh 2012; Singh and Singh 2013; Singh et al., 2014). It is likely that EDCs affect reproduction either by disrupting the synthesis or degradation of endogenous hormones or by directly activating steroid hormone receptor-mediated gene expression (Larkin et al., 2003). Exposure of E_2 mimics increases in the number of estrogen receptor as well as the induced the synthesis of Vitellogenin hormone (Bowman et al., 2002; Andersen et al., 2003). There are several authors who found the expression level of CYP19 gene after exposed endocrine disrupting compound and found that CYP19 mRNA level tend to increase (Montserrat et al., 2004, Kazeto et al., 2004 ; Kalliveretaki et al., 2006).

By histopathological analysis it is clearly said that due to endocrine disrupting compound induction of ovatestis in medaka (Gronen et al., 1999), carp (Gimeno et al., 1994,1997). Fish expose to estrogen has been reported to increased number of arteric follicles in the ovaries and decreased number of vitellogenic follicles (Belt et al., 2002; Ven et al., 2003; Singh et al., 2013). Histopathology has proven to be a useful biomarker to evaluate the risk to aquatic organisms by exposure to both natural and anthropogenic EDCs that may interfere with reproduction and development (Bateman et al., 2004). The gonads can be mostly affected by EDCs exposure a higher incidence of oocyte atresia was found in the fish gonadal tissue (Jobling et al., 2002; Karels et al., 2003; Rasmussen et al., 2005, Magadalenal et al., 2011). The histological examination showed that the majority of the fish sampled from the contaminated sites had degenerated testes in which the normal lobular arrangements were disrupted and spermatogenesis was impaired. Accumulations of yellowish-brown pigmented cells in the lumen of the lobules were also reported in these testes. Mild intersex characteristics were recently shown to be able to compete with normal males and contribute to the next generation in a competitive breeding scenario (Matthiessen et al., 2008; Singh et al., 2013) and fish kills (Ripley et al., 2008). Tetreault et al., 2011) reported alterations in gonad development compared to that of fish from reference sites during the examined of gonad sections from downstream of STP discharges. The frequency of spermatogonia was also higher with lower spermatozoan cells in male testes. Histopathology of females of both darter species did not demonstrate any site difference in oocyte development, the frequency of the observed cell types or in the size of vitellogenic oocytes. However, a series of chemical have been reported to have potential to directly or indirectly disrupt the hormonal level of fish by interrupting with the regulation of CYP19 gene expression either in vivo and in vitro (Kalliveretaki et al., 2006; Filby et al.,2007). The abnormal sexual development such as compromised reproductive function and characteristic may be due to the induction of aromatase by EDCs chemical (Larkin et al., 2003; Singh 2012; Singh et al., 2014).

Abnormal level of circulating steroid hormones have also been reported in fish exposed to pulp and paper mill effluents (Guillette et al., 1994). Estradiol (17β) also stimulate the aromatase activity in Black porgy and increase estradiol and vitellogenin hormone level in serum by the exposure of estrogen 17β Estradiol (Chang and Lin, 1998). Due to exposure of endosulphan and fenvalerate in fresh water fish *Labeo rohita* the protein subunit change (Suneetha et al., 2010).The exposure of ortho-para dichlorodiphenyltrichloroethane (op- DDT) induced the sex reversal in Medaka and also fid the higher brain

aromatase activity (Kuhl and Brouwer, 2006). Ovatestis abnormality by EDCs exposure have been reported in Medaka (Gronen et al., 1999), Carp (Gimeno et al., 1994, 1997) and Smallmouth Bass (Blazer et al., 2007) with the exposure of high concentration of synthetic environmental estrogen reproductive and developmental problem (feminization of male, masculinization of female, lower fertility). These abnormalities have been reported by the presence of organochlorine, these compounds may interact with steroid hormones and their receptors, or other hormone and transcription factor in the biochemical pathway of hormonal activity (Arukwe, 2001).

There are many reports that vitellogenin hormone level is a specific marker for assessing the effect of endocrine disrupting compound (Jobling et al., 1999; Folmar et al., 2000; Cheek et al., 2004, Singh & Srivastava 2013). Toxicants, and chemical disrupt endocrine process have potential of becoming an environmental disaster therefore it is necessary to develop biomarker of exposure to these chemicals in fish, which is useful in assessing the impact of such chemical on fish sustainability (Prakash et al., 2007).

In this review we propose several common approaches for quantifying the disruption at gene level using several molecular techniques like gene expression directly by Blotting technique, ELISA, quantitative RT-PCR and micro- arrays. Such approaches are presented and discussed below:

Blotting Technique and ELISA

Blotting technique is a technology that has been used for a number of years and is still widely used today in many molecular biology laboratories. A Southern blot is a method used in molecular biology for detection of a specific protein sequence in the sample Plasma samples were thawed on ice then separated on pre-cast Tris-HCl polyacrylamide gels in Electrophoresis system. Following electrophoresis, the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes transfer buffer for detection; PVDF membranes were incubated with the same monoclonal antibody (King and Hassell, 2008).

ELISA

In this technique, we quantify the level of the hormone during the endocrine disruptor exposure it is a Enzyme link immune sorbent assay. Slandered and sample are added to well coated IgG antibody. After that we add quantifying hormone conjugate to alkaline phosphate in the plate followed by the polyclonal antibody to quantifying hormone, after a simultaneous incubation at room temperature the antibody binds in a competitive manner after washing pNpp substrate solution is added that is catalyzed by alkaline phosphatase.(King and Hassell, 2008; Singh et al., 2013) the plasma vitellogenin, testosterone and estradiol estimated in fathead minnow (Pimephales fromelas) with this technique for regulatory ecotoxicology testing and research related to endocrine disrupting chemicals (EDCs). (Villeneuve et al., 2006; Korte et al., 2000; U.S. EPA, 2002; Jensen et al., 2001).Serum Testosterone level by ELISA in fathead minnows (Pimephales promelas) were chronically exposed to metformin for 4 wk, at 40 mg/L,(Niemuth et al., 2012)

Northern blotting

Northern blot analysis involves separating RNA sample on a denaturing agarose gel by electrophoresis and then transferring the fractionated RNA to a nylon or nitrocellulose membrane. The RNA is then probed with cDNA that is either radiolabeled or digoxigenin- labeled and that is complementary to the sequence of interest. To determine the relative abundance of the genes of interest, a specific probe to an invariant housekeeping gene is also hybridized

Quantitative RT-PCR

Quantitative real-time PCR (Q-PCR) is a now emerging technology that is more accurate and sensitive to measure gene expression. run extensive PCR optimization reactions in order to determine the log phase of a PCR reaction. The Q-PCR reaction is monitored in real time by fluorescence either with the incorporation of the SYBR green dye that fluoresces only when it is intercalated into DNA or by a fluorescent probe that is

complementary in sequence to the cDNA of interest. The fluorescent probe hybridizes with the target sequence between the gene-specific forward and reverse primers. The probe contains a reporter dye on one end and a quencher dye on the other that inhibits a fluorescent signal when the probe is intact. Adult fathead minnows (*Pimephales promelas*) were chronically exposed to metformin for 4 wk, at 40 mg/L, a level similar to the average found in WWTP effluent in Milwaukee, Wisconsin, USA. Metformin treatment induced significant up-regulation of messenger ribonucleic acid (mRNA) encoding the egg-protein vitellogenin in male fish, an indication of endocrine disruption. The expression is quantity by real time pcr (Niemuth et al., 2012) and several RT PCR results which show over expression of gene related with endocrine disruption obtained by so many authors (Akatsuka et al., 2005; Klaper et al., 2006; Daniel et al., 2007) for measuring CYP19 mRNA expression in testicular tissue from male *Xenopus laevis* the real time PCR was designed to develop and optimize a SYBR Green I-based Q-RT PCR method. (Park et al., 2006) according to so many authors the regulatory gene CYP 19 affected by Endocrine disruptor compound and the gene expression was quantified by real time PCR (Cheshenko et al., 2008; Huang et al., 2009;Kishida et al., 2001; Kortner et al., 2009; Lee et al., 2006). In small, oviparous fish, the Japanese Medaka (*Oryzias latipes*) the development and validation of a PCR array for studying by chemical-induced effects on gene expression was measured through selected endocrine pathways along the hypothalamic–pituitary–gonadal (HPG) axis. (Zhanga et al., 2008) Hilscherova et al., 2004 measured effects of chemicals on expression of ten steroidogenic genes in the H295R cell line using real-time PCR.

Micro-arrays

Microarrays are emerging technology that can be used in endocrinology research to simultaneously measure the expression of hundreds to thousands of genes. In this procedure, RNA is reverse transcribed with fluorescently or radiolabeled markers (target) and is subsequently hybridized to DNA sequences (probes) that are attached to a solid support matrix. There are two types of array, oligonucleotide arrays and cDNA arrays. In oligonucleotide arrays, probes can be designed and synthesized separately and then attached to the matrix or they can be synthesized in situ (directly on the chip). And in, cDNA clones obtained from differential display, subtractive hybridizations, or other methods are then attached to the matrix. These chips can also be constructed manually by using a handheld spotter. In both methods, the probes are spotted onto glass slides or nylon membranes. Expression analysis using glass slide arrays typically uses competitive hybridization of two different fluorescently labeled targets. For membrane arrays, scontrol and treated samples are hybridized individually on separate membranes using radioactive labeled with 32P or 33P. Altered gene expressions by endocrine disruptor compound E2 and BPA studied In humans by microarray technique, RT PCR and western Blot (Hwang et al., 2011) Moens et al., 2007 observed Gene expression analysis of estrogenic compounds in the liver of common carp (Cyprinus carpio) using a custom cDNA microarray.

CONCLUSION

While with the above discussion it is clear that in both animals and humans, exposure to EDCs can have adverse effects on reproductive physiology and behaviour, it is Important to recognition of the prevalence of these compounds in the environment and to find out their potential to affect both wildlife and human population. Now it should be an emerging topic for discussion between government and public that how they can overcome this problem. Further efforts to understand the mechanisms underlying EDC effects, particularly those seen at environmentally relevant doses by compounds with low dose. Further attention for both toxicologists and endocrinologists that these compounds have no clear mechanism of action and not clear action or result so these compounds may affect the epigenetic of the human or animal these compound have potential to destroy or do potential affect the next generation. Now researchers have challenge to obtain absolute proof of endocrine disruption by BPA, phthalates, and other organochlorine compounds in the physiology of aquatic and humans. We have need to design easy technique to find out the level of EDCs in water and soil anywhere in the environment. We consider that improvements in

Table 1: Categories of known and suspected EDCs and their primary pathways to waterways

Category	Primary pathway to waterways	Reference
Alkyl-phenolic compounds non-ionic surfactants such as nonylphenol and octylphenol	Run off from agriculture waste and house hold waste in River.	(Gronen *et al.*, 1999; Kinnberg *et al.*, 2000; Shioda and Wakabayashi, 2000; Harris *et al.*, 2001; Knorr and Braunbeck, 2002; Schwaiger *et al.*, 2002; Tanaka and Grizzle, 2002; Tijani *et al.*, 2013)
fungicide vinclozolin	Extensively used in agriculture. Insecticides, herbicides, and fungicides are included in this class.	(Makynen *et al.*, 2000; Baatrup and Junge, 2001; Bayley *et al.*, 2002; Tijani *et al.*, 2013),
Pharmaceuticals ethinylestradiol (EE2) used in oral contraceptives	Synthetic steroids mainly consist of oral contraceptives as well as steroids.	(Zillioux *et al.*, 2001; Tijani *et al.*, 2013),
Plasticizers (Phthalates, Bisphenol A)	They are found in detergents, resins, some addictives, and monomers used in the production of plastics, Excessive use of plastics and dump anywhere in open field and wastage runoff from plastic industries in the form of by products	(Haubruge *et al.*, 2000; Shioda and Wakabayashi, 2000; Sohoni *et al.*, 2001; Kang *et al.*, 2002; Kinnberg and Toft, 2003; Tijani *et al.*, 2013).
Metals (mercury)	Effluent from mills and industries	Mercury (Matta *et al.*, 2001; Tijani *et al.*, 2013).
Natural Hormones and Steroids sex hormone i.e. EE2,Estrone	Effluent from sewage treatment plants and run-off from animal farms.	(Jobling *et al.*, 2002), Ankley *et al.*, 2003; Tijani *et al.*, 2013), Singh and Srivastava, 2013.
personal care products encompassing cosmetics, fragrances, preservatives, and toiletries Pharmaceuticals include antibiotics, anti-diabetics, anti-epileptic, antimicrobials etc.	Use of this compound i.e. Personal care product includes shampoos, toiletries, toothpaste, perfume, deodorants, etc. Pharmaceutical and personal care products (PPCPs) are	Houtman, 2010; Tijani *et al.*, 2013. Singh *et al.*, 2011

measurement of EDCs are essential, together with extensive monitoring of intake and discharge water at River. Treatment technologies will need to be assessed for their capability of removing EDCs in order to ensure a safe and cost-effective drinking water supply. Making certain that drinking water is safe will require determining and implementing guidelines for maximum acceptable concentrations of known EDCs and their mixtures in drinking water. The water industry desires to identify how best to maintain a sustainable supply of safe and reliable drinking water, which requires the detection and removal of potentially harmful contaminants. Studies on the immunological effects of endocrine disruptors were also recommended, particularly for individuals exposed. Further long-term studies of populations exposed to EDCs were recommended to assess the effects on age and the structure of population as well as on altering population size.

REFERENCES

Andersen, L., Bjerregaard, P. and Korsgaard, B. 2003. Vitellogenin induction and brain aromatase activity in adult male and female zebrafish exposed to endocrine disrupters. *Fish Physiology Biochemistry,* 28: 319–321.

Baatrup, E. and Junge, M. 2001. Antiandrogenic pesticides disrupt sexual characteristics in the adult male guppy (*Poecilia reticulata*), *Environmental Health Perspectives,* 109: 1063–1070.

Brion, F., Tyler, C.R., Palazzi, X., Laillet B., Porcher, J.M., Garric, J., Flammarion, P. 2004. Impacts of 17_-estradiol, including environmentally relevant concentrations, on reproduction after exposure during embryo larval-, juvenile- and adult-life stages in zebrafish (*Danio rerio*), *Aquatic Toxicology,* 68: 193–217.

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I.L. 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *Gen. Comp. Endocrinol.* 155: 31–62

Daniel L. Villeneuve a, Ann L. Miracle , Kathleen M. Jensen , Sigmund J. Degitz , Michael D. Kahl , Joseph J. Korte , Katie J. Greene , Lindsey S. Blake , Ann L. Linnum , Gerald T. Ankley. 2007. Development of quantitative real-time PCR assays for fathead minnow (Pimephales promelas) gonadotropin β subunit mRNAs to support endocrine disruptor research. *Comparative Biochemistry and Physiology,* 145: 171–183

Evangelia Kallivretaki, Rik Eggen b, Stephan Neuhauss c, Martin Alberti d, Ulf Kausch d, Helmut Segner. 2006. Aromatase in zebrafish: A potential target for endocrine disrupting chemicalsMarine *Environmental Research* 62: 187 -190.

François Brion, Yann Le Page, Benjamin Piccini, Sok-Keng Tong, Bon-chu Chung and Olivier Kah. 2012. Brain Aromatase (Cyp19a1b) is a highly sensitive gene to estrogens and xeno-estrogens Endocrine Disruptors, Munich: Germany.

Filby, A.L., Karen L., Maack, T.G., Tyler, C.R., 2007. Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. Aquatic Toxicology, xxx) xxx–xxx. (In press).

Houtman, C. J. 2010. Emerging contaminants in surface water and their relevance for the production of drinking water in Europe. Journal of Integrative *Environmental Sciences,* 7: (4) 271–295.

Hilscherova, K., Jones, P.D., Gracia, T., Newsted, J.L., Zhang, X.W., Sanderson, J.T., Yu, R.M.K., Wu, R.S.S., Giesy, J.P. 2004. Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR, *Toxicol. Sci.* 81: 78–89.

Huang, H., Leung, L.K. 2009. Bisphenol A down regulates CYP19 transcription in JEG-3 cells, *Toxicology Letter.* 189: 248–252.

Jensen, K.M., Korte, J.J., Kahl, M.D., Pasha, M.S., Ankley, G.T. 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*), *Comp. Biochem. Physiol.* C 128: 127–141.

Jobling, S. and Tyler, C. R. 2006. Introduction: the ecological relevance of chemically induced endocrine disruption in wildlife, *Environmental Health Perspectives,* 114: 7–8.

Jobling, S., Nolam, M., Tyler, C.R., Brighty, G.C. and Sumpter, J.P. 1998. Widespread sexual disruption in wild fish, *Environmental Science Technology,* 32:2498-2506.

June-Woo Park , Markus Hecker, Margaret B. Murphy , Paul D. Jones , Keith R. Solomon , Glen Van Der Kraak , James A. Carr , Ernest E. Smith , Louis du Preez , Ronald J. Kendall , John P. Giesy. 2006. Development and optimization of a Q-RT PCR method to quantify CYP19 mRNA expression in testis of male adult *Xenopus laevis:* Comparisons with aromatase enzyme activity, *Comparative Biochemistry and Physiology,* 144: 18–28

Karels, A.A., Manning, S., Brouwer, T.H., Brouwer, M. 2003. Reproductive effects of estrogenic and antiestrogenic chemicals on sheepshead minnows (*Cyprinodon variegatus*), *Environmental Toxicology and Chemistry,* 22: 855-865.

Kathleen M. Jensen U, Joseph J. Korte, Michael D. Kahl, Mumtaz S. Pasha, Gerald T. Ankley. 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*), *Comparative Biochemistry and Physiology,* 128: 127-141.

Kinnberg, K., Toft, G. 2003. Effects of estrogenic and antiandrogenic compounds on the testis structure of the adult guppy (*Poecilia reticulata*), *Ecotoxicology Environmental Safety,* 54: 16–24.

Kuhl, A.J., Manning, S., Brouwer, M. 2005. Brain aromatase in Japanese medaka (*Oryzias latipes*): molecular characterization and role in xenoestrogen-induced sex reversal, *J. Steroid Biochem. Mol Biol,* 96: 67–77.

Kiparissis, Y., Metcalfe, T.L., Balch, G.C., Metcalfe, C.D. 2003. Effects of the antiandrogens, vinclozolin and cyproterone acetate on gonadal development in the Japanese medaka (*Oryzias latipes*), *Aquatic Toxicology,* 63: 391–403.

Kishida, M., McLellan, M., Miranda, J.A., Callard, G.V. 2001. Estrogen and xenoestrogens upregulate the brain aromatase isoform (P450aromB) and perturb markers of early development in zebrafish (*Danio rerio*), *Comp. Biochem. Physiol.* B. 129: 261–268.

Klaper R, Rees CB, Drevnick P, Weber D, Sandheinrich M, Carvan MJ. 2006. Gene expression changes related to endocrine function and decline in reproduction in fathead minnow (*Pimephales promelas*) after dietary methylmercury exposure, *Environ Health Perspect* 114:1337–1343.

Kloas, W. 2002. Amphibians as model for the study of endocrine disruptors, *International Review of Cytology,* 216:1-57.

Korte, J.J., Kahl, M.D., Jensen, K.M., Pasha, M.S., Parks, L.G., LeBlanc, G.A., Ankley, G.T. 2000. Fathead minnow vitellogenin: complementary DNA sequence and messenger RNA and protein expression after 17β-estradiol treatment, *Environ. Toxicol. Chem.* 19:972–981.

Kyung-A Hwang, Se-Hyung Park, Bo-Rim Yi and Kyung-Chul Choi. 2011. Gene Alterations of Ovarian Cancer Cells Expressing Estrogen Receptors by Estrogen and Bisphenol A Using Microarray *Analysis Lab Anim Res:* 27(2), 99-107. DOI: 10.5625/lar.2011.27.2.99

Lavado, R., Thibuat , R., Raldua, D., Martin, R., Porte, C. 2004. First evidence of endocrine disruption in feral carp from the Ebro River, *Toxicology and Applied Pharmacology,* 196:247-257.

Lee, Y.M., Seo, J.S., Kim, I.C., Yoon, Y.D., Lee, J.S. 2006. Endocrine disrupting chemicals (bisphenol A, 4-nonylphenol, 4-tert-octylphenol) modulate expression of two distinct cytochrome P450 aromatase

genes differently in gender types of the hermaphroditic fish *Rivulus marmoratus, Biochem. Biophys. Res. Commun,* 345: 894–903.

Loomis, A.K., Thomas, P. 2000. Effects of estrogens and xenoestrogens on androgen production by Atlantic croaker testes in vitro: evidence for a nongenomic action mediated by an estrogen membrane receptor, *Biol. Reprod.* 62: 995–1004.

MacLatchy, D.L., Courtenay, S.C., Rice, C.D., Van der Kraak, G.J. 2003. Development of a short-term reproductive endocrine bioassay using steroid hormone and vitellogenin end points in the estuarine mummichog (*Fundulus heteroclitus*), *Environ. Toxicol. Chem,* 22: 996–1008.

Moens, L.N., van der Ven, K., Van, R.P., Del-Favero, J., De Coen, W.M. 2007. Gene expression analysis of estrogenic compounds in the liver of common carp (*Cyprinus carpio*) using a custom cDNA microarray, *J. Biochem. Mol. Toxicol,* 21: 299–311.

Magadalenal, N.N., Prabhu, R., Inbaraj, R.M. 2011. In vitro effect of endocrine disrupting chemical on the gonadal steroidogenesis in an Indian major carp, *Labeo rohita. Indian Journal of Science and Technology.* (9th ISRPF issue).

Makynen, E.A., Jensen, K.M., Tietge, J.E. Wells, K.L., Van Der Kraak, G. and Ankley, G.T. 2000. Effect of mammalian antiandrogen vinclozolin on the development and reproduction of the Fathead Minnow (*Pimephales Promelas*), *Aquatic Toxicology,* 48: 461-475.

Malik, A. 2006. Studies on the trace organics and major ion chemistry of the Gomati River. Ph.D. Thesis submitted to the Gurukul Kangri University, Hardwar, India.

Malik, A., Singh, K.P., Mohan, D., Patel, D.K. 2004. Distribution of polycyclic aromatic hydrocarbons in Gomti river system of India, Bulletin of *Environmental Contamination Toxicology,* 72:1211–1218.

Mandiki, N.M., Virginie G., Koen, M., Dirk, E., Els, D.R., Tom, D.B., Steven, A.S., Declerck, L.D.M., Catherine, B., Edwige, V.H., Marie-Louise, S., Patrick, K. 2014. Effect of land use on pollution status and risk of fish endocrine disruption in small farmland ponds. *Syaghalirwa, Hydrobiologia,* 723:103–120.

Mandiki, S.N.M., Babiak, I., Bopopi, J.M., Leprieur, F., Kestemont, P. 2005. Effects of sex steroids and their inhibitors on endocrine parameters and gender growth differences in Eurasian perch (*Perca fluviatilis*), *juveniles. Steroids,* 70: 85–94.

Mandiki, S.N.M., Babiak, I., Bopopi, J.M., Leprieur, F., Kestemont, P. 2005. Effect of sex steroids and their inhibitors on endocrine parameters and gender growth differences in Eurasian perch (*Perca fluviatilis*), *juveniles, Steroids,* 70:85-94.

Marciniak, A.T. and Witczak, A. 2010. Distribution of endocrine-disrupting pesticides in water and fish from the oder river Poland, *Acta Ichthyologica et piscatorial,* 40(1): 1–9 doi: 10.3750/aip2010.40.1.01.

Mastin, J.P. 2005. Environmental cardiovascular disease, *Cardiovascular Toxicology,* 5: 91–94.

Matta, M.B., Linse, J., Cairncross, C., Francendese, L., Kocan, R.M. 2001. Reproductive and transgenerational effects of methylmercury or Aroclor 1268 on *Fundulus heteroclitus, Environ. Toxicol. Chem,* 20: 327–335.

Montserrat, N., Montserrat, A., Gonzalez, E., Mendez, F., Piferrer and Planas, J.V. 2004. Effects of follicle stimulating hormone on estradiol-17beta production of P-450aromatase (CYP19) activity and mRNA expression in brown trout vitellogenic ovarian folliclesin vitro, *General and Comparative Endocrinology,* 137: 123–131.

Nash, J.P., Kime, D.E., Van der Ven, L.T., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R. 2004. Long-term exposure to environmental concentrations of the pharmaceutical

ethinylestradiol causes reproductive failure in fish, *Environmental Health Perspectives,* 112: 1725–1733.

Nathalie Hinfray, Ksenia Cheshenko, Sélim Ait-Aissa, Olivier Palluel, Jean-Marc Porcher, Farzad Pakdel, Rik I.L. Eggen, Olivier Kah and François Brion. 2008. Brain and gonadal aromatases as molecular and biochemical targets of endocrine disrupters in a model species, the zebrafish (*Danio rerio) Cybium,* 32(2)34-36.

Nayak, A., Raha, R. and Das, A.K. 1995. Organochlorine pesticide residues in middle stream of the Ganges River, India, *Bulletin of Environmental Contamination and Toxicology,* 54: 68–75.

Naoya Akatsuka, Etsuko Komatsuzaki, Azusa Ishikawa, Ikue Suzuki, Naoko Yamane, Shohei Miyata. 2005. Expression of the gonadal p450 aromatase gene of Xenopus and characterization of the 5-flanking region of the aromatase gene, *Journal of Steroid Biochemistry & Molecular Biology* 96: 45–50

P.D. Hansen, H. Dizer B. Hock, A. Marx J. Sherry, M. McMaster Ch. Blaise. 1998. Vitellogenin a biomarker for endocrine disruptors. *Trends in analytical chemistry,* vol. 17: 7

Paech, K., Webb, P., Kuiper, G.G., Nilsson, S., Gustafsson, J.A., Kushner, P.J. 1997. Differential ligand activation of estrogen receptors ERa and ERb AP1 sites. *Science,* 277: 1508–1510.

Pawlowski, S., Van Aerle, R., Tyler, C.R., Braunbeck, T. 2004. Effects of 17_-ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay, *Ecotoxicology Environmental Safety,* 57: 330–345.

Pratap, B., Singh V. S., Singh, V., Nigam, K.S., Singh, H. K. 2008. Sperm motility in the fishes of pesticide exposed and from polluted rivers of Gomti and Ganga of north India, *Food and Chemical Toxicology,* 46(12): 3764-3769.

Pratap, B.S., Singh, V. 2008. Bioaccumulation of hexachlorocyclohexane, dichlorodiphenyltrichloroethane, and estradiol-17b in catfish and carp during the pre-monsoon season in India, *Fish Physiology and Biochemistry,* 34: 25–36. DOI 10.1007/s10695-007-9142-9.

Propper, R. C. 2005. The study of endocrine disruption compounds past approaches and new directions, *Integrative and Comparative Biology,* 45: 194-200.

Rasmussen, T.H., Teh, S.J., Bjerregaard, P., Korsgaard, B. 2005. Anti-estrogen prevents xenoestrogen-induced testicular pathology of eelpout (*Zoarces viviparus*), *Aquatic Toxicology,* 72: 177-194.

Ray, E.J. 2006. Effect of p,p' DDE on reproduction and biomarkers of endocrine disruption in fatherad minnows (*pimephales promelas*). MSc. Thesis University of florida.

Ripley, J.L, L.R. Iwanowicz, V. S. Blazer, and C. M. Foran. 2008. Utilization of protein profiles as indicators of environmental impairment of smallmouth bass (*Micropterus dolomieu*) from the Shenandoah River, VA, USA. Environmental Toxicology and Chemistry 27:1756-1767.

Samir, K.K., Xie, B., Thompson, M. L., Sung, S.W., Ong, S. K., & Leeuvent, J.V. 2006. Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. *Environmental Science and Technology,* 40: 6537–6546.

Schinder, W.C. 1957. Determenation of nucleic acid in tissue by pentose analysis: Methods in enzymology (Eds:S.P. Colowick and N.O.kaplon). Academic press, New York.111:680-684.

Schug, T.T., Janesick, A., Blumberg, B., & Heindel, J.J. 2011. Endocrine disrupting chemicals and disease susceptibility, *The Journal of Steroid Biochemistry and Molecular Biology,* 127: 204–215.

Singh A.K., P.P. Srivastava, Ruchi Singh and Abubakar Ansari 2014. Effect of dietary administration of letrozole and tamoxifen on gonadal development, sex differentiation and biochemical changes in

common carp (*Cyprinus carpio L*). Reproduction, Fertility and Development.

Srivastava, A., Sharma, P.V. Tripathi, R. Kumar, R., Patel, D.K., Mathur P.K. 2010. Occurrence of phthalic acid esters in Gomti River Sediment, India. *Environ Monit Assess,* 169:397–406. DOI 10.1007/s10661-009-1182-4.

Singh, A.K. 2013. Introduction of modern endocrine techniques for the production of monosex population of fishes. *General & Comparative Endocrinology* 181: 146–155.

Singh A.K. and Ruchi Singh 2013. In vivo response of melatonin, gonadal activity and biochemical changes during aromatase inhibited sex reversal in common carp *Cyprinus carpio* (L). *Animal Reproduction Science* 136: 317– 325.

Singh A.K. and Ranjana Srivastava 2013. An overview of gonadal disruption in fishes inhabiting natural aquatic environments and future research need. In Advances in Fish Research, Ed. U.C.Goswami Vol V, Narendra Publishing House, Delhi pp 103-115.

Singh A.K., Abubakar Ansari, Ruchi Singh 2011. Possible impact of pharmaceutical wastes on aquatic environment. In: Water Pollution and Management (Eds) D.S. Malik, Sunil Kumar and Umesh Bharti. Biotech Books, New Delhi. Pp 371-386.

Singh, K.P., Malik, A., Sinha, S. 2007. Persistent organochlorine pesticide residues in soil and surface water of northern Indo- Gangetic alluvial plains, *Environmental Monitoring and Assessment,* 125:147–155.

Singh, K.P., Singh, Malik, A., Mohan, D. and Takroo, R. 2005. Distribution of persistant organochlorine pesticide residues in Gomti river, India, *Bulletin of Environmental Contamination and Toxicology,* 74: 146–154.

Singh, P.B. and Singh, V. 2008. Pesticide bioaccumulation and plasma sex steroids in fishes during breeding phase from north India, *Environmental Toxicology and Pharmacology,* 25: 342–350.

Singh, P.B. and Canario, A.V.M. 2004. Reproductive endocrine disruption in the freshwater catfish, Heteropneustes fossilis, in response to the pesticide c-hexachlorocyclohexane, *Ecotoxicological and Environmental Safety,* 58(1):77–83.

Singh, S., Singh, P.B. and Singh, V. 2007. Exposure and recovery response of isomers of HCH, metabolites of DDT and estradiol-17β in the female catfish, Heteropneustes fossilis, *Environmental Toxicology and Pharmacology,* 24: 245–251.

Singh, S., Singh, P.B. and Singh, V. 2008. Bioaccumulation of hexachlorocyclohexane, dichlorodiphenyltrichloroethane, and estradiol-17β in catfish and carp during pre-monsoon season in India, *Fish Physiology and Biochemistry,* 34: 25–36.

Suneetha, K., Kumar, K.G. and Veeriah, K. 2010. Change in protein subunit induced by endosulphan and fenvalerate in fresh water fish *Labeo rohita* through SDS PAGE, *Journal of Environmental biology,* 31(5): 759-763.

Susan Codi King and Kathryn Hassell 2008. Preliminary Assessment of an Indirect Elisa and Western Blot Techniques to Screen Vitellogenin in Barramundi as a Potential Biomarker of Exposure to EDCs in Australian Tropical Waters, *Australasian Journal of Ecotoxicology* , 14: 109-116.

U.S. EPA, 2002. A short term test method for assessing the reproductive toxicity of endocrine disrupting chemicals using the fathead minnow (Pimephales promelas). ubiquitous water pollutant, 4-nonylphenol, Gen. Comp. Endocrinol. 164: 91–99 *U.S. Environmental Protection Agency,* Washington, DC. EPA/ 600/R-01/067.

Valerio Matozzo, François Gagné, Maria Gabriella Marin, Francesco Ricciardi, Christian Blaise. 2008. Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A

review, *Environment International.* 34 :531–545

Van den Belt, K., Wester, P.W., van der Ven, L.T., Verheyen, R., Witters, H. 2002. Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): time dependency and reversibility, *Environmental Toxicology and Chemistry.* 21: 767-775.

Van der Ven, L.T., Wester, P.W., Vos, J.G. 2003. Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (*Danio rerio*), *Environmental Toxicology and Chemistry.* 22: 908-913.

Verma, R. and Singh, A.K. 2013. Endocrine disruptor in aquatic bodies: Issues and challenges, *Journal of Ecology Environment and Biology.* 1:17-21.

Verma, Y. and Rana, S.V.S. 2009. Endocrinal toxicity of industrial solvents A Mini Review, *Indian Journal of Experimental Biology.* 47:537-549.

Xiaowei Zhanga, Markus Hecker, June-Woo Parka, Amber R. Tompsett, John Newstedb, Kei Nakayamaf, Paul D. Jonesb, Doris Auh, Richard Kongh, Rudolf S.S.Wuh, John P. Giesya.2008. Real-time PCR array to study effects of chemicals on the Hypothalamic–Pituitary–Gonadal axis of the Japanese medaka, *Aquatic Toxicology.* 88: 173–182

Ying, G., Kookana, R.S., Ru, Y. 2002. Occurrence and fate of hormone steroids in the environment, *Environment International.* 28: 545–551.

Yukinori Kazeto, Allen R. Place, John M. Trant. 2004. Effects of endocrine disrupting chemicals on the expression of CYP19 genes in zebrafish (*Danio rerio*) juveniles, *Aquatic Toxicology.* 69: 25–34

Zhang, Y., & Zhou, J.L. 2008. Occurrence and removal of endocrine disrupting chemicals in wastewater treatment. *Chemosphere.* 73:848–853.

Zillioux, E.J., Johnsonl, C., Kiparissis, Y., Metcalfe, C.D., Wheat, J.V., Ward, S.G. 2001. The sheepshead minnow as an in vivo model for endocrine disruption in marine teleosts: a partial life-cycle test with 17 alphaethynylestradiol. *Environ. Toxicol. Chem.* 20: 1968–1978.

Aceh Journal of Animal Science (2016) 1(1): 16-20
DOI: 10.13170/ajas.1.1.3800

RESEARCH ARTICLE

Preliminary study on diminution level of RNA/DNA ratio in tissue of *Labeo rohita* by exposure to some endocrine disrupting compounds (EDCs)

Rita Verma[1*], Atul K Singh[2] , Kamal Jaiswal[1]
[1]Department of Applied Animal Sciences, Babasaheb Bhimrao Ambedkar (A Central) University, Lucknow, 226025, Uttar Pradesh, India; [2]Directorate of Coldwater Fisheries Research (ICAR), Bhimtal 263136 Nainital, Uttarakhand, India. *Corresponding author email: aksingh56@rediffmail.com

ABSTRACT

Effects of EDCs particularly on RNA/DNA ratio are yet to be investigated to manage the effluents in natural waters. We investigated exposure effects of endocrine disrupting compounds (EDCs) phthalic acid ester (PAE) and hexachlorocyclohexane (HCH) on the RNA/DNA ratio in tissue of an Indian major carp *Labeo rohita*. Fish were exposed to pre-determined sublethal concentrations of phthalic acid ester (Di-methyl phthalate (DMP), di-butyl phthalate (DBP), and di-(2- ethylhexyl) phthalate (DEHP) and also HCH for determining the tissue RNA/DNA ratio after 30, 60 and 90 days of exposure in the doses of 0.2 mg L^{-1}, 0.3 mg L^{-1}, and 0.5 mg L^{-1} respectively. All these tested chemicals significantly ($P<0.05$) inhibited RNA/DNA ratio. The ratio gradually significantly ($P<0.05$) decreased after DEHP where it was 1.9±0.51 F1, 18=15.8 P=0.014 n=19; in case of DBP it was 1.92±0.62 F1, 20=6.5 P=0.012 n=19 and for HCH it was 0.94±0.21 F1, 18=18.08 P=0.0012 n=19 at treatments concentrations of 0.3 mg L^{-1} and 0.5 mg L^{-1}, compared to control (2.9±0.2) after 90 days. However, there was no statistical significance ($P>0.05$) in RNA/DNA ratio after the DMP (F1, 20=2.4 P=0.15n=21) treatment.
Keywords: DMP, DEHP, DBP, HCH, endocrine disruptors, growth, reproduction

INTRODUCTION

Currently, residual pharmaceutical compounds, agricultural runoff, domestic effluents, livestock waste, personal care products, industrial waste are reported to be present in aquatic environment and are generally recognized as a source of environmental pollutants (Gehring *et al.,* 2002; Ying *et al.*, 2002; Ankley *et al.*, 2003; Cespedes *et al.,* 2004; Harris *et al.*, 2005; Singh and Srivastava, 2013; Tijani *et al.*, 2013). Due to the presence of these compounds, there occurs a change in the chemical composition of natural aquatic environment which may affect the non-target aquatic organisms, particularly fish impairing with its reproductive physiology. Such contaminants can lead to interactions between the chemicals and biological systems, including physiological changes in fish (Schwaiger *et al.*, 2004). Many of these compounds are categorized as endocrine disrupting compound (EDCs). In our Indian River, presence of compounds like HCH, Bisphenol-A, polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs) are reported plasticizers like phthalic acid ester (Singh *et al.*, 2005; Singh *et al.,* 2007; Srivastava *et al.,* 2010; Verma *et al.,* 2013).

The muscle of the fish contaminated with photochemical ozone creation potential (POCPs (Organochlorine pesticide (OCPs) has been published to range between 2.58 and 22.56 ng g^{-1} (Malik *et al.*, 2004; Singh *et al.*, 2005a). Although, these compounds are most frequently detected in rivers, its potential toxic effects on aquatic organisms (e.g., fish) remains principally unknown. It is well known that many environmental contaminants could produce severe damage in different organs of fishes and alter the activities of enzymes. Toxic pollutants interfere with energy yielding reactions indirectly inhibiting the synthesis of RNA, DNA and protein (Kim and Kang, 2004; Li *et al.*, 2010).

RNA/DNA ratio is useful indicators of natural or anthropogenic impacts in river, marine population and communities, such as upwelling or dredge fisheries (Chícharo and Chícharo, 2008). Ratio provides a measure of synthetic capacity of cell, but also could be a potential tool for reflecting environmental stress (Li *et al.,* 2009a; Li *et al.*, 2010) and nutritional level of cell (Chícharo and Chícharo, 2008). The objectives of this study were to investigate the effects of different doses of EDCs such as Phthalic acid ester (DMP, DBP, and DEHP) and HCH on the RNA/DNA ratio in muscle of *Labeo rohita*.

MATERIALS AND METHODS

Time and Location

Actively moving healthy immature *Labeo rohita* (total length, 22.5±2 cm, weight, 158±10.2 g, n=150) were collected from one of the culture ponds, Lucknow during the period from Dec 2013 to Dec 2015. Immediately after the collection, specimens were maintained in glass aquaria having 150 L water and they were acclimatized to the laboratory conditions for two weeks.

Experimental Fish and Feed

After the acclimation period, 150 immature *Labeo rohita* of 158±10.2 g weight and 22.5±2 cm length were divided equally and kept into four glass aquaria containing 150 L water in triplicate. Fish of each aquarium were given 0.2 mg L^{-1}, 0.3 mg L^{-1} and 0.5 mg L^{-1} of all Phthalic acid ester similarly, in other group of experiment fish of similar length and weight were maintained in triplicate and were exposed to 0.2 mg L^{-1}, 0.3 mg L^{-1}, 0.5 mg L^{-1} HCH. Every 10^{th} day, the water of each aquarium was replaced with fresh water containing similar dose of phthalic acid ester and HCH. The test chemical phthalic acid ester was diluted in carrier solvent DSMO (1%) whereas HCH carrier solvent used was acetone (Naciff *et al.,* 2005). These diluents were also mixed in control group as vehicle solution. After 90 days of exposure, length and weight of each group of fishes was recorded with the help of digital calipers scale and digital electronic balance respectively.

Fish were fed with pond collected zooplankton twice daily and also with commercially available fish food pellets (Tyio Pvt. Ltd. India).Twelve treatment groups were set up to study the effect of 99% pure Phthalic acid esters DMP, DBP and DEHP, and HCH (procured from Sigma Aldrich USA) on the RNA/DNA ratio in *Labeo rohita.*

RNA/DNA Ratio Analysis

The muscle samples (100 mg ml^{-1}, w/v) of each experimental group of fish was homogenized for 5 min in 5% trichloroacetic acid (TCA) at 90°C and then centrifuged at 5000 rpm for 20 min. For the determination of RNA, 2.0 ml of distilled water and 3.0 ml of orcinol reagent (1g orcinol +100 ml HCL+ 0.5g Ferric chloride) was added in 1.0 ml of supernatant. The reaction mixture was kept in boiling water bath for 20 min. The greenish-blue colour thus developed was read at 660 nm in a spectrophotometer. For DNA determination, 1.0 ml of distilled water and 4.0 ml of freshly prepared diphenylamine reagent (1 g Diphenylamine reagent + 100 ml glacial acetic acid + 2.75 ml con $H_2 SO_4$) were added to 1.0 ml of the supernatant. The reaction mixture was kept on a boiling water bath for 10 min. The blue colour developed was measured with spectrophotometer at 600 nm. Standard curves for RNA and DNA were drawn using different concentrations of yeast RNA and calf thymus DNA, respectively. The values were expressed as μg 100 mg^{-100} fish muscle tissue on dry basis. For the calculation of results, average of the duplicate readings for each standard, control and samples were obtained after subtracting the average zero standard optical density. Created a standard curve on log-log graph paper, with concentration on the y-axis and absorbance on the x-axis. Then drew the best fit straight line through the standard points and it was determined by regression analysis.

Statistical Analysis

All data are reported as means ± standard deviation (SD). Differences between the control and each exposure treatment group were evaluated by one-way analysis of variance (ANOVA) and parametric multiple comparisons with group 1 as control performed by Dunnett test. $P< 0.05$ was considered statistically significant. All analyses were performed with SPSS 16.0 (SPSS, Chicago, IL, US).

RESULTS AND DISCUSSION

The group of fish treated with Di-methyl phthalate (DMP) showed significant ($P<0.05$) decrease of RNA/DNA ratio in high dose during the experimental period when compared with the control (Figure 1a). However, DBP and DEHP treated groups showed significant decrease only in the dose of 0.3mg/L, 0.5mg/L when compared to control. (Figure 1b and Figure 2a). In control group RNA/DNA ratio ranged from 2.2±0.01 to 2.12±0.09, which decreased to 1.9±0.48 ($F1, 20 =6.5$ $P=0.012$) in DBP treated the dose of 0.3mg/L after 60 days of exposure, and 1.9±0.02 ($F1, 20=7.4$ $P=0.01$) after 90 days of exposure. In DEHP group the RNA/DNA ratio was 1.9± 0.14 ($F 1, 20 =11$ $P= 0.07$) after 60 days and 1.85±0.9 ($F1, 20$) 11.8$P= 0.04$) ($P<0.05$) after 90 days.

The experimental group of fish treated with HCH showed a gradual and significant decrease of RNA/DNA ratio in different exposure concentration (Figure 2b) RNA/DNA ratio ranged from 2.6±0.09 to 2.74±0.05 in control group and in HCH treated group it was significantly decreased in 0.2mg L^{-1},0.03mg L^{-1},0.5mg L^{-1} it was 1.9±0.13 (F1,20)=5.8 P=0.02, n=21) 1.3±0.14 (F1, 20=6.5 P=0.012 n=21) 1.04±0.07 (F1, 20=5.4 P=0.015) after 30 days. It gradually decreased after long time exposure significantly (P<0.05) and the reduction was 1.6±0.22 (F1,18 =18.08 P=0.0012), in 0.02mg L^{-1}, 1.8±0.17 (F1, 18=15.3 P=0.018) 0.3mg L^{-1}, 1.45±0.07(F1,20 8.5 P=0.01) 0.5mg L^{-1} after 60 days. There was also significant (P>0.01 Dunnet test) reduction after 90 days where the values were 1.9±0.13 (F1, 18=4.41 P=0.05), 1.4±0.18 (F1, 20=8.8 P=0.01) and 0.89±0.05 (F1, 20 8.4 P=0.014) after 0.02mg L^{-1} 0.3mg L^{-1} and 0.5mg L^{-1} exposures respectively.

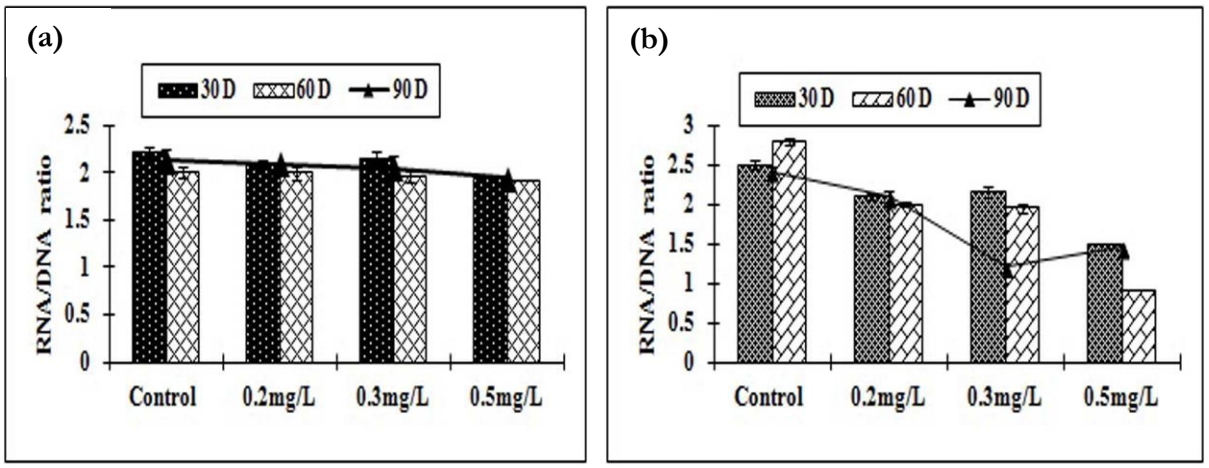


Figure 1. (a) Value of RNA/DNA ratio in *Labeo rohita* exposed to 0.2 mg L^{-1}, 0.3 mg L^{-1} and 0.5 mg L^{-1} DMP for 90 days. (Y axis) represents the ratio, (b) Value of RNA/DNA ratio in *Labeo rohita* exposed to 0.2mg L^{-1}, 0.3mg L^{-1} and 0.5mg L^{-1} DBP for 90 days (Y axis) represents the ratio. The data are presented as the mean ± standard deviation (n= 20) statistically significant differences according to a one–way ANOVA (P < 0.05) Dunnett test compared to control.

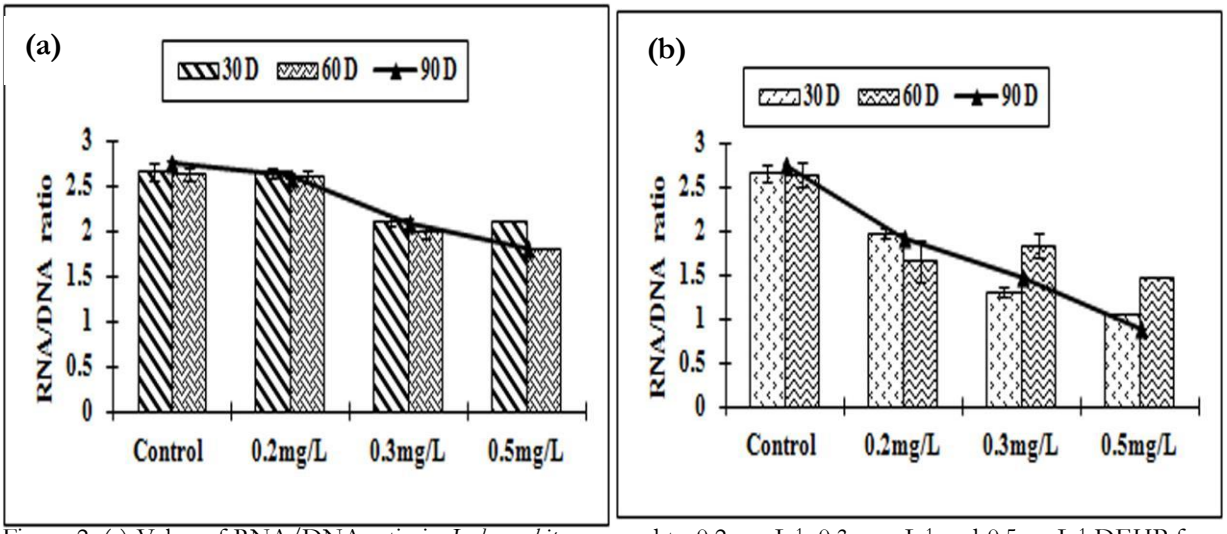


Figure 2. (a) Value of RNA/DNA ratio in *Labeo rohita* exposed to 0.2 mg L^{-1}, 0.3 mg L^{-1} and 0.5mg L^{-1} DEHP for 90 days (Y axis) represents the ratio, (b) Value of RNA/DNA ratio in *Labeo rohita* exposed to 0.2mg L^{-1}, 0.3mg L^{-1} and 0.5mg L^{-1} HCH for 90 days (Y axis) represents the ratio. The data are presented as the mean ± standard deviation (n = 20) statistically significant differences according to a one–way ANOVA (P < 0.05) Dunnett test compare to control.

The results of this study did not show any change in RNA/DNA ratio in DMP exposed group. Unlike the observations in this study, there are many earlier studies showing that several endocrine disrupting compound, phthalic acid ester and, HCH effect on GSI, cell death, sex differentiation,

reproductive defects, oxidative stress, steroid hormone concentration and RNA/DNA ratio in tissue (Tripathi *et al*., 2000; Rakshekhar, 2012).

Similarly, environmentally relevant concentrations of organotin compounds (OTs) was found to trigger sex changes in marine invertebrates (An *et al.,* 2013) where it was reported that the RNA/DNA ratio was significantly ($p<0.05$) lower, and a slight increase in DNA damage was observed in females bringing about imposex individuals rather males. Parallel results were obtained by Li *et al*., (2010), after long-term exposure to carbamazepine (CBZ) on the enzymatic alterations and RNA/DNA ratio in intestine tissue of rainbow trout. In an another study, the application of RAN/DNA ratio to evaluate the effects of toxicants on fish was reported, because a depressed RNA/DNA ratio was found in fish exposed to metal and organic contaminants (James and Sampath, 1999).

However, there are some studies which did not find any relation between the RNA/DNA ratio and the changes of environmental contaminants (Kim and Kang, 2004). Analogous revision done by Rathod and Kshirsagar *et al*. (2010) investigated the effect of sublethal concentration of two different kinds of pesticides Fenvalerate (synthetic pyrethroid) and Monocrotophos (organophosphate) for 24, 48, 72 and 96 hrs and observed declined level of DNA and RNA contents in selected tissues like gills, liver, kidney and muscle of freshwater fish *Puntius arenatus* (Day) due to exposure of different concentration of two different kinds of pesticides. Tripathi *et al*. (2003) also reported that fish exposed to Dimethoate (organophosphate) exhibited decreased nucleic acid (DNA and RNA) content. Related results was also reported by Wu and Or (2005), and the RNA:DNA ratio was most sensitive, and decreased significantly (by 50 to 86%) following exposure to 4.5 and 3.5 mg O2 l–1 for 1 week. Peakall (1992) reported that RNA/DNA ratios are useful but non-specific indicators of recent growth and general nutritional conditions in a variety of animals including mollusks, crustaceans and fish. Furthermore, RNA/DNA ratios were positively correlated with the somatic growth (Sambhu and Jayaprakas, 1997).

CONCLUSIONS

From the above results and interpretation it can be concluded that the RNA/DNA ratio is precious indicator for the well being of the fishes i.e., growth and development, as it was observed that ratio was highly affected due to exposure of pesticides and endocrine disrupting compounds. The group of fish treated with DEHP showed significant decrease in RNA/DNA ratio in high dose. This needs to be explored further requiring a keen research to justify the relation of fish growth and the reproductive development in fishes after exposure of several EDCs compound. Moreover from the above study it can be concluded that due to exposure of toxic EDCs there is gradual loss in RNA/DNA content in muscle which alleviate the nutrition level of fishes.

ACKNOWLEDGEMENTS

Authors are thankful to Parasitology Lab, Department of Applied Animal Sciences, Babasaheb Bhimrao University (A Central) University Vidhya Vihar Lucknow, 226025 for giving us an opportunity to complete the research work.

REFERENCES

Ankley, G.T., K.M. Jensen, E.A. Makynen, M.D. Kahl, J.J. Korte, M.W. Hornung, T.R. Henry, J.S. Denny, R.L. Leino, V.S. Wilson, M.C. Cardon, P.C. Hartig, L.E. Gray. 2003. Effects of the androgenic growth promoter 17-trenbolone on fecundity and reproductive endocrinology of the Fathead minnow. Environmental Toxicology and Chemistry, 22: 1350–1360.

Cespedes, R., M. Petrovic, D. Raldua, U. Saura, B. Pina, S. Lacorte. 2004. Integrated procedure for determination of endocrine-disrupting activity in surface waters and sediments by use of the biological technique recombinant yeast assay and chemical analysis by LC-ESI-MS. Analytical and Bioanalytical Chemistry, 378: 697–708.

Chícharo, M.A., L. Chícharo. 2008. RNA: DNA ratio and other nucleic acid derived indices in marine ecology. International Journal of Molecular Science, 9: 1453-1471.

Gehring, M., D. Weltin, L. Tennhardt, D. Vogel, B. Bilitewski. 2002. Elimination of bisphenol A during wastewater treatment: detailed mass balances derived from laboratory experiments. Proceedings of the AWWA Endocrine Disruptors & the Water Industry Symposium, April 18 – 20, Cincinnati, Ohio, USA.

Harris, R.M., C.J. Kirk, R.H. Waring. 2005. Non-genomic effects of endocrine disruptors: inhibition of estrogen sulfotransferase by phgenols and chlorinated phenols. Molecular and Cellular Endocrinology, 244: 72–74.

James, R., K. Sampath. 1999. Effect of zeolite on the reduction of cadmium toxicity in water and a freshwater fish *Oreochromis mossambicus*. Bulletin of Environmental Contamination and Toxicology, 62: 222–229.

Kim, S.G., J.C. Kang. 2004. Effect of dietary copper exposure on accumulation, growth and hematological parameters of the juvenile rockfish *Sebastes schlegeli*. Marine Environmental Research, 58: 65–82.

Li, Z., V. Zlabek, R. Grabic, J. Velisek, J. Machova, T. Randak. 2010. Enzymatic alterations and RNA/DNA ratio in intestine of rainbow trout, *Oncorhynchus mykiss*, induced by chronic exposure to carbamazepine. Ecotoxicology, 19: 872–878.

Li-hui An, Z. Yanqiang, S. Shuang-shuang, L. Yue, L. Zi-cheng, C. Hao, Z. Xing-ru, L. Kun, G. Junmin, Z. Bing-hui. 2013. Imposex effects on the veined rapa whelk (*Rapa navenosa*) in Bohai Bay, China. Ecotoxicology, 22: 538–547.

Mathers, E.M., D.F. Houlihan, L.J. Burren. 1994. RNA/DNA and protein concentrations in fed and starved herring *Clupea harengus* larvae. Marine Ecology and Progress Series, 107: 223-231.

Naciff, J.M., J.A. Hess, G.J. Overmann, S.M. Torontali, G.J. Carr, J.P. Tiesman, L.M. Foertsch, B.D. Richardson, J.E. Martinez, G.P. Daston. 2005. Gene expression changes induced in the testis by transplacental exposure to high and low doses of 17a-ethynyl estradiol, genistein, or bisphenol A. Toxicological Sciences, 86(2): 396–416.

Peakall, D. 1992. Animal biomarkers as pollution indicators. Chapman and Hall, London.

Raksheskar, G.A. 2012. Influenced of Cypermethrin on DNA, RNA and RNA/DNA ratio in gills of the freshwater fish *Channa striata*. Bioscience Discovery, 3(1): 17-19.

Rathod, N.D., R.V. Kshirsagar. 2010. Quantification of nucleic acid from fresh water fish *Punctius arenatus* (Day) exposed to pesticides. International Journal of Advanced Biotechnology and Research, 11(1): 43-51.

Sambhu, C., V. Jayaprakas. 1997. Dietary supplementation of testosterone propionate on growth performance of white prawn, *Penaeus indicus* (M. Edwards). Indian Journal of Experimental Biology, 35: 1353–1358.

Schwaiger, J., U. Mallow, H. Ferling, S. Knoerr, T. Braunbeck, W. Kalbfus, R.D. Negele. 2002. How estrogenic is nonylphenol? A transgenerational study using rainbow trout (*Oncorhynchus mykiss*) as test organism. Aquatic Toxicology, 59: 177-189.

Singh, A.K., R. Srivastava. 2013. An overview of gonadal disruption in fishes inhabiting natural aquatic environments and future research need. In Advances in Fish Research (Ed.), Narendra Publishing House, Delhi, pp. 103-115.

Singh, K.P., A. Malik, D. Mohan, R. Takroo. 2005a. Distribution of persistent organo-chlorine pesticide residues in Gomti River, India. Bulletin of Environmental Contamination and Toxicology, 74: 146–154.

Singh, K.P., A. Malik, S. Sinha. 2007. Persistent organochlorine pesticide residues in soil and surface water of northern Indo- Gangetic alluvial plains. Environmental Monitoring and Assessment, 125: 147–155.

Singh, K.P., A. Malik, D. Mohan, R. Takroo. 2005. Distribution of persistent organochlorine pesticide residues in Gomti River, India. Bulletin of Environmental Contamination and Toxicology, 74: 146–154.

Srivastava, A., P.V. Sharma, R. Tripathi, R. Kumar, D.K. Patel, P.K. Mathur. 2010. Occurrence of phthalic acid esters in Gomti River sediment, India. Environmental Monitoring and Assessment, 169: 397–406.

Tijani, J.O., O.O. Fatoba, L.F. Petrik. 2013. A review of pharmaceuticals and endocrine-disrupting compounds: sources, effects, removal, and detections. Water, Air and Soil Pollution, 224: 1770-1798.

Tripathi, G. 1992. Relative toxicity of aldrin, fenvalerate, captan and diazinon to the freshwater food fish, *Clarias batrachus*. Biomedical Environmental Science, 5: 33-38.

Tripathi, G., S. Harsh, P. Verma. 2001. Fenvalerate induced macromolecular changes in the catfish, *Clarias batrachus*. Environmental Biology, 23: 143-146.

Tripathi, P.K., V.K. Srivastava, A. Singh. 2003. Toxic effect of dimethoate (organophosphate) on metabolism and enzyme system of fresh water teleost fish *Channa punctatus*. Asian Fisheries Science, 16: 349-359.

Verma, R., A.K. Singh. 2013. Endocrine disruptor in aquatic bodies: Issues and challenges. Journal of Ecology Environment and Biology, 1(1): 17-21.

Wu, R.S.S., Y.Y. Or. 2005. Bioenergetics, growth and reproduction of amphipods are affected by moderately low oxygen regimes. Marine Ecology Progress Series, 297: 215–223.

Ying, G.G., R.S. Kookana, Y.J. Ru. 2002. Occurrence and fate of hormone steroids in the environment. Environment International, 28: 545-551.

Received: 17 March 2016 Accepted: 24 April 2016