A toxicological study of organophosphorous pesticide, chlorpyrifos exposure on reproductive performance of freshwater catfish "Heteropneustes fossilis"

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE IN FULFILLMENT OF

Doctor of Philosophy

IN

APPLIED ANIMAL SCIENCES

Under the Supervision of

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Submitted By

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Dedication

I dedicate this thesis to my dear parents, my husband and my brother and people who have always been there to support me, congratulate me and show me always the best path to follow. To my parents, I will never finish thank you for everything you do every day for me.

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Firstly I would like to offer my heartfelt salutation to almighty god for the unbroken health and vigor, bestowed upon me, all through the span of my studies in accomplishing this arduous work.

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"The pen is the tongue mind, gratitude is the heart's memory" although 'thanks' is a poor expression of the deep sense of gratitude, one feels in the heart, yet there is no better way to express it.

Sneha

Sneha Verma

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CERTIFICATE

This is to certify that the thesis titled **"A toxicological study of organophosphorous pesticide, chlorpyrifos exposure on reproductive performance of freshwater catfish "Heteropneustes fossilis"**submitted by Ms. SnehaVerma is an 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 any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University Lucknow satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) regulations-1999 as amended in 2010* andit is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Date: 18/8/17

Supervisor

Head of the Department

DECLARATION

I hereby declare that this thesis entitled **"A toxicological study of organophosphorous pesticide, chlorpyrifos exposure on reproductive performance of freshwater catfish "Heteropneustes fossilis"** submitted by me under the supervision of Dr. Abha Mishra, in accomplishment of the degree of Ph.D. in Applied Animal Sciences in the Department of Applied Animal Sciences, School for Biosciences and Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow (U.P.). It is an outcome of my own efforts and is an original research work.

Sneha

Date: 18/8/17 *(SnehaVerma)*

Place: Lucknow

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General Introduction

Agriculture remains the backbone of India. It is most important sector in the economy upon which the Indian population depends. To increase the agriculture crops, pesticides are widely used which constitute an important component in agriculture development and protection of public health. Pesticides are being used by the human beings for their benefits like control of insect vectors and increased crop yield (Warren, 1998; Prakasam et al., 2001). It makes a significant contribution to maintaining world food production. However, the extensive use of pesticides has caused severe environmental and health hazards to many organisms also (Abdollahi et al., 2004; Tuzmen et al., 2008). Pesticides, including herbicides, insecticides, fungicides, bactericides and rodenticides, are widely used to control pests and pest-induced diseases (Damalas and Eleftherohorinos, 2011).

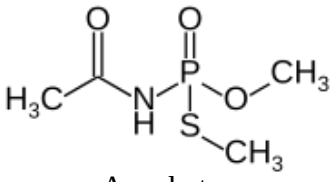
1. Pesticide classification

Based on chemical properties, pesticides can be divided into two main group viz., inorganic and organic group. Inorganic pesticides are derived from natural resources and don't contain carbon for example arsenic, cyanide, mercury, thallium etc. (Hassall, 1990). However organic pesticides are synthetic compounds either aliphatic or aromatic hydrocarbons. They are further classified on the basis of chemical structure and their physical state viz., pyrethroids, organophosphorous, organochlorine, carbamate and sulfonylurea pesticides (Wasswa, 2009). The organophosphorous pesticide further classified in two major categories which include: organophosphate and organothiophosphate.

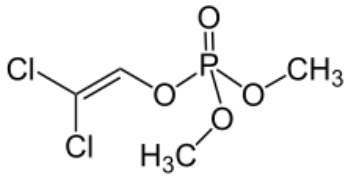
2. Organophosphorous pesticide

Organophosphorous pesticides (OPs) are ubiquitous in the environment, commonly used as agricultural and vector control. OPs are phosphate esters derived from

phosphoric acid comprising of a central phosphate atom and three organic side chains (R), two of which are usually alkyl substitutions and third group is more labile to hydrolysis (termed leaving group). The P=S bond in OPs require metabolic activation by oxidative desulfuration to their corresponding oxon (P=O) metabolite. They are not very stable either chemically or biochemically, but they are readily hydrolysed in soil, sediment or surface water (Walker, 2001). OPs are most preferred pesticides because of their low persistence in the environment as economically useful pesticide by agriculturists to eradicate insect pests (Raman et al., 1983). This group of pesticides has virtually replaced the persistent organochlorine compounds (Briggs, 1992). Through many pathways, these pesticides leave their particular sites of application and distribute throughout the environment and enter the aquatic ecosystem. The major route to contaminate water ecosystems in urban areas is through rainfall, runoff and atmospheric deposition, and another source of water contamination is municipal and industrial dischargers. They ultimately find their way into rivers, lakes and ponds (Tarahi, 2001; Honarpajouh, 2003; Bagheri, 2007; Shayeghi et al., 2007; Vryzas et al., 2009; Werimo et al., 2009; Arjmandi et al., 2010). Further, it may contaminate the environment and accumulate in food chains there by posing hazardous effect to human and aquatic animal health (Pesticides Action Network, 2001; Blasco et al., 2003; Khogali et al., 2005; Ali et al., 2009). The application of pesticides to flooded areas, along with accidental spillage, spray drift, leaching, runoff or drainage from treated agricultural lands has the potential to expose the non-target organisms especially the fish. Nearly 90% of pesticides were dispersed through air, soil and water (Moses et al., 1993). OPs are highly toxic in acute poisoning than other classes of pesticides (Mansour, 2004; Collins, 2006). There are several pesticides belong to organophosphorous category such as, acephate, dichlorvos, dimethoate, ethion, malathion, mevinphos, chlorfenvinphos, parathion, chlorpyrifos and diazinon. The chemical structures of OPs compounds are given below.



Acephate



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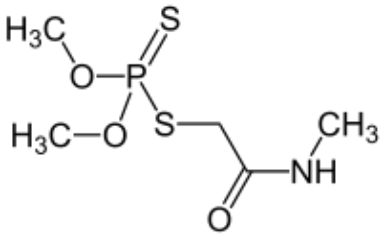
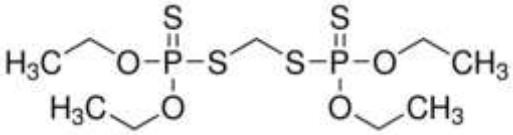
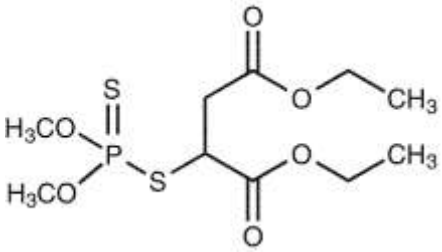


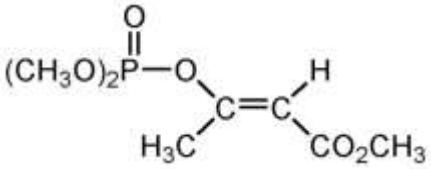
Dimethoate



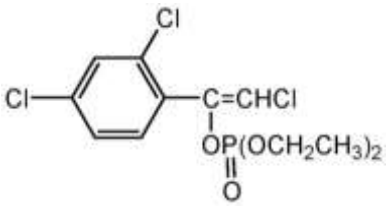
Ethion



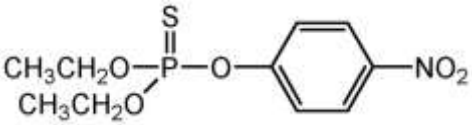
Malathion



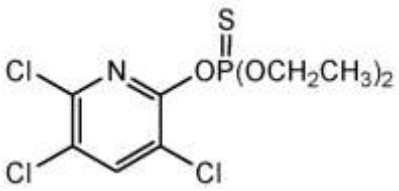
Mevinphos



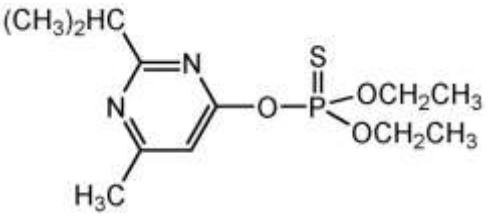
Chlorfenvinphos



Parathion



Chlorpyrifos



Diazinon

Chemical structure of organophosphorous compounds

The primary mechanism of action of OPs is as neurotoxic agents. It can affect both CNS (central nervous system) and PNS (peripheral nervous system) (Ecobichon, 1996; Keifer and Firestone, 2007). They showed their toxic activity by inhibiting acetylcholinesterase enzyme (AChE) through the interaction of nucleophilic active site serine of the enzyme with the OP to form a phosphorylated enzyme derivative (Habig and DiGiulio, 1991; Li and Han, 2004). The phosphorylated derivative is much more resistant to subsequent hydrolysis than the normal acetylated derivative. AChE is responsible for hydrolysis of acetylcholine, a neurotransmitter that conducts nerve impulses across neuromuscular junction in the nervous system. Acetylcholine acts as a neurotropic factor in brain development, promotes cell differentiation, in cholinergic targets sites (Hohmann et al., 1988; Navarro et al., 1989; McFarland et al., 1991). Inhibition of AChE causes accumulation of neurotransmitter acetylcholine in sympathetic and parasympathetic fibres, neuromuscular junction and some synapses within the vertebrate central nervous system (Koelle, 1970) leading to generalized cholinergic action and muscles. This inhibition eventually leads to paralysis, respiratory failure and death (Fulton and Key, 2001; Rendon et al., 2004; Podolska and Napierska, 2006). Acute exposure of OP caused acute toxicity which includes dizziness, headache, sweating, fatigue, numbness, vomiting neurological effects, coma or death (Turgut, 2007). Chronic exposure to low doses of pesticide through air, water and food may lead to chronic toxicity via accumulation of residues in the body over a long period of time which resulted in chronic pesticide toxicity including cancer, congenital malfunction, neurological disorders, infertility, impotence, immunological disorders, liver and kidney damage, skin alterations and worsening of existing health conditions (Jobling et al., 1995).

The present thesis was dealt with widely used compound i.e., chlorpyrifos (CPF) which comes under classification of organothiophosphate under the pyridine category which comes under heterocyclic organothiophosphate. Pesticides are extensively used to protect agriculture crops against the damages caused by pests. However these chemicals may reach other ecological compartments as lakes and rivers through rain and wind affecting other organisms away from primary target only 0.10% reaches the specific target. The significant increase of chemical emissions of the water resources had led to

deleterious effect for aquatic organism (Bailey et al., 1997; Foe et al., 1998; Livingstone, 2001; Sarder et al., 2001; Varo et al., 2002; Matsumoto et al., 2006). Their widespread use not only brought adverse effect on agro ecosystem but also caused alteration in physiological processes of non-target organisms that inhabit natural environment close to agricultural fields (Werimo et al., 2009).

2.1. Chlorpyrifos (CPF)

Chlorpyrifos (O,O-diethyl-O-3,5,6-trichlor-2-pyridyl) phosphorothioate: CPF) is a emulsifiable broad spectrum organophosphorous insecticide (OP), that has been widely used all around the world. It has been ranked third among all pesticides in use for household and commercial applications (Aspelin, 1994; Richardson, 1995). It was first introduced into the marketplace in 1965 by DowElanco, formerly by Dow Chemical Company. It is currently produced by Dow Chemical Company, India Medical Corp. It is the second largest selling OP and found to be more toxic to aquatic animals than other organochlorine and organophosphorus pesticide compounds (Tilak et al., 2001). CPF has an average half-life of 30 days in soils and two months in less alkaline soils (Howard, 1991). Chlorpyrifos enhanced the half-life of soil about in water (Wauchope et al., 1992; Racke, 1993). The principle metabolite of chlorpyrifos, TCP (3,5,6-trichloro-2-pyridinol), absorbs weekly to soil particle and persistent in soils. It also can persist indoors for weeks to months (Arcury et al., 2007). According to world health organization (WHO) pesticide classification system, CPF is ranked in classII and regarded as only moderately hazardous (WHO, 2004). The indoor residential use of CPF was terminated by United States Environmental Protection Agency (USEPA) since 2000, however, it is widely use in agriculture field in US (USEPA 2011a, b). Its use for agricultural purposes is one of the most anxious issues (Chen et al., 2009; Sun and Chen, 2008).

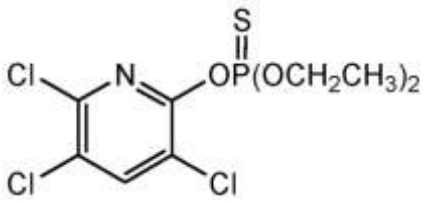
2.1.1. Common name

An organophosphorus pesticide, chlorpyrifos (CPF) is registered for use in over 30 products under its several names viz., Brodan, Detmol UA, Dowco 179, Dursban,

Hilban, Empire, Eradex, Lorsban, Paqeant, Piridane, Stipend (Eisler, 2000; EXTOXNET, 1996).

2.1.2. Physical and Chemical properties of CPF

Chemical structure of chlorpyrifos:



CAS Registry number:	2921-88-2
Empirical formula:	$C_9H_{11}Cl_3NO_3PS$
IUPAC name:	O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)-phosphorothioate
Molecular weight:	350.6
Colour:	white
Nature:	granular, crystalline solid
Melting point:	41-42.5^{o}C
Vapour pressure:	3.35×10^{-3} Pa at 25^{o}C (EFSA, 2005), 2.54×10^{-3} Pa at 25^{o}C (WHO, 2009)
Odour:	Mercapton (thiol) similar to sulphur compounds
Solubility:	Moderately soluble in water (Kidd and James, 1991; Mackay et al., 1999) and readily soluble in acetone, benzene, iso-octane and methanol (Tomlin, 1997)

Log k_{ow} (Octanol-water partition coefficient): 4.7 at 20^{o}C (EFSA, 2005; Tomlin, 2006), 5.0 at 25^{o}C (WHO, 2009)

Soil sorption coefficient, K_{oc}: 652-30,381 L/kg (Gebremariam et al., 2012)

Aquatic sediment sorption, K_{oc}: 3,000-25,656 L/kg (higher affinity for aquatic sediment than soil; Gebremariam et al., 2012)

2.1.3. Toxicologically relevant metabolites

- 3,5,6-trichloro-2-pyridinol (TCP) (EFSA, 2005)
- 3,5,6-trichloro-2-methoxypyridine (TMP) (EFSA, 2005)
- O-ethyl-O-(3,5,6-trichloro-2-pyridoyl) phosphorothioic acid (phosphorothioate) (EFSA, 2005)
- Chlorpyrifos oxon (US EPA, 2009)

2.1.4. Uses of chlorpyrifos

It is used to control many types of insects and pests in a wide range of crops, ornamentals and also for indoor purposes (EPA, 1997; PMRA, 2000). In agricultural settings, it is being used to protect a number of important agricultural crops such as corn, citrus, alfalfa and peanuts from pest insect attack and food crops viz., cranberries, strawberries, apples, figs, pears, nectarines, cherries, peaches, plums, grapes, almonds, pecans, walnuts, onions, peppers, kale, broccoli, Brussels sprouts, cabbage, cauliflower, collards, cucurbits, asparagus, roots/tubers, tomatoes, lentils, beans, peas, sorghum, tobacco, wheat, soybeans, sunflower, cotton, sugar beets, mint, and bananas (Whitmore et al.,1992; Reinecke and Reinecke, 2007). It is also being used to control over 250 non-agricultural insects and arthropod pests, including subterranean termites, cockroaches, fleas, ants, and others, that are found in and around household items, on lawns, trees and shrubs. CPF uses have changed drastically over the last ten years. The agricultural applications have been reduced and commercial applications around residential locality have increased.

2.1.5. Mechanism of CPF metabolism

The primary mechanism of chlorpyrifos (CPF) toxicity is cholinesterase (ChE) inhibition. The inhibition results in the buildup of acetylcholine (ACh) at choline receptors, causing continual nerve stimulation (Giesy et al, 1999). CPF is transformed inside animals to chlorpyrifos-oxon which is about 3000 times as potent against nervous system as chlorpyrifos itself (Sultatos, 1991; Chambers and Carr, 1993; El-Merhibi et al.,

2004). CPF pesticide is bio activated by the microsomal cytochrome P450 (CYP)-mediated monooxygenase system to their more potent cholinesterase inhibitor, chlorpyrifos-oxon (CPO). Thus toxicity is initiated by the formation of chlorpyrifos-oxon by oxidative desulfuration (Giesy et al., 1999; Eisler, 2000). When CPF was exposed to UV light or to sunlight, it underwent hydrolysis in the presence of water to liberate 3,5,6-trichloro-2-pyridinol, which underwent further decomposition to diols and triols and ultimately cleavage of the ring to fragmentary products (Smith, 1968). Hydrolysis in water occurs least readily at about pH 6 and very readily above pH 8. Chlorpyrifos and chlorpyrifos-oxon kill insects and other animals, including humans (Chambers and Carr, 1993). These two OPs pesticide inhibit acetylcholinesterase (AChE), which plays an important role in neurotransmission at cholinergic synapses by rapid hydrolysis of neurotransmitter acetylcholine to choline and acetate (Kwong, 2002). The inhibition of AChE activity caused by chlorpyrifos is more persistent than that caused by other organophosphates (Sakai, 1990; Pope, 1992; Bushnell et al., 1993). Its inhibitory effect depend on its binding capacity to the enzyme active site and by its rate of phosphorylation in relation to the behavior and age of spp. (Richmonds and Dutta, 1992; Dutta et al., 1995) and is widely used for rapid detection to predict early warning of pesticide toxicity (Dutta and Arends, 2003). Most bio-activation takes place in the liver, while detoxification takes place in the liver and plasma.

2.1.6. CPF toxicity

CPF is highly toxic to varied vertebrates and invertebrates. CPF inhibits the acetycholinesterase (AChE) enzyme which caused accumulation of acetylcholine resulting in acute toxicity (Pope et al., 1991, 1992; Pope and Chakraborti, 1992; Chakraborti et al., 1993; Chambers and Carr, 1993), delayed neuropathy (Kaplan et al., 1993; Richardson et al., 1993) and ultimately death (Cremlyn, 1991) of an organism. Symptoms of delayed neuropathy include cramps, weakness, tingling and numbness of extremities, a high stepping gate, paralysis (Fikes, 1992; Lotti, 1992). The CPF toxicity was influenced by number of factors include metabolic rate, number of target sites available for CPF metabolism (Chambers and Carr, 1995), organism surface area, and life

stage (El-Merhibi et al., 2004). Chlorpyrifos affects the activity of ATPase, an enzyme important in cellular respiration (Sakai, 1990) thus impending cellular respiration. Its metabolite, chlorpyrifos-oxon inhibits the enzyme cholesterol-ester-hydrolase thus eliminating normal reactions to stress (Civen et al., 1977a). Researchers believed that this is because chlorpyrifos is lipophilic, which causes changes in behavior of animal along with AChE inhibition. Reduction in working memory and a slowing of motor activity were documented two to three weeks following exposure. Weekly injections of smaller amount of chlorpyrifos caused similar effect (Bushnell et al., 1993). Intoxication of CPF caused a significant decrease in the reduced glutathione (GSH), catalase (CAT) and glutathione S-transferase (GST) activities (Goel et al., 2005).

Human exposures to organophosphates are broadly classified into two categories: occupational and environmental (Racke, 1993). Occupational exposures occur among agricultural and industrial workers and pest control exterminators. Environmental exposure affects a large population. It includes residential exposures, dietary and accidental exposures via breathing contact with CPF treated soil, CPF exposed farms area, and exposures in chemical warfare (Jaga and Dharmani, 2003). CPF has been associated with chronic effects in humans, including chronic neurobehavioral effects and multiple chemical sensitivities. The neurobehavioral effects included persistent headaches, blurred vision, unusual fatigue or muscle weakness and problems with mental function including memory, concentration, depression, and irritability (Jobling et al., 1995). It can cause acute poisoning and well known symptoms include myosis, increased urination, diarrhoea, diaphoresis, lacrimation and salivation in rats (Samsum et al., 2005; Turgut, 2007). Several studies reported the CPF involvement in multiple mechanisms like causing hepatic dysfunction (Poet et al., 2003), genotoxicity (Mehta et al., 2008), neurobehavioral and neurochemical changes (Slotkin et al., 2005). Earlier study concluded that CPF was reported to be activated by dermal contact, air inhalation, ingestion of contaminated food or water, and vapour action, causing convulsions and paralysis.

CPF also affect male reproductive system in animals (Akbarsha et al., 2000). Exposure to a chlorpyrifos product causes death of cells in male testes and a decrease in sperm production in cattle (Everett, 1982). This chemical has been detected in cervical mucous, semen and human milk in various studies conducted by German scientists (Wagner 1994). Immune system abnormalities have been reported in patients exposed to chlorpyrifos. Many individuals reported developing sensitivities to a broad array of substances following chlorpyrifos exposure (Thrasher et al., 1993). Chlorpyrifos has been responsible for causing genetic damage in lymph and blood cells, mice spleen cells and hamster bone marrow cells (Amer and Fahmy, 1982). The developmental effects cannot always be visible at birth or even in later life. The disturbances of nervous system and brain may be expressed in terms of how an individual functions and behaves throughout life with considerable variation from birth through adulthood. Functional deficiencies range from very mild to very severe to totally debilitating, consequently, it is difficult to quantify neuro-developmental impairment.

CPF spread through air drift or surface runoff into surrounding waters and gets accumulated in different aquatic organisms, particularly fish, and adversely affecting them via several sources (Tarahi, 2001; Varo et al., 2002; Honarpajouh, 2003; Bagheri, 2007; Shayeghi et al., 2007; Vryzas et al., 2009; Werimo et al., 2009; Arjmandi et al., 2010). Fish are particularly very sensitive to the environmental contamination of water. Hence, pesticides may significantly damage certain physiological and biochemical processes when they enter into the fish organs (John, 2007; Banaee et al., 2011). Earlier studies reported that there are different kinds of pesticides which can cause serious impairment to physiological and health status of fish (Begum, 2004; Monteiro et al., 2006; Siang et al., 2007; Banaee et al., 2009). Fish are the non-target organism of pesticidal exposure as they play an important in food chain. Recent evidences indicate that fish, an extremely valuable resource, are quickly becoming scares. One consequence of this scarcity is the increasing concern for fish survival and growing interest in identifying the level of various chemical pollutants. Earlier investigation reported that the incident of fish killing was highest in chlorpyrifos toxicity (AbdelHalim et al., 2006). Fish are exposed to aquatic toxicants via the extensive and delicate respiratory surface of

the gills and inhaling sea water (Wendelaar, 1997). Hence, for the study of toxicological impacts, fish serves as an excellent bio assay animal and have been widely used for this purpose (Ruggieri, 1975; Swarup et al., 1977; Blaxter and Hallers, 1992; Espelid et al., 1996; Wendellar, 1997; Hollis et al., 1999; Pratap, 1999; Kumar et al., 2007). There are number of studies reporting the pathological changes or toxic effects in fish exposed to different organochlorine, organophosphorous, carbamate and pyrethroid pesticides (Scott, 1967; Jackson, 1968). These toxic effects include both lethal and sublethal, which may change the growth rate, development, reproduction, histopathology, biochemistry, biochemistry, physiology and behavior (Rand and Petrocelli, 1985). The extent of severity of tissue damage of a particular compound as toxicant depends on its toxic potentiality in the tissue of organism (Murty, 1986). Several study reported the acute toxicity of CPF in response to mortality, LC_{50} and behavioral study in several fish (Barron and Woodburn, 1995; Kavitha and Venkateswara, 2008; Oruc, 2010; Sharbidre et al., 2011; Xing et al., 2012). CPF caused developmental dysfunctions (Levin et al., 2003; Levin et al., 2004; Richendrfer et al., 2012) and also produce neurological effects (Braquenier et al., 2010; Eddins et al., 2010; Middlemore-Risher et al., 2010; Sledge et al., 2011). CPF is popular for its AChE inhibitory nature in fish (Straus and Chambers, 1995; Taylor and Brown, 1999; Kavitha and Venkateswara, 2008; Leticia and Derardo, 2008). AChE activity in fish is essential for normal behavior and muscular function (Kirby et al., 2000). It also interferes with hormone synthesis (Hontela et al., 1997; Oruc, 2010). CPF has also been reported to alter brain development and neuronal morphogenesis in absence of ChE inhibition (Campbell et al., 1997; Das and Barone, 1999). CPF also affect the biochemical properties of fish tissues (Gupta et al., 1987; Khan et al., 1992; James and Sampath, 1995; Das et al., 1999; Khare and Singh, 2002; Sobha et al., 2007; Hadi et al., 2009).

3. Animal model

For the investigation, the freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) was selected. The freshwater catfish is one of the important groups of fish in our country

and is getting increasingly popular showing a promising future for commercial culture (Barua, 1989). Freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) locally known as "Shinghi" is an important air breathing catfish, due to presence of accessory respiratory organ which extends through the dorsal muscle of the body on either side of the vertebral column. This air-breathing adaptation enables it to survive in water of low oxygen content and also out of water on wet ground. This species found in ponds, pools, swamps and rivers of India, Sri Lanka, Burma, Laos, Thailand and Vietnam (Day, 1989; Dutta-Munshi and Hughes, 1992). This catfish is very hardy and can be cultured in swamps and derelict ponds of our country where carp culture is not possible. This species is ideal for wastewater aquaculture.

3.1. Systematic position

Group: Pisces
Phylum: Chordata
Subphylum: Vertebrata
Superclass: Gnathostomata
Class: Actinopterygii
Division: Teleostei
Superorder: Ostariophysi
Order: Siluriformes
Family: Heteropneustidae
Genus: *Heteropneustes*
Species: *fossilis* (Bloch, 1794)

The freshwater catfish, *Heteropneustes fossilis* is good in taste, highly nutritious and medicinal quality, and rich in protein and minerals (Islam, 1989). The chemical composition of the fish is 72% water, 19% protein, 8% fat, 0.15% calcium, 0.25% phosphorus and 0.10% vitamin A, B, C and D (Shahidullah, 1964). The muscles of the fish have been reported to have very high content of iron (226 mg/ l0 gm) and fairly high content of calcium (Saha and Guha, 1939).

3.2. Fish Synonyms

As listed by IUCN (The International Union for Conservation of Nature), there are many synonyms reported for freshwater catfish, *Heteropneustes fossilis* (Jha and Rayamajhi, 2010):

- *Clarisilurus kemratensis* (non Fowler, 1937)
- *Heteropneustes microps* (non Gunther, 1864)
- *Heteropneustes microps* (Gunther, 1864)
- *Saccobranchus fossilis* (Bloch, 1794)
- *Saccobranchus microcephalus* (Gunther, 1864)
- *Saccobranchus singio* (Hamilton, 1822)
- *Silurus biserratus* (Swainson, 1839)
- *Silurus fossilis* (Bloch, 1794)
- *Silurus singio* (Hamilton, 1822)

4. Objectives

On the basis of previously investigated information about chlorpyrifos toxicity, the present work was focused on the chlorpyrifos toxicity on reproductive potential of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). The main objectives of the presented work were as followed:

1) Effect of organophosphorous pesticide, chlorpyrifos on mortality, behavior, morphology and ovarian histology of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794).
2) Effect of organophosphorous pesticide, chlorpyrifos on oocyte maturation and follicular steroids in freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794).
3) Effect of organophosphorous pesticide, chlorpyrifos on embryogenesis of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794).

The thesis is divided into 3 chapters, besides a general introduction, consolidated summary and conclusion. The chapters were written in research manuscript form and references of all chapters were compiled at the end of the thesis to avoid repetition.

CHAPTER – I

Effect of organophosphorous pesticide, chlorpyrifos on mortality, behavior, morphology and ovarian histology of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)

ABSTRACT

The present investigation was undertaken to explore the toxic effect of organophosphorous pesticide, chlorpyrifos (CPF) to assess the mortality, behavior, morphology and ovarian histology of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). This study was conducted by exposing the fish to different concentrations of CPF (0.002-2.28 mM/l) for 96 hr experimental duration along with control group of fish in different reproductive phases (resting, preparatory and pre-spawning phase). The finney's probit analysis (1971) was used to determine median lethal concentration (LC_{50}). The safe concentration of the studied pesticide was calculated as per Hart et al. (1945) and Henderson et al. (1959) in all respective seasons. The obtained LC_{50} were found to 1.547, 0.678, 0.299 and 0.174 mM/l in resting phase, 0.332, 0.193, 0.152 and 0.123 mM/l in preparatory phase and 0.296, 0.107, 0.044 and 0.026 mM/l in pre-spawning phase for 24, 48, 72 and 96 hr respectively. The safe concentrations were 0.039 mM/l in resting phase, 0.19 mM/l in preparatory phase and 0.004 mM/l in pre-spawning phase. Susceptibility of freshwater catfish, *Heteropneustes fossilis* to CPF was found to be duration and concentration dependent. Their tolerance to CPF was increasing with inactiveness of gonadal activity (resting season). At higher concentration of CPF, fish showed alterations in morphological and behavioral responses, especially erratic and jerky swimming,

frequent surfacing and ingulping, mucus secretion, an increase in opercular movement and copious secretion of mucus all over the body. The histological assessment resulted that CPF caused reduction in size of oocytes, vacuolation in cytoplasm, damaged ovarian follicles, increase in inter-follicular space, breaking of ovarian wall, occurrence of necrosis, damaged structure of ovarian follicles, formation of fragmented ova, atretic oocytes and nucleolar extrusion. It is concluded that CPF is highly toxic to catfish and severely affect their physiology and behavior.

Keywords: Chlorpyrifos, LC_{50}, mortality, *Heteropneustes fossilis*, ovarian histology.

1. Introduction

In the midst of most recent one decade, a colossal progress has been made in the improvement of new compounds with better toxicity, in this way, a great deal of work has been done on impact of pesticides on non-target aquatic organisms (Battaglin and Fairchild, 2002**;** Prashanth et al., 2005). Fish are considered to be most significant biomonitors in aquatic systems for the estimation of metal pollution level (Rashed, 2001; Authman, 2008), they offer several specific advantages in describing the natural characteristics of aquatic systems and in assessing changes to habitats (Lamas et al., 2007). A vast scale mortality of fish in various water bodies has been recorded and the reason behind was the huge application of pesticides and insecticides in agricultural practices which further contaminate the aquatic environment (Talebi, 1998; Stephen, 2004; Banaee et al., 2008). Aquatic contamination of pesticides cause acute and chronic poisoning in fish and other organisms directly or indirectly via food chain (Heger et al., 1995; Omitoyin et al., 2006; Velmurugan et al., 2007). The reactions of fish towards the lethal chemicals are wide gone relying upon the toxicant type, exposure duration, water quality and the species (Fisher, 1991; Richmonds and Dutta, 1992; Venkateswara, 2004).

Toxicity tests are a vital segment in creating awareness and evaluating the potential effect of chemicals on aquatic communities since they illustrate the toxic impacts of chemicals in living beings by controlling their morphology, behavior and

survival rate (Adedeji et al., 2008; Onyedineke et al., 2010). It may be interpret as an estimation of the concentration of biologically active substances by the level of their impact on living beings (Chapman and Long, 1983). Acute toxicity of different pesticides is influenced by the age, sex, genetic properties, body size of fish, water quality and its physico-chemical parameters, and purity or formulation of insecticides. The frequency or intensity of injury or abnormalities to fish in pesticide toxicity may be more or even less depends on exposure duration.

In addition to mortality test, study of behavioral markers to assess the toxicant affects are the most promising and sensitive indicator of ecotoxicology (Drummond and Russom, 1990; Scherrer, 1992; Cohn and MacPhail, 1996). The behavioral study is becoming prominent in toxicity assessment in an unicellular organism (Tahedl and Hader, 2001), insects (Martin, 2004; Venkateswara et al., 2005), fish (Hansen et al., 1999; Rao et al., 2005) and rodents (Dell'Omo et al., 1997). It is a unique perspective of an organism that produced a link between the physiology and environmental ecology (Little and Brewer, 2001). It serves as significant tool in toxicology to perceive and assess impacts of natural stressors that cause variations in the responses and range of sensitiveness towards contaminants. However, these variations are recognized as an adaptive mechanism which permits fish to cope with genuine or perceived stressors so that the typical homeostatic state could be kept up maintained (Barton, 2002).

Organophosphate compounds (OPs) are powerful neurotoxins, since they inhibit AChE activity (Coppage et al., 1975; Klaverkamp and Hobden, 1980) results in overwhelming of post-synaptic acetylcholine receptors and hyper-stimulation, which leads to physiologic aberrations ranging from behavioural impairment to death (Garcia et al., 2002; Howard et al., 2005; Eaton et al., 2008). Several factors seem to be involved in affecting the AChE activity caused by OPs such as duration and concentration of exposed compounds (Adedeji, 2011). CPF directly inhibits acetyl cholinesterase enzyme activity in fish and invertebrates (Fulton and key 2001; Rao et al., 2005) which may lead to decreased mobility of fish (Bretaud et al., 2000). Therefore, it primarily works as nerve poison which is reflected in uncoordinated abnormal fish behavior after the toxicant

application (Scott and Sloman, 2004; Krian and Jha, 2009; Nwani et al., 2010; Nagaraju et al., 2011; Prashanth et al., 2011; Satyavardhan, 2013; Rani and Kumaraguru, 2014; Ullah et al., 2014).

The main clinical internal sing of fish includes neurological disorder, disruption of nerve functions, respiratory dysfunction and suffocation (Banaee et al., 2011) that lead to death of fish. It has been found that different fish species, even from the same family, show differences in the sensitivity to high concentrations of insecticides in water.

Fish are used as an indicator of environmental suitability. So that any change in fish behavior including change in swimming (Brewer et al., 1999) or respiratory pattern, indicated the water quality deterioration (Olla et al., 1983; Byrne and O'Halloran, 2001). Swimming pattern of fish is frequently assessed as a response of toxicity because altered locomotive activity can indicate pesticidal effect on nervous system. The different behavioral manifestations are reported by many researchers (Brewer et al., 1999; Ayuba and Ofojekwu, 2002; Pandey et al., 2011). The pesticides also caused changes in respiratory functions, frequency of opercular movement (Shivakumar and David, 2004; Lawrence and Temiotan, 2010), surfacing (Katja et al., 2005) and gulping of air (Shedd et al., 2001; Scott and Sloman, 2004).

The assessment of morphological deformities is another method to study the effects of contamination on fish (Thiyagarajah et al., 1996; Sun et al., 1998; Tricklebank, 2001; Lemos et al., 2005; Liao et al., 2005; Linde et al., 2008; Hart et al., 2008; Yap et al., 2009). Different studies reported that fish exposed to a wide range of pesticides exhibited abnormal behavioral and morphological alterations (Devi and Mishra 2013). There are different types of morphological alterations reported in fish of contaminated waters, including fin necrosis (Sindermann, 1979; Reash and Berra, 1989; Sun et al., 1998), skull deformation (Lindesjoo and Thulin, 1992), opercular deformity (Handwerker and Douglas, 1994; Sun et al., 1998) and fin splitting (Sun et al., 1998; Almeida et al., 2008). Various studies were reported on the toxicity of different organophosphate

pesticides on fish (Dikshit and Raizada, 1981; Verma et al., 1982; Srivastava and Singh, 2001; Gul, 2005; Pandey et al., 2005).

Fish are very sensitive to a wide variety of toxicants in water (Herger et al., 1995). Due to accumulation of pesticides in tissues, it produces many physiological, histological and biochemical changes in the fish and freshwater fauna by influencing the activities of several enzymes and metabolites (Nagarathnamma and Ramamurthi, 1982). The histopathology proves to be a sensitive and effective biomarker to study lesions or abnormalities on a cellular level and hence reflect the health of the entire aquatic ecosystem (Mumford, 2004; Camargo and Martinez, 2007; Datta et al., 2007; Monsefi et al., 2010; Nikalje et al., 2012). It constitutes a useful tool for assessing the degree of pollution, particularly for sublethal and chronic effects (Bernet et al., 1999; Pieterse, 2004). It is a mechanism which includes study of diseased or injured cell after death of the aquatic animal may serves to identify the cause of death of an aquatic animal (Pieterse, 2004).

The earlier histopathological observations of fish exposed to pollutants revealed that fish organs are an efficient indicators of water quality (Cardoso et al., 1996; Cengiz et al., 2001). Therefore, it is necessary to study the histopathological alterations in different organs of fish to assess the extent of damage (Palmes, 1976; Singh and Sahai, 1985; Rastogi and Kulshrestha, 1990; Mumford, 2004; Pieterse, 2004; Deshmukh and Kulkarni, 2005; Camargo and Martinez, 2007; Verma and Srivastava, 2008; Pugazhvendan et al., 2009; Monsefi et al., 2010). Many Literatures have been reported on the impact of pesticides on various fish tissues but studies of reproductive organ in fish are not as many. The pesticide cause several damages to the reproductive organ as observed earlier by Boyd (1964), Burdick et al. (1972), Carlson (1972), Freeman and Idler (1975), Saxena and Garg (1978), Singh and Singh (1980b), and Dey and Bhattacharya (1989).

The selection of organism for toxicity test is mainly based on certain criteria like its ecological status, position within the food chain, suitability for laboratory studies,

genetically stable, uniform population and adequate background data of the organism (Buikema at al., 1982). The species selected for current study viz. freshwater catfish, *Heteropneustes fossilis* satisfied most of the above criteria. The objective of the present study was to examine acute toxicity level of chlorpyrifos with special emphasis on behavioral, morphological responses and histopathological changes in the ovary of a freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in resting, preparatory and pre-spawning phases of annual reproductive cycle.

2. Materials and Methods

2.1. Chemicals and Instruments

An organophosphorous pesticide, chlorpyrifos (CPF) was procured from the local market of Lucknow, Uttar Pradesh, India, under the trade name Hilban® (20% EC CPF), supplied by Hindustan insecticide limited. All other chemicals used for this study was of analytical grade of HiMedia Laboratory Pvt. Ltd., India company. The instruments were used in current analysis was rotary microtome (Weswox) and brightfield microscope Olympus equipped with CX41 camera.

2.2. Test Animal and their acclimatization

An experiment was performed in accordance with local/ national guidelines of ethical committee for experimentation in animals.

The live freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) of relatively same size and weight were collected from commercial fisherman of Lucknow, Uttar Pradesh, India in resting (December), preparatory (February), and pre-spawning (April) phase of annual reproductive cycle. Fish were brought to the laboratory in wide mouthed large plastic containers in natural water avoiding stresses and injuries as possible. Before introducing in the aquarium, fish were treated with 0.05% $KMnO_4$ solutions to remove any dermal infection (Figure 1). Later, they were acclimatized in 120 L glass aquaria containing water having constant physico-chemical characteristic as standardized by APHA (1998) techniques, for two weeks. Water was renewed daily to remove fecal

matter and waste metabolite of fish during acclimatization. During this period, fish were fed regularly with commercial fish food pellets and goat liver. Diseased and dead individuals were removed immediately if any.

2.3. Experimental design

2.3.1. Seasonal median lethal concentration bioassay

The toxicity test was performed under natural photoperiod for 96 hr to determine LC_{50} values of CPF in resting, preparatory and pre-spawning phase of reproduction. For this bioassay, normal and healthy fish of uniform weight and length were selected. Fish were exposed in the batch of ten to varying concentrations (0.002, 0.005, 0.028, 0.057, 0.085, 0.114, 0.142, 0.171, 0.199, 0.228, 0.285, 0.313, 0.342, 0.37, 0.399, 0.456, 0.513, 0.57, 0.627, 0.684, 0.741, 0.798, 0.855, 1.14, 1.425, 1.71, 1.99 and 2.28 mM/l) of chlorpyrifos (CPF). The control group was also maintained concurrently in all the seasons. Acute toxicity test were carried out for period of 96 hr. Mortality data was recorded after 24, 48, 72 and 96 hr to get LC_{50} of respective intervals. The concentrations of the test compound used were selected on the basis of our trial experiments that caused 0-100% mortality in respective reproductive phase. The experiment was set in five replicates having ten fish each in all reproductive phases. All solutions (control and test) were renewed daily and dead fish were removed immediately. Fish were not given any food during the experiment.

2.3.2. Estimation of safe concentration

The safe concentration of any compound was presumed harmless. It was calculated as per Hart et al. (1945) and Henderson et al. (1959). This calculation was based on the 24 and 48 hr LC_{50} values. The safe concentration was obtained from the following formula:

$$\mathbf{C = 48\ hours\ LC_{50} \times Application\ factor\ (A) \times S^2}$$

Where: C = Safe concentration

A = application factor i.e. 0.3 according to Henderson et al. (1959)

S= 48 h LC_{50} ÷ 24 h LC_{50}

2.3.3. Observation of behavioral and morphological responses

The behavioral and morphological alterations of healthy and exposed fish to various concentrations of CPF were recorded up to 24 hr. These alterations were consistently monitored and recorded as Gupta and Dua (2010), Nimila and Nandan (2010), AL-Aker and Shamsi (2000). The frequency of occurrence of different behavior was counted for group of exposed fish.

2.3.4. Ovarian histology of freshwater catfish, Heteropneustes fossilis (Bloch,1794)

2.3.4.1. Experimental design

To study the concentration effect of chlorpyrifos (CPF) on ovarian tissue of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794), different concentrations of CPF were selected in different reproductive phase. In resting phase, seven different concentrations of CPF were selected that were 0.057, 0.085, 0.114, 0.171, 0.228, 0.285 and 0.57 mM/l. In preparatory phase, 0.028, 0.057, 0.085, 0.114, 0.142, 0.171 and 0.199 mM/l concentrations were selected. In pre-spawning phase, 0.002, 0.005, 0.028, 0.057, 0.085, 0.114 and 0.142 mM/l doses of CPF were selected. Those all concentrations were selected on the basis of their mortality response (Earlier experiment). A control group of fish was also maintained concurrently. Experiment was set in triplicate with ten numbers of fish each. The water was changed daily with the required amount of the CPF.

To study the chronic or duration effect of CPF, short term and long term experiments were designed. In short term assay, fish were exposed to $1/3^{rd}$ of LC_{50} of 96hr of CPF (0.058, 0.041 and 0.008 mM/l in resting, preparatory and pre-spawning phase) for 15 days exposure period. For long term exposure, $1/10^{th}$ of LC_{50} of 96 hr of

CPF (0.017, 0.012 and 0.002 mM/l in resting, preparatory and pre-spawning phase) for 30 day exposure duration was selected. The LC_{50} of CPF for 96 hr were found 0.174 mM/l in resting period, 0.123 mM/l in preparatory period and 0.026 mM/l in pre-spawning period.The control set of fish was running side by side with experimental group. In each set of experiment ten fish were taken. During both of the period, dead fish were removed immediately.

At the end of concentration (acute) and duration (chronic) experiment, the desired tissue (ovary) was dissected out by using stainless steel dissecting kit to avoid metal contamination for histo-pathological process in different reproductive phases.

2.3.4.2. Process of histological assessment

Tissue Fixation and trimming

The ovary tissues were dissected out, washed in normal saline and fixed in Bouin's fixative. After 5 hr of fixation, the tissues were trimmed as in square pieces of about 3-5 mm. Then further for 19 hr, the tissues were fixed in same fixative. After 24 hr of completion, tissues were washed with tap water, distilled water, 30% alcohol, 50% alcohol and 70% alcohol.

Block preparation

The block preparation includes two processes: dehydration and embedding. The tissues were dehydrated in ascending order of different concentrations of alcohol i.e., 90% alcohol and 100% alcohol for 15 min. with two changes in each grade of alcohol. After dehydration, tissues were transferred in xylene+abs. alcohol (1:1) and xylene for 15 min. each. The embedding of tissues was performed in hot air oven at 60^0C temperature. In this process, the tisues were put in xylene+filtered wax (1:1) for 15 min. with two changes, and further processed in filtered wax_1, wax_2 and wax_3 (100%) for 30 min with two changes each. At the end of embedding, the tissues were taken out and placed in a metal L shaped angle kept ready by filling with wax and the air bubble arising were removed by using hot spatula.

Sectioning of block

The excess of wax was trimmed with the help of spatula and knife till the material is slightly visible and then it was fixed on the wooden block holder for sectioning. Before sectioning, the Mayer's egg albumin was spread on the slides and leaves it up to dry. Sectioning of the tissue was performed by rotary microtome (Weswox). The thickness of the sections was 7 µm and the sections (ribbon) were spread on the slides having albumin by putting on hot plate having approximately 30-35^{0}C temperature. All sections were allowed to cool at 37^{0}C overnight when they spread properly.

Slide preparation

The procedures of slide preparation include three steps viz., staining, dehydration and mounting. For tissue anatomical analysis, standard double staining (haematoxylin and eosin) technique was used. The procedure was shown in Figure 2.

2.4. Data analysis

The fish mortality was presented in percentage. It was defined as number of dead fish divided by total number of exposed fish in aquarium multiplied by hundred. It was recorded every 24 hr up to 96 hr study period. The data were analyzed statistically by one-way analysis of variance (ANOVA) among different annual reproductive phases. The LC_{50} with 95% confidence limits of 24 hr, 48 hr, 72 hr and 96 hr was estimated by probit analysis (Finney, 1971) with the statistic software (IBM SPSS version 20). For tabulation of behavioral and morphological alterations, the frequency of occurrence was considered as behavior/min. and morphological alterations were observed in each experimental set. These responses were recorded in different phase of annual reproductive cycle. The histological sections of ovarian tissues were examined at 10X magnification under Bright field microscope (Olympus CX41) using micropublisher 3.3 RTV camera.

Figure 1

A: Acclimatization of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in laboratory condition.

B: Experimental control freshwater catfish *Heteropneustes fossilis* (Bloch, 1794).

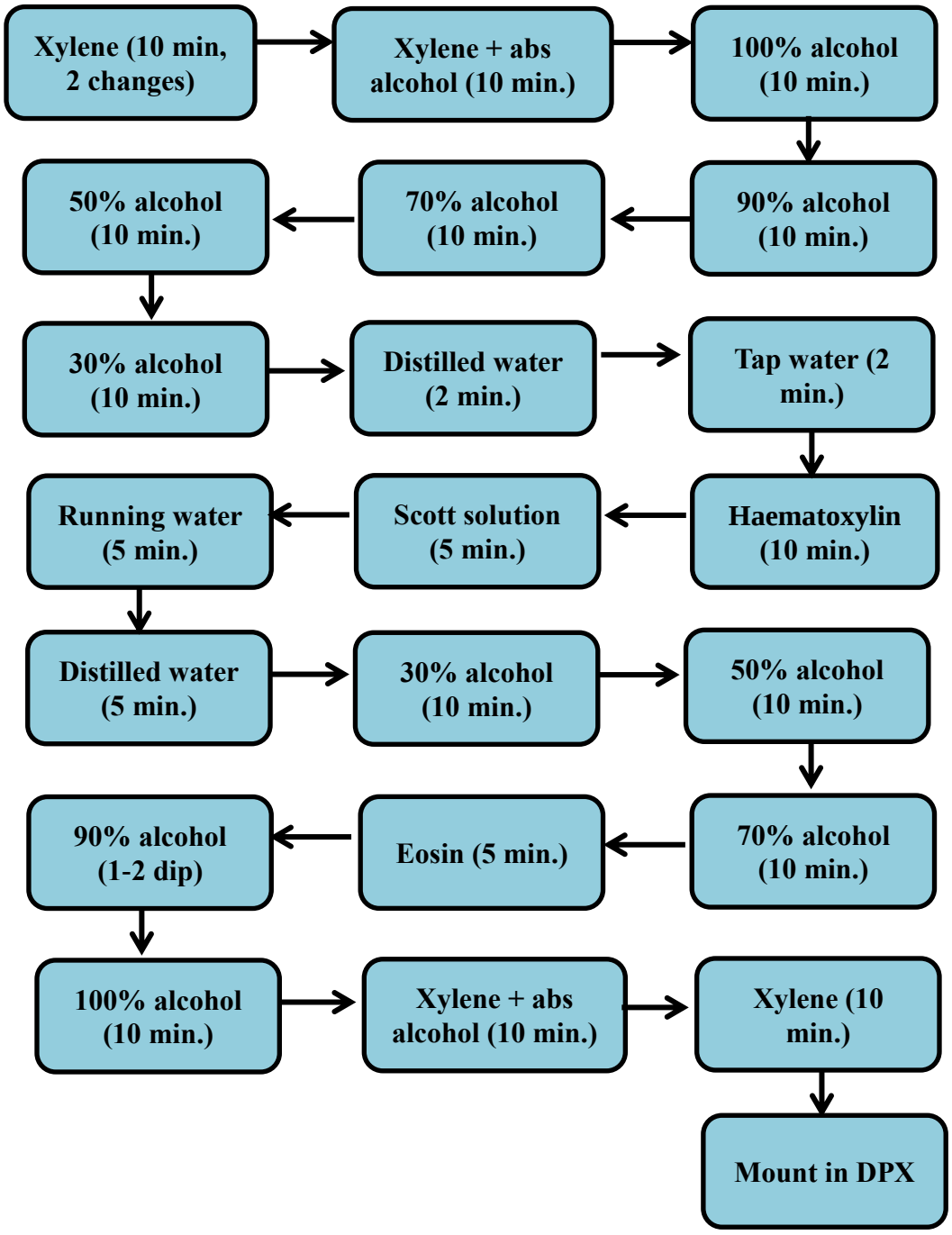


Figure 2: Detailed procedure of staining and slide preparation with its estimated duration.

3. Result

3.1. Lethal toxicity test (LC_{50})

The result showed that CPF was highly toxic to fish. Exposure of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) to different concentrations of CPF showed varied degree of mortality in different reproductive season with a wide range of concentrations. The percentage of mortality was significantly increased with the increase in concentration of the toxicant as well as duration of the experiment (F=52.27 in pre-spawning phase, F=134.24 in the preparatory phase, F=211.35 in resting phase; $p < 0.05$; Figure 3-4). In lower concentration the adult (0.002 mM/l) showed 0% mortality while in highest concentration (0.142 mM/l), 100 % mortality was recorded in 96 hr duration in pre-spawning phase (Figure 4B). However, in the preparatory phase, 0% mortality was occurred at 0.005 mM/l and 100% mortality at 0.199 mM/l in 96 hr exposure (Figure 3B). In the resting phase, 0% mortality was found at 0.057 mM/l and at 0.57mM/l, 100% mortality was recorded in 96 hr toxicity duration (Figure 3A). On the basis of mortality, LC_{50} value was found higher in resting phase (1.547, 0.678, 0.299 and 0.174 mM/l), moderate in preparatory phase (0.332, 0.193, 0.152 and 0.123 mM/l), whereas minimum in pre-spawning phase (0.296, 0.107, 0.044 and 0.026 mM/l) for 24, 48, 72, and 96 hr respectively (Table 1). The observation of the safe concentration value study represented that in resting phase, the safe concentration was higher as compared to preparatory and pre-spawning phase of reproductive cycle (Table 2).

3.2. Behavioral alterations

The behavior responses (Table 3) are the most sensitive indicator of potential toxic effects. CPF influences the behavioral pattern of fish. The observation of behavioral changes was started within 30 min after treatment with CPF. The response time was decreased as the toxicant concentration was increased. The behavioral alterations were positively correlated with the concentration of toxicant in different seasons of reproduction (Table 4-6). These alterations were very clear in all the reproductive phases.

But the occurrence of alterations in treatment groups were higher even in low concentrations during pre-spawning phase than preparatory and resting phase of reproductive cycle as compared to their control groups may be due to maturity aspects.

The control group showed a normal behavior and tends to move together. They came to the surface at intervals to gulp air. In low concentrations of CPF in all reproductive phase viz., in resting phase (0.057 and 0.085 mM/l), in preparatory phase (0.005, 0.028 mM/l) and in pre-spawning phase (0.002, 0.005 mM/l), fish showed similar behavior as of control group. The exposed fish showed a number of abnormalities in their swimming pattern. The swimming speed of fish was frequent and become erratic, and fish appeared hyper excitable with jerky movements in higher concentration and this behavior was shown with immediate exposure of toxicant that slow down with the passage of treatment duration. The schooling behavior was also disturbed in comparison to control, followed by hyperactivity, pectoral fin forward, convulsions, ingulping, avoidance behavior, escaping tendency and loss of buoyancy by vertically hanging in the test aquaria after treatment and moreover progressively became sluggish, lethargic and finally lead to death as compared to control group.

Convulsion prior to death were most evident, the severity of which paralleled with the concentration of CPF. Fins drooping were also observed inexposed fish. On initial exposure at higher concentration, the fish exhibited characteristic avoidance behavior by rapid swimming, stretching half of their body out of the water surface and trying to jump out. The opercular movements or beats are major indicators of stress. The opercular beats increased to 130/min in CPF exposed fish group from 85-90/min obtained in the control group. When the oxygen was reduced in toxic medium of different concentration fish were showed surfacing and air ingulping.

3.3. Morphological alterations

The exposed fish to CPF showed various abnormal morphological features which include pale yellow body colour, discoloration of skin, lesion of skin, eye deformities

(Pop eye and Anapthalmia), fin deformities and high mucus secretion and its coagulation all over the body as compared to control (Figure 5-6). Barbels were also losing their colour. These deformities were positively correlated with CPF concentrations and exposure duration among all phase of reproduction (Table 7-9).

3.4. Histological analysis

The histological observation of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) presented clear cut evidence of pesticidal toxicity. The changes were severe in all treated groups of chlorpyrifos, and both short term and long term study also when compared with control fish tissue in all reproductive phases' viz., resting, preparatory and pre-spawning phase.

In this study a variety of histo-pathological changes were observed in the ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) after CPF treatment. In the control group of animal, the ovaries were surrounded by germinal cuboidal epithelium. There was a connective tissue called tunica albugina which in turn surrounded by ovarian stoma at which female cell was embedded. The ovary of control fish showed large number of mature and maturing oocytes in pre-spawning phase. The immature oocytes were transparent with nucleus and cytoplasm. Mature oocytes are round opaque and contain large and small yolk globules. The vitelline membrane and follicular layer become more prominent.

3.4.1. Ovarian histology of control catfish (Heteropneustes fossilis)

The histological examination of control freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) ovary of resting phase showed an oogonia or primary oocytes and chromatin nucleolus oocytes (Figure 7). The entire oocyte was covered by a layer of follicular cells. They were present along the ovigerous lamellae and characterized by a homogeneous basophilic cytoplasm with a large nucleus. The chromatin nucleolus

oocytes were originated from oogonia. They had little cytoplasm large and stained nucleus and central nuclei.

The preparatory phase of reproduction was clearly characterized by the presence of perinucleolar and cortical alveoli or yolk vesicle oocytes- an indication of vitellogenesis in preparatory phase (Figure 9). These oocytes were larger oocyte in relation to earlier oocytes. The number of nucleoli had increased, and the nuclear membrane acquired an irregular outline. The cytoplasm now appeared dense and granular, and there was a slight reduction in its basophilia. These oocytes were covered by prominent follicular layer. The yolk vesicle oocytes were characterized by the presence of yolk in the cytoplasm. Initially these vesicles were deposited along the periphery in the form of minute granules which were interspersed with the vacuoles. As the diameter of the oocyte increased, these granules coalesced to form platelets which impart the characteristic greenish-brown colour to the ovary. The fully formed primary oocyte was invested with an outer-most layer of thin and elongated thecal cells which abuts against an inner layer of granulosa cells. The yolk was separated from the granulosaa layer by a thin vitelline membrane or chorion. The nucleus was smaller and compare to the earlier stages and chromatin threads still occurred in the nucleus, and the nuclear membrane began to degenerate. The zona radiate layer was very evident and follicle epithilium was more developed.

During pre-spawning phase, the ovaries were enlarged and various cytological changes were observed in the oocytes indicating rapid growth and maturation (Figure 11). This phase was observed with the presence of yolk granule and primary vitellogenic oocytes. The growth during this phase was mainly due to formation of yolk vesicles and deposition of yolk. The nuclear membrane was completely degenerated. The follicular layer and zona radiate were now well developed and distinct. In *Heteropneustes fossilis* during this phase, oocytes proliferated and all types of oocytes were visible except the matured ones.

3.4.2. Ovarian histology of CPF exposed Heteropneustes fossilis: Concentration study

The experimental fish exposed to different concentrations of chlorpyrifos (CPF) and two sub-lethal concentrations of CPF, showed marked histological changes in the ovary. In acute exposure with different concentrations of CPF, ovary showed significant changes in all reproductive phases viz., resting, preparatory and pre-spawning phase. The changes observed in ovarian section of freshwater catfish, *Hetreopneustes fossilis* (Bloc, 1794) were dose-dependent. The histological changes were partial disruption of ovarian follicles, cytoplasmic vacuolization of germinal cells and reduction in number of maturing ovum and oocytes (Figure 7-12). The inter-follicular connective tissues were damaged. The cytomorphological structure of ovarian follicles got deformed and elongated, losing their typical configuration. Necrosis and fibrosis in connective tissue and damage to yolk vesicles of maturing oocytes were seen. Degenerative oocytes became phagocytic and formed atretic oocytes. The greater degree of histopathological changes was marked in the ovary exposed to higher concentration of CPF in all reproductive seasons. During resting phase, the cytoplasmic agglutination was noticed in increasing concentration of chlorpyrifos from 0.085 mM/l to 0.57 mM/l (Figure 7). The oocyte showed vacuole formation and disintegration of nucleus and more number of immature oocytes was noticed in higher concentration of CPF (Figure 7). At 0.57 mM/l, ovary showed the degenerative oocyte and the presence of vacuolization in cytoplasm. During preparatory phase, the formation of inter follicular spaces was noticed in exposed fish to different concentrations of CPF (Figure 9). Large number of oogonia and perinucleolar oocytes were present with proliferation of ovigerous lamella and thecal and granulosa layer. Oocyte nucleous exhibited degenerative changes (liquefaction of the perinuclear cytoplasm) followed by the condensation of the nucleous. After dissolution of the nucleoli, the oocytes become atretic. There was disruption in follicular wall by CPF exposure during pre-spawning phase (Figure 11). These all manifestations were occurred according to the potentiality of toxicant in the environment. At higher concentration of CPF, inter-follicular spaces were larger and vacuolation in developing oocytes were also observed. In certain oocytes inversion of granulosa layer was also observed (Figure 9). The above result showed the retardation of oocytes, proliferation and increase in the number of atretic follicles, cytoplasmis liquefaction, fibrosis and necrosis or degeneration

in the ovary was more in higher concentration exposure. At lower concentration of CPF (0.057, 0.028 and 0.002 mM/l in resting, preparatory and pre-spawning phase), ovary showed no more histological changes as compared to higher concentrationsof CPF.

3.4.3. Duration study of chlorpyrifos

In short term study of CPF, fish were exposed to 0.058 mM/l, 0.041 mM/l and 0.008 mM/l concentrations of CPF ($1/3^{rd}$ of LC_{50} of 96 hr) in resting, preparatory and pre-spawning phase of reproduction for 15 days. CPF caused histological changes in ovary viz., primary follicles began to show adhesion and as well as cytoplasmic retraction in oocyte was also occurred. There was partial disruption of ovarian follicles and vacuolation in cytoplasm of germinal cells. The ovarian follicles were losing their typical configuration. The inter-follicular connective tissue was damaged. Number of atretic oocytes increased. Cytoplasmic retraction, degeneration and clumping were more visible in oocyte. Partial destruction of the ovigerous lamellae and vitellogenic membrane was noticed. Necrosis and fibrosis in connective tissue and damage to yolk vesicles of maturing oocytes was observed (Figure 8, 10, 12). In pre-spwaning phase, in maturing oocytes, the granulosa layer got separated and complete or partial rupture was observed. Overall deformed ovarian tissue was observed in the section of the ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794).

In the long term study, the exposure duration was 30 days. The tested concentrations of chlorpyrifos (CPF) in different phase of reproduction were 0.017 mM/l, in resting phase, 0.012 mM/l in preparatory phase and 0.002 mM/l in pre-spawning phase. These all concentrations were $1/10^{th}$ of LC_{50} of 96 hr of CPF in their respective phases. The noticed changes were severe damage of the ovigerous lamellae, increased intrafollicular spaces, vacuolated cytoplasm, extrusion of karyoplasm and necrosis in the cytoplasm. The ovarian wall became frayed and broken. Additionally, a marked increase of atretic follicles, shrinkage, and embedded nucleoli into the surrounding cytoplasm in oocyte were observed (Figure 8, 10, 12). This study revealed that oocytes at their different stages of maturation get affected differently. Based on observations of the

ovarian tissue, it becomes evident that consistent $1/10^{th}$ of sub-lethal doses of CPF can bring a considerable change in the ovarian histological structure of the freshwater catfish *Heteropneustes fossilis* (Bloch, 1794),

Table 1: LC_{50} values with its 95% confidence limit (upper and lower) at various exposure periods in different reproductive phases in freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) exposed with organophosphorous pesticide, chlorpyrifos through Finney Probit analysis.

Reproductive phase	**Test duration (hr)**	**LC_{50} (mM/l)**	**95% confidence limits**	
			Upper	**Lower**
Resting phase	24	1.547	2.564	1.103
	48	0.678	0.969	0.514
	72	0.299	0.347	0.261
	96	0.174	0.191	0.159
Preparatory phase	24	0.332	0.41	0.283
	48	0.193	0.21	0.179
	72	0.152	0.166	0.14
	96	0.123	0.135	0.113
Pre-spawning phase	24	0.296	0.356	0.243
	48	0.107	0.143	0.074
	72	0.044	0.066	0.023
	96	0.026	0.051	0.01

Table 2: Safe concentrations of organophosphorous pesticide, chlorpyrifos (CPF) exposed to freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in different reproductive (resting, preparatory and pre-spawning) phase, calculated by Hart et al. (1945) and Henderson et al. (1959).

	Resting phase	**Preparatory phase**	**Pre-spawning phase**
Safe Concentration of CPF (mM/l)	0.039	0.019	0.004

Table 3: Operational descriptions of observed behavioral and morphological responses caused by organophosphorous pesticide, chlorpyrifos during current investigation

Behavioral and morphological responses	**Descriptions**
Opercular movement	Opening and closing of operculum
Ingulping	fish suddenly came to the water surface and in gulp air and just back to the bottom to settled down
Surfacing	fish swims below 20 mm from the water surface
Avoidance behavior	defense mechanism by which fish removes themself from unpleasant situations
Pectoral fin forward	pectoral fins are held perpendicular or in a more forward posture towards the head and no movement of pectoral fins
Convulsions	no swimming; continuous ataxia with intermittent body spasms, violent shaking
Loss of buoyancy	fish roll over on side or back by vertically hanging
Discoloration of skin	depigmentation of colour of skin
Fins drooping/ necrosis	damage and deterioration in fins
Pop eye	Protruding eyes, eyes become white or cloudy
Anapthalmia	One side of eye absent or lost

Table 4: Effect of different concentrations of organophosphorous pesticide, chlorpyrifos (CPF) on the behavioral pattern of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in resting phase of annual reproductive cycle. The represented data was of five independent experiments and number of fish in each experiment was ten.

Behavioral alterations	**Control**	**Concentration of CPF (mM/l)**								
		0.057	**0.085**	**0.114**	**0.142**	**0.171**	**0.199**	**0.228**	**0.285**	**0.57**
Opercular movement	-	-	-	+	+	++	++	+++	+++	+++
Ingulping	-	-	-	+	+	++	++	+++	+++	+++
Surfacing	-	-	-	+	+	++	+++	+++	+++	+++
Frequent swimming	-	-	-	-	++	++	++	+++	+++	+++
Avoidance behavior	-	-	-	-	+	++	++	+++	+++	+++
Hyperactivity	-	-	-	+	+	++	++	+++	+++	+++
Pectoral fin forward	-	-	-	+	+	++	++	+++	+++	+++
Convulsions	-	-	-	+	+	++	+++	+++	+++	+++
Abrupt swimming	-	-	-	+	+	++	+++	+++	+++	+++
Escaping tendency	-	-	-	+	++	++	++	+++	+++	+++
Loss of buoyancy	-	-	-	+	++	++	++	+++	+++	+++

Normal (-), Mild (+), Moderate (++), Maximum behavior (+++)

Table 5: Effect of different concentrations of organophosphorous pesticide, chlorpyrifos (CPF) on the behavioral pattern of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in preparatory phase of annual reproductive cycle. The represented data was of five independent experiments and number of fish in each experiment was ten.

Behavioral alterations	**Control**	**Concentrations of CPF (mM/l)**							
		0.005	**0.028**	**0.057**	**0.085**	**0.114**	**0.142**	**0.171**	**0.199**
Opercular movement	-	-	-	+	+	++	+++	+++	+++
Ingulping	-	-	-	+	+	++	+++	+++	+++
Surfacing	-	-	-	+	+	++	+++	+++	+++
Frequent swimming	-	-	-	-	+	++	+++	+++	+++
Avoidance behavior	-	-	-	+	+	++	+++	+++	+++
Hyperactivity	-	-	-	+	+	++	+++	+++	+++
Pectoral fin forward	-	-	-	+	+	++	+++	+++	+++
Convulsions	-	-	-	+	+	++	+++	+++	+++
Abrupt swimming	-	-	-	+	+	++	+++	+++	+++
Escaping tendency	-	-	-	+	++	++	+++	+++	+++
Loss of buoyancy	-	-	-	+	++	++	+++	+++	+++

Normal (-), Mild (+), Moderate (++), Maximum behavior (+++)

Table 6: Effect of different concentrations of organophosphorous pesticide, chlorpyrifos (CPF) on the behavioral pattern of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in pre-spawning phase of annual reproductive cycle. The represented data was of five independent experiments and number of fish in each experiment was ten.

Behavioral alterations	**Control**	**Concentrations of CPF (mM/l)**						
		0.002	**0.005**	**0.028**	**0.057**	**0.085**	**0.114**	**0.142**
Opercular movement	-	-	-	+	++	++	+++	+++
Ingulping	-	-	-	+	+	++	+++	+++
Surfacing	-	-	-	+	+	++	+++	+++
Frequent swimming	-	-	-	-	++	++	+++	+++
Avoidance behavior	-	-	-	+	+	++	+++	+++
Hyperactivity	-	-	-	+	+	++	+++	+++
Pectoral fin forward	-	-	-	+	+	++	+++	+++
Convulsions	-	-	-	+	+	++	+++	+++
Abrupt swimming	-	-	-	+	+	++	+++	+++
Escaping tendency	-	-	-	+	++	++	+++	+++
Loss of buoyancy	-	-	-	+	++	++	+++	+++

Normal (-), Mild (+), Moderate (++), Maximum behavior (+++)

Table 7: Effect of different concentrations of organophosphorous pesticide, chlorpyrifos (CPF) on the morphological alterations of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) at 96 hr of exposure in resting phase of annual reproductive cycle. The represented data was of five independent experiments and number of fish in each experiment was ten.

Morphological observations	**Control**	**Concentrations of CPF (mM/l)**								
		0.057	**0.085**	**0.114**	**0.142**	**0.171**	**0.199**	**0.228**	**0.285**	**0.57**
Mucus secretion	-	-	-	-	++	++	+++	+++	+++	+++
Discoloration of skin	-	-	-	-	++	++	+++	+++	+++	+++
Fins drooping/ necrosis	-	-	-	+	+	++	+++	+++	+++	+++
Lesions of skin	-	-	-	+	++	+++	+++	+++	+++	+++
Pop eyes	-	-	-	-	-	+	++	++	+++	+++
Anapthalmia	-	-	-	-	+	+	++	++	+++	+++

Normal (-), Mild (+), Moderate (++), Maximum behavior (+++)

Table 8: Effect of different concentrations of organophosphorous pesticide, chlorpyrifos (CPF) on the morphological alterations of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) at 96 hr of exposure in preparatory phase of annual reproductive cycle. The represented data was of five independent experiments and number of fish in each experiment was ten.

Morphological observations	**Control**	**Concentrations of CPF (mM/l)**							
		0.005	**0.028**	**0.057**	**0.085**	**0.114**	**0.142**	**0.171**	**0.199**
Mucus secretion	-	-	-	+	++	++	++	+++	+++
Discoloration of skin	-	-	-	+	++	++	++	+++	+++
Fins drooping/ necrosis	-	-	-	++	++	++	+++	+++	+++
Lesions of skin	-	-	-	+	++	++	+++	+++	+++
Pop eyes	-	-	-	-	+	+	++	+++	+++
Anapthalmia	-	-	-	-	+	+	++	++	+++

Normal (-), Mild (+), Moderate (++), Maximum behavior (+++)

Table 9: Effect of different concentrations of organophosphorous pesticide, chlorpyrifos (CPF) on the morphological alterations of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) at 96 hr of exposure in pre-spawning phase of annual reproductive cycle. The represented data was of five independent experiments and number of fish in each experiment was ten.

Morphological observations	**Control**	**Concentrations of CPF (mM/l)**						
		0.002	**0.005**	**0.028**	**0.057**	**0.085**	**0.114**	**0.142**
Mucus secretion	-	-	-	+	++	++	+++	+++
Discoloration of skin	-	-	-	+	++	++	+++	+++
Fins drooping/ necrosis	-	-	-	+	+	++	+++	+++
Lesions of skin	-	-	-	+	++	+++	+++	+++
Pop eyes	-	-	-	-	-	+	++	+++
Anapthalmia	-	-	-	-	+	+	++	++

Normal (-), Mild (+), Moderate (++), Maximum responses (+++)

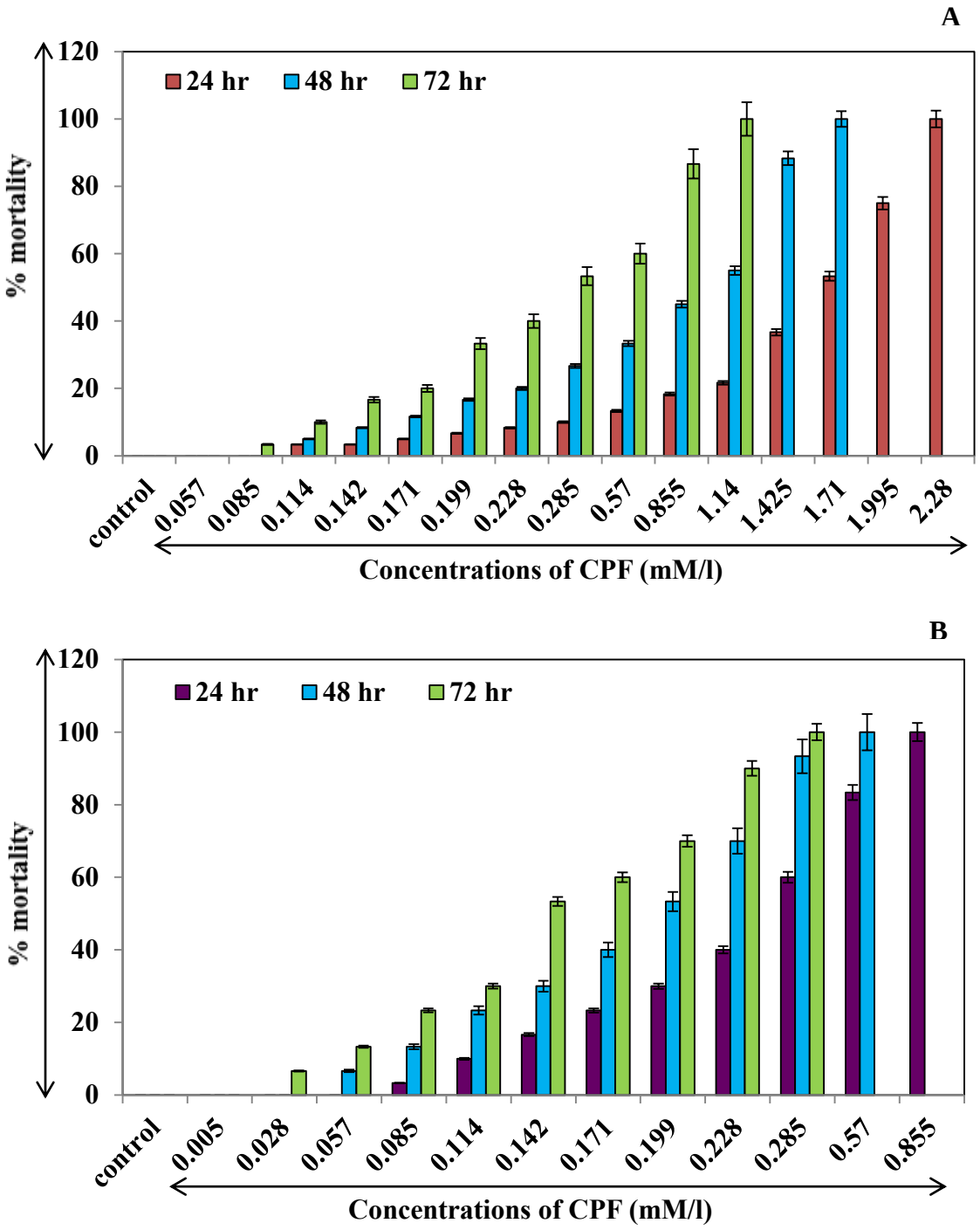


Figure 3: Effect of different concentrations of chlorpyrifos for 24 hr, 48 hr and 72 hr on % mortality of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in different phase of reproduction viz., A: *Resting phase* and B: *Preparatory phase*. Values were represented as means±SEM of percentage of mortality. Data were analyzed by one way ANOVA ($p < 0.001$).

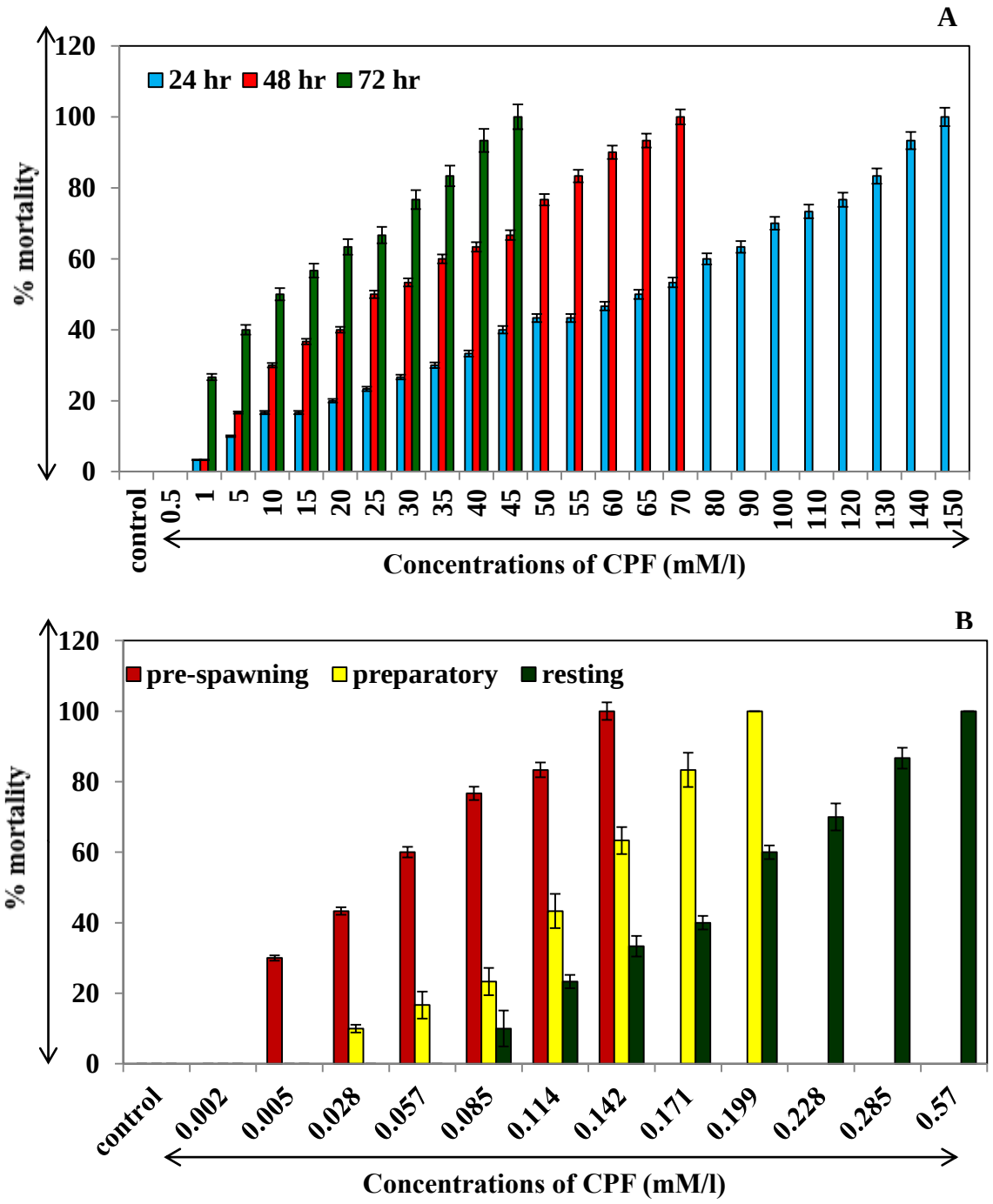


Figure 4: (A) Effect of different concentrations of chlorpyrifos (CPF) for 24 hr, 48 hr and 72 hr on % mortality of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in *pre-spawning phase* of reproduction. (B) Effect of different concentrations of CPF for 96 hr on % mortality of freshwater catfish, *Heteropneustes fossilis* in *pre-spawning, preparatory* and *resting phase.* Values were represented as means±SEM. Data were analyzed by one way ANOVA ($p < 0.001$), followed by Newman-Keuls test ($p < 0.05$) for comparison within the groups.

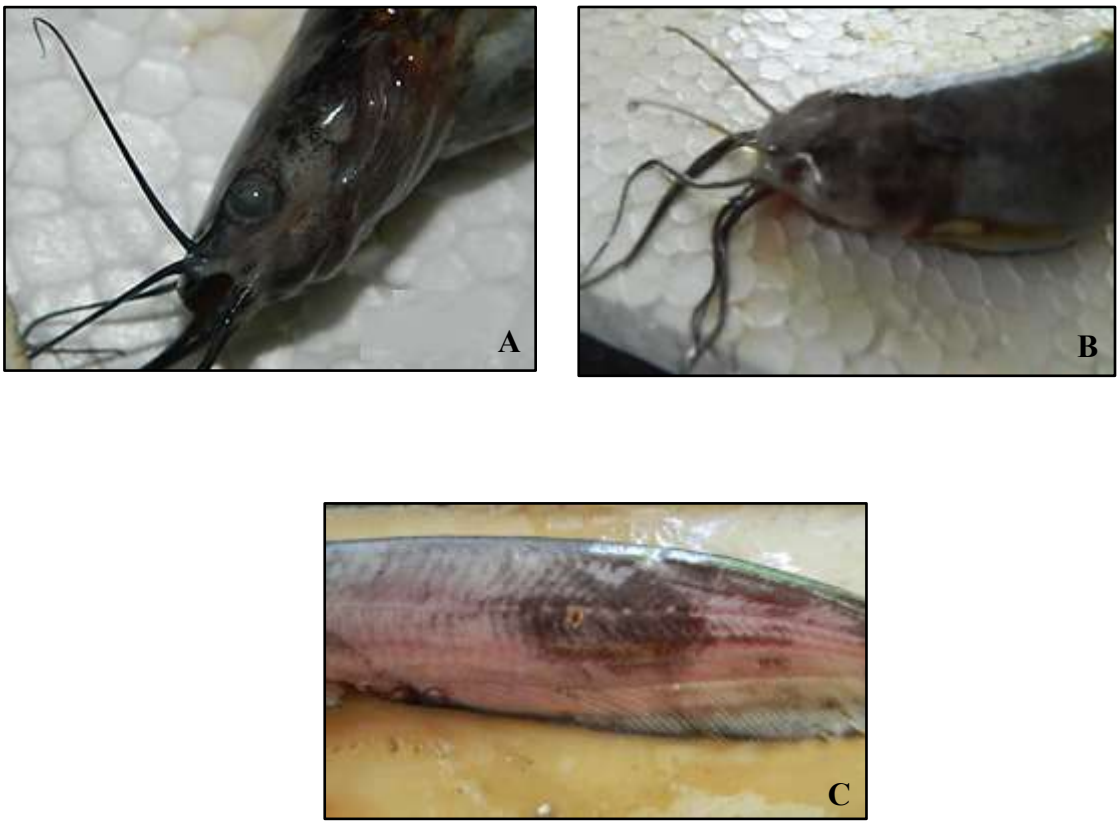


Figure 5: Effect of organophosphorous pesticide, chlorpyrifos (CPF) on the morphological appearance of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) up to 96 hr exposure period. Fish showed (A): Pop eye, (B): Anapthalmia, (C): skin lesions.

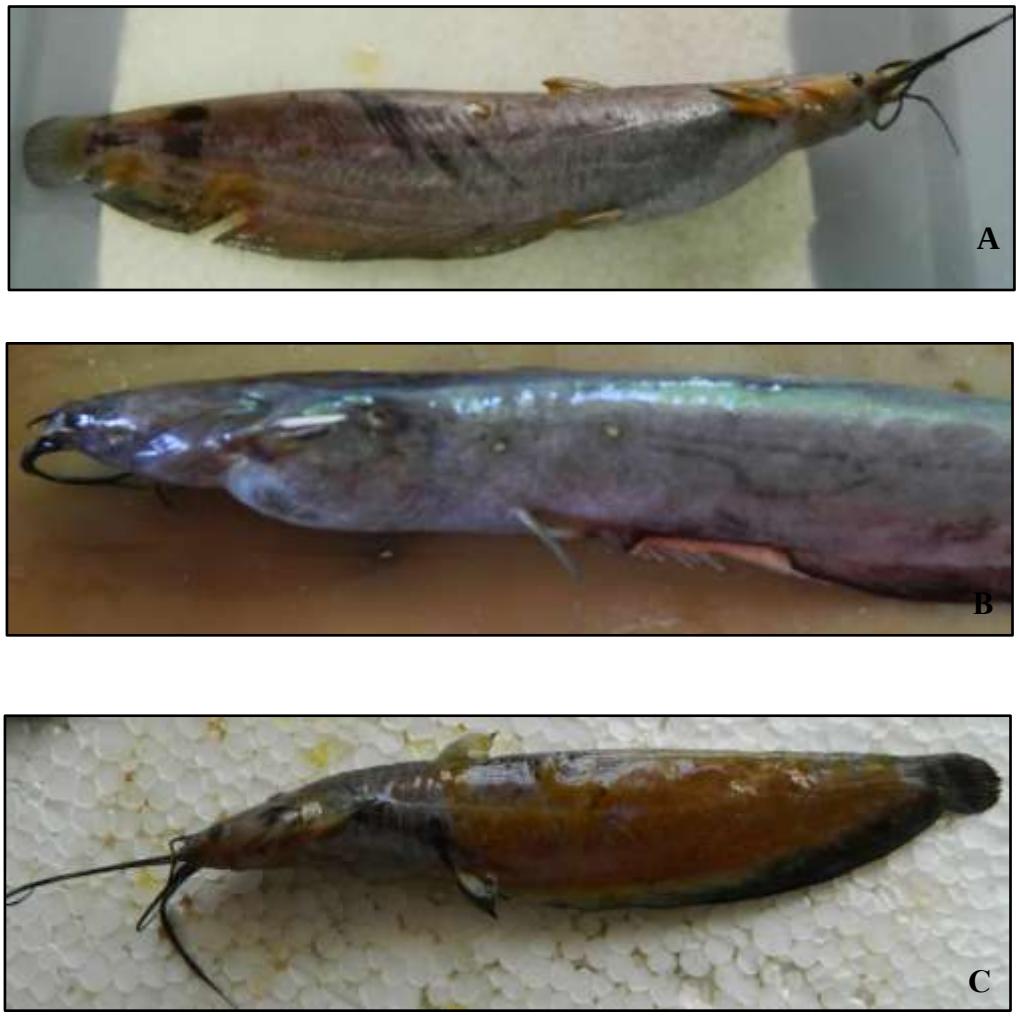


Figure 6: Effect of organophosphorous pesticide, chlorpyrifos (CPF) on the morphological appearance of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) up to 96 hr exposure period. Fish showed (A): fin splitting, (B): fin necrosis, skin discoloration and skin lesions, (C): heavy mucus secretion.

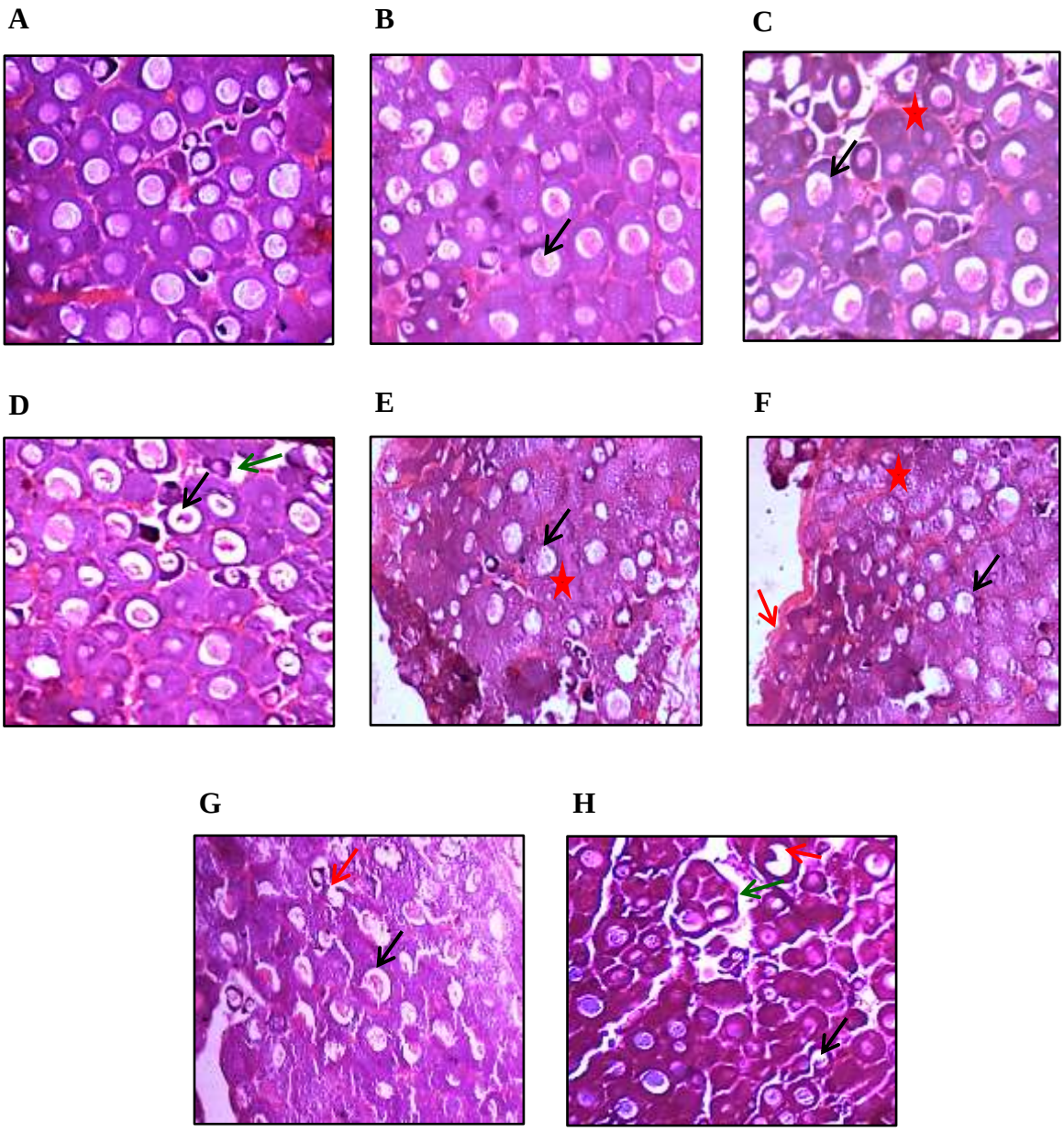
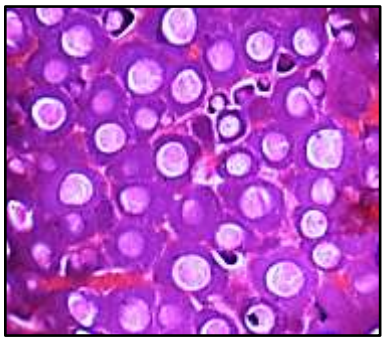
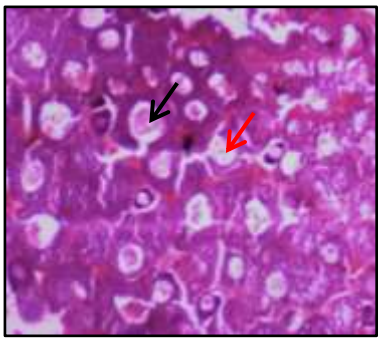


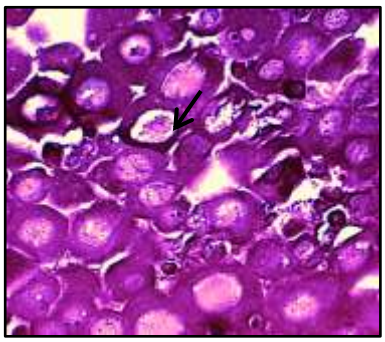
Figure 7: Transverse section of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) exposed to different concentrations of chlorpyrifos for 96 hr in *resting phase.* Histological photographs showed ruptured ovarian wall (red arrow), cytoplasmic retraction of oocyte (black arrow), cytoplasmic liquefaction (red arrow), wrinkled oocyte (red star) and inter-follicular space (green arrow). A- Control, B- 0.057 mM/l, C- 0.085 mM/l, D- 0.114 mM/l, E- 0.171 mM/l, F- 0.228 mM/l, G- 0.285 mM/l, H- 0.57 mM/l. Images were captured at 10X magnification.



Control fish



Short term ($1/3^{rd}$ of LC_{50})



Long term ($1/10^{th}$ of LC_{50})

Figure 8: Transverse section of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) exposed to $1/3^{rd}$ and $1/10^{th}$ of LC_{50} of 96 hr of CPF (0.058 mM/l and 0.0174 mM/l) in *resting phase* for 15 and 30 days of exposure duration. Histological photographs showed cytoplasmic retraction of oocyte (black arrow) and cytoplasmic liquefaction (red arrow). Images were captured at 10X magnification.

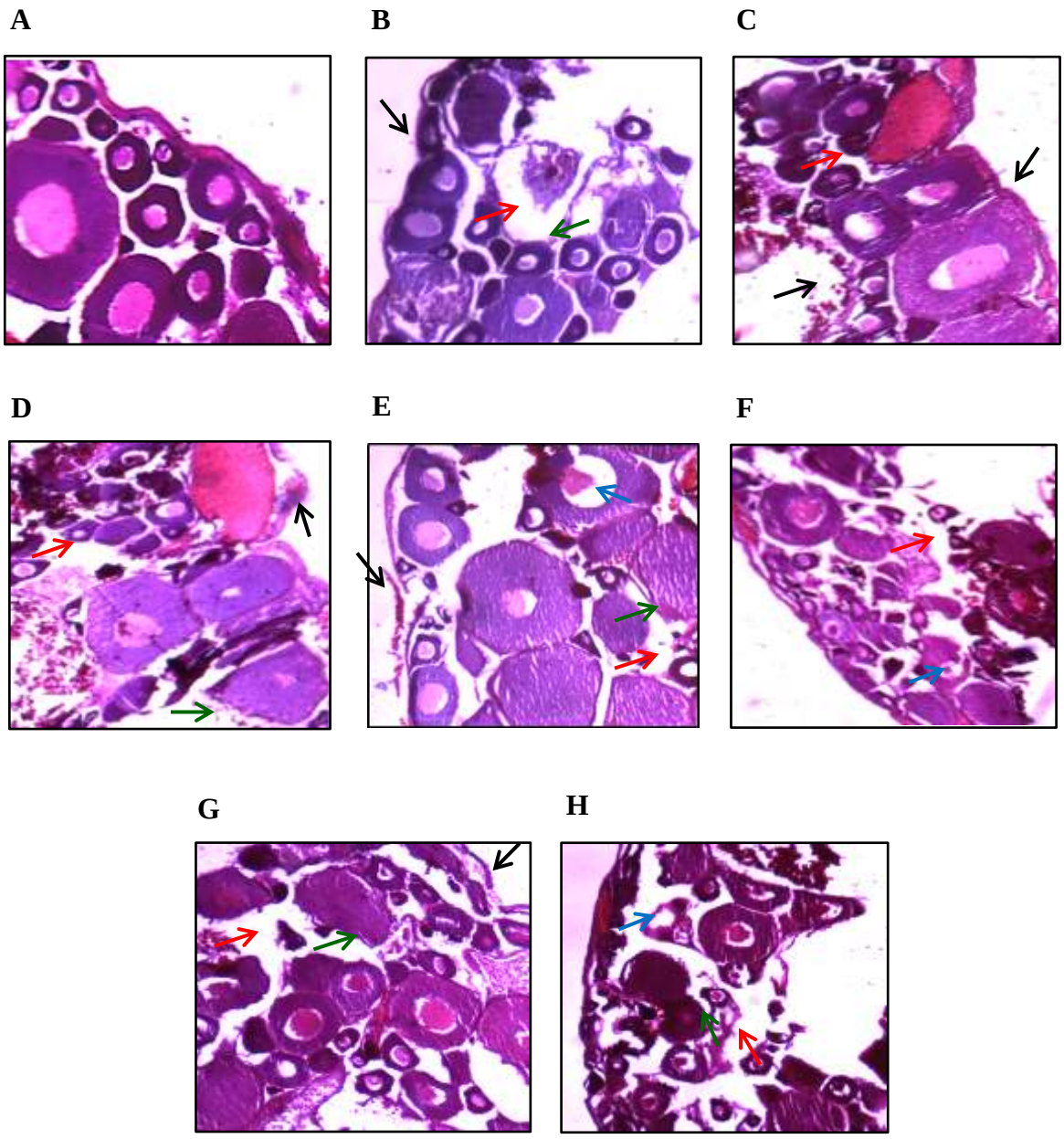
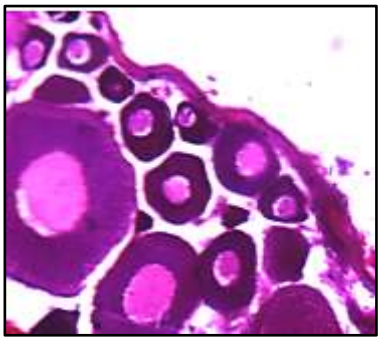
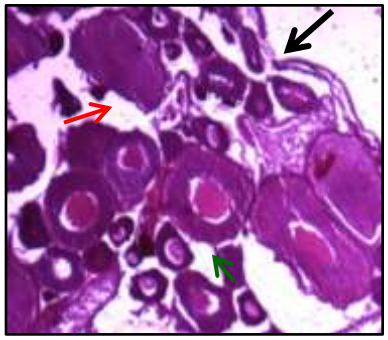


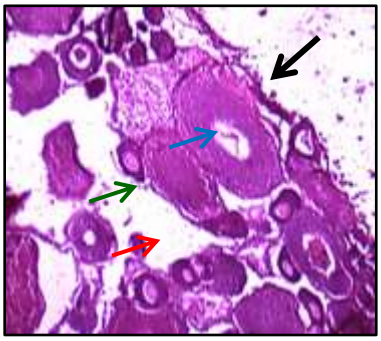
Figure 9: Transverse section of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) exposed to different concentrations of chlorpyrifos for 96 hr in *preparatory phase.* Histological photographs showed ruptured ovarian wall (black arrow), inter-follicular space (red arrow), cytoplasmic liquefaction (blue arrow) and degenerated granulosa layer (green arrow). A- Control, B- 0.028 mM/l, C- 0.057 mM/l, D- 0.085 mM/l, E- 0.114 mM/l, F- 0.142 mM/l, G- 0.171 mM/l, H- 0.199 mM/l. Images were captured at 10X magnification.



Control fish



Short term (1/3rd of LC_{50})



Long term (1/10th of LC_{50})

Figure 10: Transverse section of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) exposed to 1/3rd and 1/10th of LC_{50} of 96 hr of CPF (0.041 mM/l and 0.12 mM/l) in *preparatory phase* for 15 and 30 days of exposure duration. Histological photographs showed ruptured ovarian wall (black arrow), inter-follicular space (red arrow), cytoplasmic liquefaction (blue arrow) and degenerated granulosa layer (green arrow). Images were captured at 10X magnification**.**

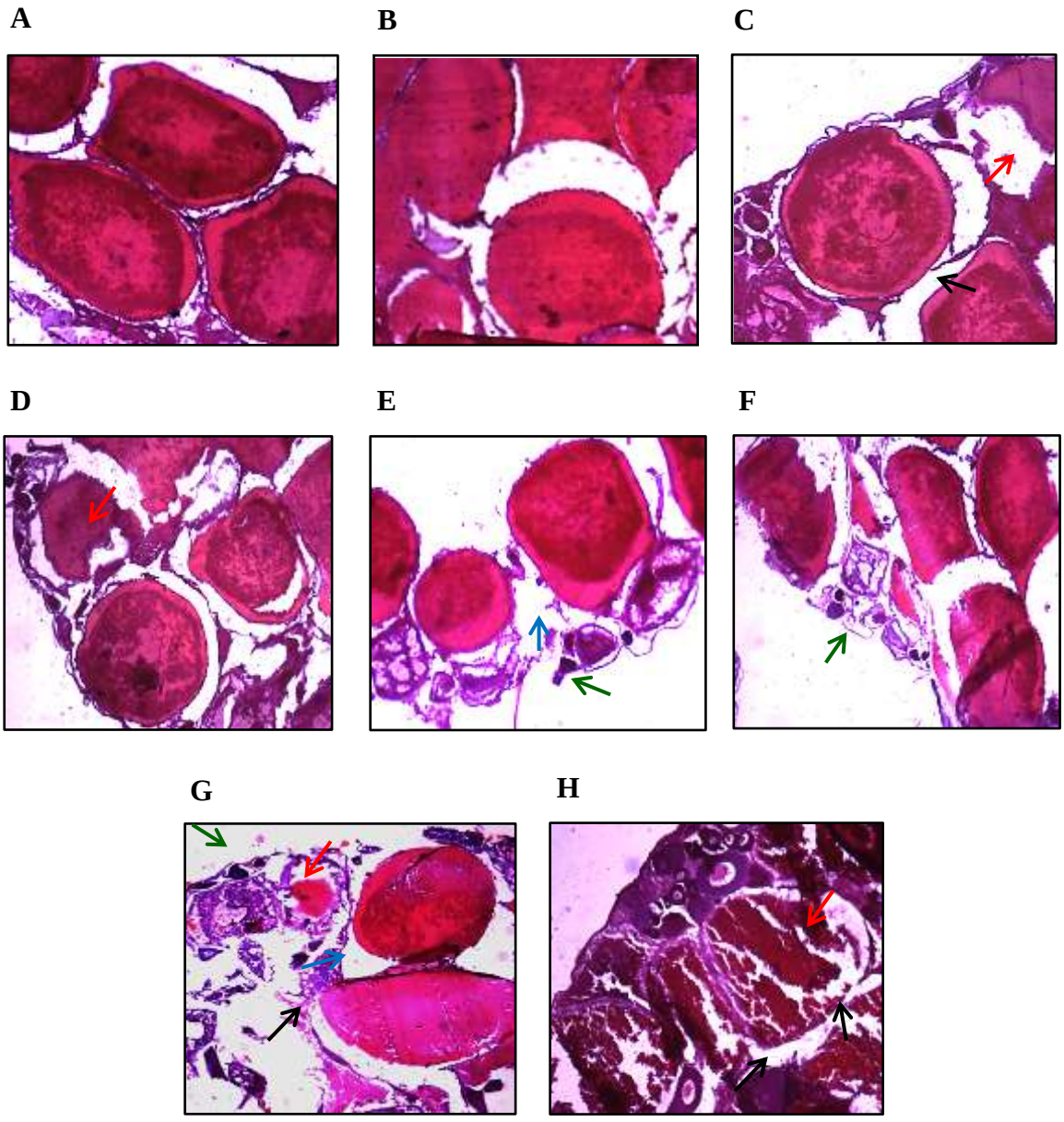
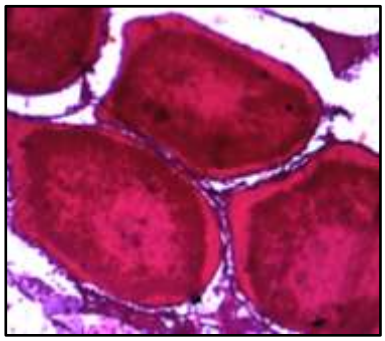
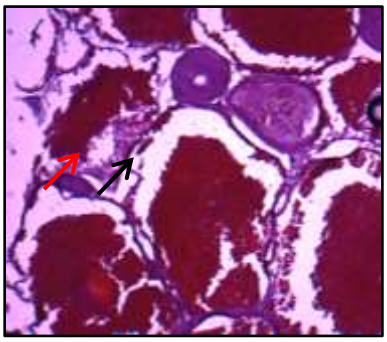


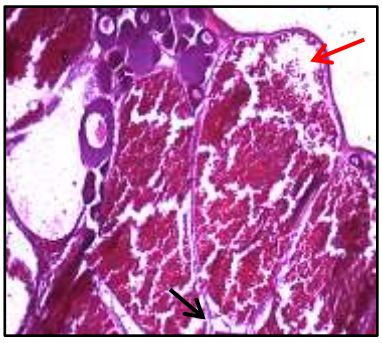
Figure 11: Transverse section of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) exposed to different concentrations of chlorpyrifos for 96 hr in *pre-spawning phase.* Histological photographs showed degenerated granulosa layer (black arrow), ruptured oocyte (red arrow), inter-follicular space (blue arrow) and degenerated of ovarian wall (green arrow). A- Control, B- 0.002 mM/l, C- 0.005 mM/l, D- 0.028 mM/l, E- 0.057 mM/l, F- 0.085 mM/l, G- 0.114 mM/l, H- 0.142 mM/l. Images were captured at 10X magnification.



Control fish



Short term ($1/3^{rd}$ of LC_{50})



Long term ($1/10^{th}$ of LC_{50})

Figure 12: Transverse section of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) exposed to $1/3^{rd}$ and $1/10^{th}$ of LC_{50} of 96 hr of CPF (0.008 mM/l and 0.002 mM/l) in *pre-spawning phase* for 15 and 30 days of exposure duration. Histological photographs showed degenerated granulosa layer (black arrow) and ruptured oocyte (red arrow). Images were captured at 10X magnification**.**

4. Discussion

The toxicity study is essential to find out toxicants limit and safe concentration, so that there will be minimum harm to aquatic fauna in the near future. The assessment of toxicity of chlorpyrifos (CPF) with reference to aquatic biota, especially fish is crucial in establishing the toxicity evaluation. In last few decades a lot of attention has been paid towards bioassay due to its proved potential to evaluate toxicity of chemicals. Several workers investigated the toxicity of organophosphorous pesticides in fish (Koundinya and Ramamurthi, 1979; Johnson and Finley, 1980; Kumar and Gupta, 1997; Santhakumar et al., 2000; Singh et al., 2009; Srivastava et al., 2010; Zhang et al., 2010; Barbieri and Ferreira, 2011; Maniyar et al., 2011).

In the present study, an attempt has been made to investigate the toxicity of CPF to freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) by estimation of the median lethal concentration (LC_{50}). The investigation of its impacts on behavior, morphology and ovarian histology were also observed. The lethal concentration (LC_{50}) of CPF was varied with different reproductive phases in an annual cycle which clearly indicate that the acute toxicity of CPF varied with the fish species and even same species under the influence of number of factors including size and sensitivity to the toxicant, its concentration and duration of exposure and reproductive season as well. The safe concentration of chlorpyrifos for experimental fish, freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) suggested that in resting phase, fish enjoy high tolerance limit (safe value) as compare to preparatory and pre-spawning phase. During pre-spawning phase even small concentration, i.e. safe in resting phase, may be lethal to fish. This suggested that as the fish moved toward their gonadal maturity, became more vulnerable to health hazards by aquatic toxicants.

The present investigation reported that as the maturity of fish was increased LC_{50} value become decreased. The sub-lethal concentration of CPF was different in different exposure duration and reproductive phases (Table 1). It is difficult to compare the toxicity of individual pesticides to different fish species (Schimmel et al., 1976).

Variations in the LC_{50} depend upon many factors such as animal weight, developmental stages (Kamaldeep and Joor, 1975), period of exposure and temperature (Pathan et al., 2009) and number of biological and physiological factors which have been reported by earlier workers (Srivastav et al., 2002; Jaroli and Sharma, 2005; Ali et al., 2008; Banaee et al., 2013; Padmanabha et al., 2015). A wide range of toxicant was found to increase the toxicity at higher temperature (Macek et al., 1969; Muirhead, 1971). It was related to the higher metabolic and respiration rate, which may largely be involved in toxicant response in an annual cycle (Macek et al., 1969; Wedemeyer et al., 1976; Gordon and McLeay, 1977). Fish in general have low metabolic rate in resting phase (Pickford and Atz, 1957). This low metabolic activity results in slowdown toxicant absorption and its bioaccumulation (Hussein et al., 2006). The result found that highest LC_{50} value was recorded in resting phase as compared to preparatory and pre-spawning phase. The toxicant absorption and its bioaccumulation were increased comparatively in preparatory and pre-spawning phase with increase in metabolic activity (Pickford and Atz, 1957).

The previously studies of acute toxicity of chlorpyrifos for freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) revealed that LC_{50} value for 96 hr was found to be 1.2 µl/L (Barbhuiya and Dey, 2014a). The value of LC_{50} 36 hr of chlorpyrifos was determined as 2.84 ppm for *H. fossilis* in the laboratory condition (Khatun and Mahanta, 2014). LC_{50} value of deltamethrin to *H. fossilis* was 1.86 µg/l (Srivastav et al., 2002). This is in agreement with Sprague (1969) who observed variation in LC_{50} for the same species and toxicant depending on size, age, sex and condition of test species along with experimental factors.

Reports on the acute toxicity tests by earlier workers revealed a clear idea of comparative lethal values of organophosphorous pesticide (CPF) for different fish species (Santhakumar and Balaji, 2000; Mathivanan, 2004; Ramasamy et al., 2007). The LC_{50} of chlorpyrifos and other organophosphates varied considerably with different fish species and environment viz., 297.63 µg/l in *Gambusia affinis* (Rao et al., 2005), 154 µg/l and 0.022 ppm in *Oreochromis mossambicus* (Rao et al., 2003; Padmanabha et al., 2015), 0.365 ppm and 811.98 µg/l for *C. punctatus* (Jaroli and Sharma, 2005; Ali et al.,

2008), 0.002 mg/l in *Tilapia guineensis* (Chindah et al., 2004) and 0.16 mg/l, and 203 µg/l in *Cyprinus carpio* (Halappa and David, 2009; Banaee et al., 2013). The range of 96 hr LC_{50} of CPF was 0.57-3270 ppb for mosquito fish, Bluegill, Fathead minnow, Rainbow trout, Nila tilapia, Goldfish (Davey et al., 1976; Holcombe et al., 1982; Bowman, 1988a, b; Gul, 2005; Wang et al., 2009). Ramesh and Saravanan (2008) reported that 24 hr LC_{50} of CPF in *Cyprinus carpio* was 5.28 ppm. The calculated LC_{50} for 96 hr of chlorpyrifos-methyl, for adult male Guppy *Poecilia reticulata* was 1.79 mg/l and for *O. niloticus* larvae was 0.92 mg/l (Ali, 2005). Different LC_{50} values were reported in previous studies for different organophosphate pesticides. LC_{50} value of malathion was 9 µl/l for *Labeo rohita* (Patil and David, 2008) and 16 µl/l for *Ophiocephalus punctatus* (Pugazhvendan et al., 2009). 96 hr LC_{50} of different organophosphate pesticides- monocrotophos, diazinon, nuvan and dimethoate in *Channa punctatus, Silurus glanis, Ctenopharyngodon idella* and *Heteropneustes fossilis* were 18.56 ppm (Agrahari et al., 2006), 4.142 mg/l (Koprucu et al., 2006), 6.5 mg/l (Tilak and Kumar, 2009) and 11.34 mg/l (Srivastava et al., 2010) respectively. The organophosphorous compound Coroban (20% EC- CPF) LC_{50} for 96 hr was 2.2 mg/l of *H. fossilis* (Srivastav et al., 1997). 24 hr, 48 hr, 72 hr and 96 hr LC_{50} value of Tricel (20% EC-CPF) for *Labeo bata* was as 257.03 µg/l, 208.92 µg/l, 177.82 µg/l and 109.64 µg/l respectively (Samajdar and Mandal, 2015). The LC_{50} value of other organophosphate pesticide, dimethoate for *Clarius batrachus* was 65 mg/l (Begum and Vijayaraghavan, 1995), 17.9 mg/l in *Channa punctatus* (Srivastav and Singh, 2001), 2.98 mg/l in *H. fossilis* (Pandey et al., 2009), 0.007 ppm of *Catla catla* (Kumar and Singh, 2000) and 1.61 mg/l for *Cyprinus carpio* (De Mel et al., 2005). Thus LC_{50} value of organophosphorous pesticides showed considearble variation reflecting different tolerance limit of different fish species.

Omoregie et al. (1990) observed that toxicants in the aquatic environment result in mortality of aquatic organisms and several physiological dysfunctions in fish. The fish response towards toxic medium is important since it reflects the internal changes and also externally as behavioral and morphological toxicity. Behavioral changes as a result of stress are accepted as the most sensitive indication of potential toxic effects. The level of

aquatic toxification can be assessed by behavioral changes at very first and by morphological changes in a while. These are considered as first signal of any kind of stress (Rapport et al., 1985).

In the present study, the observation of behavioral changes was started immediately after CPF treatment as compared to control group. The pesticides were influenced the behavior pattern of fish by interfering with the nervous systems and sensory receptors (Keizer et al., 1995; Pan and Dutta, 1998; Cong et al., 2009). The degree of distress was increased as the toxicant concentration increased. Noticeable changes in behavior were found at various concentrations of CPF.

Upon exposure to the toxicant, the immediate response of the fish was to drive to the bottom of the test chamber and stay there for few minutes of time. Diving to the bottom resembles the approach to the fish interpreted as avoidance behavior (Painter et al., 2009). The swimming pattern of exposed fish exhibited irregular, abrupt, erratic, jerky swimming movements, hyper excitability and loss of equilibrium. Fish also showed lateral swimming with their pectoral fin forward towards the head and the movement of fins was concentrations dependent however it was reduced with increasing exposure period. The exposed group of fish showed increased locomotor activity and entails increased expenditure of energy (Rathnamma et al., 2008; Halappa and David, 2009) due to stress. Abnormal behaviors such as convulsion (fish was showed no swimming, continuous ataxia with intermittent body spasm and violent sacking to avoidance of toxicant), restlessness, surface to bottom movement and sudden quick movement observed in the current study were similar to the observations of Omoniyi (2002), and Halappa and David (2009). The observed behavioral changes showed by the exposed fish to the chlorpyrifos were similar to those observations in other fish exposed to organophosphate pesticides (Ahmed, 1975; Ghosh, 1986; Shukla, 1995).

The physiological changes of any organism altered neurotransmitter levels or hormones that can affect external behavioral features (Shah, 2002; Scott and Sloman, 2004). Altered behavior may result from nervous system damage, because of

neurotransmitters are sensitive to contaminant exposure. Neurotoxicants that alter dopaminergic systems can affect swimming behavior and memory in fishes (Panula et al., 2006). Some pesticides, target the enzyme cholinesterase that breaks down the neurotransmitter cholinesterase for example mosquito fish *Gambusia affinis* exposed to sublethal concentrations of the pesticide CPF for 20 days reduced their locomotary behavior and swimming speed and accumulated acetylcholine at synaptic junctions (Rao et al., 2005). Abnormal swimming and loss of balance was caused by the deficiency in nervous and muscular coordination by toxicant (Rathnamma et al., 2008; Halappa and David, 2009). It might be due to some neurological impairment in central nervous system as evident by inhibition of AChE when they exposed fish to acute concentrations of different toxicants (Devi and Fingerman, 1995; Lata et al., 2001; Patro, 2006; Ricceri et al., 2006; Ezike and Ufodike, 2008; Oshode et al., 2008; Angelis et al., 2009). The behavioral changes observed in the present study indicated the loss of physiological equilibrium may be due to inhibition of the acetylcholinesterase enzyme in the brain and leading to acetylcholine accumulation at synaptic junctions (Fulton and Key, 2001; Rao et al., 2005; Agrahari et al., 2006; Patil and David, 2008; Ramesh and Saravanan, 2008; Anita et al., 2010) which was likely to caused prolonged excitatory post synaptic potential. This may first lead to stimulation and later caused a block in the cholinergic system. The stimulation of peripheral nervous system resulted in to increased metabolic activities and more oxygen utilization (Rao, 1989).

The control groups of fish were calm, quiet and preferred to confine themselves to the bottom of the aquarium. They swam horizontally and slowly as compared to the treated fish that swam in a slanting manner from a lower level to an upper level. It is likely that the region in the brain (telencephelon) which is associated with the maintenance of equilibrium should have been affected.

In the present study, the freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) showed an increment in the opercular movements and in surfacing behavior with the increasing concentration of CPF, to avoid breathing in the poisoned water (Lata et al., 2001) as observed by Shwetha and Hosetti (2009). The increase in opercular movement

and corresponding increase in rate of surfacing of fish clearly indicated that fish adaptively shift towards aerial respiration (by obtaining atmospheric oxygen surfacing) and fish tried to avoid contact with CPF through gill chamber. Opercular movement is one of the early warning systems as an indicator of respiratory stress. It gives direct relation towards oxygen stress. Since fish breath in water in which they live, changes in chemical properties there will surely be reflected in their respiratory activity (Almeido et al., 2009). The increased opercular movements in the initial period of exposure might be to support enhanced physiological activities in stressful habitat and later decreased with exposure duration, may be due to accumulation of mucus over the gill filaments. Similar findings were observed by Pandey et al. (2005), Omitoyin et al. (2006), Yadav et al. (2007), Koprucu et al. (2006), Srivastava et al. (2010) in *Channa punctatus, Clarias gariepinus, Channa straita, Silurus glanis,* and *Heteropneustes fossilis* exposed to mercuric chloride and malathione, lindane, fertilizer, diazinon and dimethoate respectively. Rapid opercular movement was also confirmed by Wasu et al. (2009) in *Clarias batrachus* treated with carbaryl and malathion. Shivakumar et al. (2006) also observed increased opercular movement in *Cyprinus carpio* exposed to endosulfan, cypermethrin and fenvalerate. Chindah et al. (2004) reported an initial increase in opercular beat frequency in chlorpyrifos exposed tilapia, followed by a marked decline with exposure time and explained the initial increase as sudden response to shock.

Treated fish slowly reached the water surface, probably due to gill damage that caused respiration malfunctions (Rathnamma et al., 2008) or as a result of difficulties in gas exchange due to mucous accumulation. Moreover, CPF cause severe damage to the gill membranes (Johal et al., 2007; Velmurugan et al., 2007), hampering the branchial exchange of gases and leading to asphyxia. Surfacing and gulping of air might be a compensatory device to cope with the oxygen deficiency (Israeli and Kimmel, 1996). The increased gulping activity by the exposed fish may be the reflection of an attempt by the fish to extract more oxygen to meet the increased energy demand to with stand the CPF toxicity. These behavioral patterns are the indication of respiratory impairment due to the effect of toxicant (Shwetha and Hosetti, 2009). Gulping of air may help to avoid contact of toxic medium (Katja et al., 2005). Similar observation has been reported by Patil and

David (2008) in malathion treated fish *Labeo rohita* and by Parithabhanu (2013) in cypermethrin treated *Orechromis mossambicus*.The surfacing phenomenon of fish might be a demand for higher oxygen level during the exposure period due to hydro toxic condition (Katja et al., 2005) and these results are supported by Cook et al. (2005), Shivakumar et al. (2006), Balasubramani and Pandian (2008), Charjan et al. (2008), Appa et al. (2009), Halappa and David (2009). According to Gabriel et al. (2010), this behavior may have resulted from enzyme dysfunction and paralysis of respiratory muscle or depression of respiratory center and disturbances in energy pathways leading to depletion of energy.

Fish were lethargic and at the time of death they exhibited transient hyperactivity before collapsing. The fish were found to be lying down motionless at the bottom before death and died by opening their mouth. Same behaviors were found by Koprucu et al. (2006), Patil and David (2008), Susan et al. (2010) and Barbhuiya and Dey (2014b).

Among morphological deformities, fish showed fin necrosis, depigmentation in body colour, eye deformities and cupous mucous secretion. The occurence of these deformities was increased with increasing concentration and duration of CPF. The fade body colour can be considered as symptoms of stress and it has also been reported by Ree and Paney (1997), Omitoyin et al. (2006), Koprucu et al. (2006) and Wasu et al. (2009) in fish like zebra fish, *Clarias gariepinus, Silurus glanis* and *Clarias batrachus* exposed to different pesticides. Pandey et al. (1990) also reported depigmentation in exposed fish and attributed it to reduction in number and size of chromatophores. The mucus accumulation also was observed all over the body surface of the exposed and dead fish in all concentrations which was found maximum at higher concentration of chlorpyrifos. This may due to the interference of toxicant with the gaseous exchange, secretion, waste products and osmoregulation (Oti, 2002; Adeyemo, 2005; Ayuba and Ofojekwu, 2005; Omitoyin, 2007; Okomoda et al., 2010). Fish secrete copious amount of mucus, as a defence mechanism to neutralize the insecticide effect, which gradually covers all over the body, gills and the buccal cavity. Moreover, Bisht and Agarwal (2007) observed hypertrophy and hyperplasia of mucous cells in $CuSO_4$ exposed fish and suggested it as

adaptive and defense mechanism to prevent cutaneous entry of toxicant by coagulation through increased mucus production.

Researchers have reported the same alterations in *Oryzias latipes* exposed to CPF, permethrin, phenol, strychnine and 2,4-dinitrophenol (Rao et al., 1997), *Cyprinus carpio* exposed to CPF (Halappa and David, 2009), *Labeo rohita* malathion exposure (Patil and David, 2008), *Oncorhynchus tshawytscha* diazinon exposure (Scholz et al., 2000), *Oryzias latipes* endosulfan exposure (Gormley and Teather, 2003), *Cirrhinus mrigala* fenvalerate exposure (Mushigeri and David, 2005), *Oreochromis niloticus exposed to* fenitrothion (Benli and Ozkul, 2010), *Oreochromis niloticus* exposed to dimethoate (Auta et al., 2002) *Heteropneustes fossilis* exposed to dimethoate (Pandey et al., 2009).

The two type of eye malformations were also noticed in CPF treated group of fish such as pop eye and anapthalmia. Such abnormalities were found may be due to the apoptosis of cells in the eye or reduce in the diameter of eye socket (Tyor et al., 2012). The fin necrosis, skin lesions and discoloration of skin were also noticed in CPF exposed fish. Similar reports were investigated by lger et al. (1994) in cadmium exposure treatment.

Further to know about the toxicological activity of chlorpyrifos on the ovarian tissue of freshwater catfish, *Heteropneustes fossilis*, the histological tool was used. CPF inhibited the growth of the ovary at different stages of maturation. Histological manifestations in ovaries may be caused by several factors and by a variety of effluents and aquatic pollutants (Sarojini and Victor, 1985; Davis and Cook, 1993; Farmer et al., 1995; Kumar et al., 2000). Fish exposed to different concentrations of CPF reported significant damage in ovarian tissue in all reproductive phases indicating that it was a useful methodology for monitoring the effects of pesticides on fish (Banaee, 2013). The result showed that the frequency of oocyte degenerations was more in higher concentration of CPF which was dose-dependent. The ovaries were predominated by oocytes which were at perinucleor stage with large nuclei and many nucleoli of various sizes. In *H fossilis*, after CPF exposure, oocytes exhibited degenerative changes,

liquification of perinuclear cytoplasm, condensation of nucleus, disappearance of nuclear membrane, cytoplasmic clumping, degenerated granulosa layer, and degenerated ovarian wall and wrinkled oocytes. Similar results were also found by Khillare (1992), Sukumar and Karpagaganpathy (1992), Ramachandra (2000) and Hossain et al. (2002). Partial disruption of the ovarian follicles, vacuolation in the cytoplasm of germinal cells, loss of connective tissue and secondary oocytes were reported in *H. fossilis* exposed to BHC (Hazarika and Das, 1998). Saxena and Agrawal (1986) have shown that cadmium chloride blocked all the oogonial activity at the vitellogenic phase in *C. btrachus* and suggested it that it might be due to retardation, oocytes proliferation and increase in number of atretic follicles. Similar finding were observed by Pawar and Katdare (1983), Khillare (1992), Sukumar and Karpagaganpathy (1992), Dutta (1996), Adityakumar et al. (2002), Lee and Yang (2002), Chandra et al. (2004) and Ghosh and Nath (2005). Jyothi and Narayan (1999) reported the impact sub lethal concentration of carbaryl on the ovary of *Clarias batrachus*, resulted in vacuolation, necrosis, arrested ovarian recrudescence and inter-follicular oedema.

Benarji and Rajendranath (1991) studied cyto-architectural changes in the oocytes, including pronounced vacuolation, degeneration and deformation, clumping of the cytoplasm and karyohypertrophy in *Clarias batrachus* exposed to dichlorvos. Degeneration of follicular walls, connective tissues and vacuolisation in the ooplasm of oocyte was observed in *A. testudineus* treated to carbofuran (Chatterjee et al., 1997). The formation of nucleolar extrusion has attracted the attention of many researchers because of its significance for the formation of proteins as reported by Khanna (1956). Muley and Mane (1987) observed ruptured follicles, completely dissolute nucleus and nucleoli in the gonads of Lamellibranch mollusks under cythion and malathion toxicity. Rastogi and Kulshrestha (1990) reported necrosis and fibrosis in connective tissue along with dilation of blood vessels and damage to yolk vesicles of maturing oocytes in carp minnow *Rasbora daniconius* under endosulfan, carbofuran and methyl parathion toxicity. The carbamates pesticides were also reported to induce similar degenerative changes in fish ovary (Kulshrestha and Arora, 1984; Sukumar and Karpagaganpathy, 1992).

During present study atretic oocytes and increase in the interfollicular spaces were observed. Same result was supported by findings of Nath and Kumar (1990) in *C. fasciatus* under nickel toxicity. Ramachandra (2000) reported that lower dose of Malathion caused reduction in ovarian weight and also retard the growth of the pre-vitellogenic oocytes, and on the other hand a higher dose resulted in the degeneration of the immature oocytes and rupture of follicular epithelium. Fish ovary exposed to other organophosphate compounds such as monocrotophos, methylparathion, phenthoate, malathion, and fenitrothion also resulted in follicular atresia and other degenerative changes in ovary (Pawar and Katdare, 1983; Shukla et al., 1984; Kumar and Pant, 1988; Dey and Bhattacharya, 1989; Rastogi and Kulshrestha 1990).

The present findings suggested that the histopathological changes in the ovary might be a reflection of disturbance in the endocrine/hormonal imbalance. Chatterjee et al. (1997) found that carbofuran altered both the area and the percentage occurrence of the various types of primary oocytes in the ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). He observed the degeneration of follicular walls, connective tissues and vacuolization in the ooplasm of the stage II and III oocytes. He stated that carbofuran at sub-lethal concentrations inhibits oocyte maturational processes in catfish. Giri et al. (2000) studied the effect of basathrin on the ovarian tissue of *H. fossilis* and he reported marked damage in germinal epithelium, atresia of oocyte, stromal hemorrhage, vacuolization of oocytes and general inflammation. Kling (1981) reported that cells assimilation of yolk granules during the exposure and simultaneous arrest of vitellogenesis caused the reduction in size of oocytes, resulting in a total atresia of ovaries of Tilapia.

Dutta et al. (1994) reported notable microscopic changes in ovigerous lamellae, oocytes at different stages of development and the nucleus of the immature oocyte of the catfish *Heteropneustes fossilis*. He observed clumping of cytoplasm, degeneration in the follicular cells, shrinkage of nuclear material, increased atretic oocytes, along with ruptured follicular epithelium. Saxsena and Saksena (1996) observed the toxicity of two organophosphorous pesticides Nuvan and Dimecron in freshwater murrel, *Channa*

orientalis. He found that both pesticide decreased gonadosomatic index, reduced diameter of different stages of oocytes and number of later stages of oocyte development and significant increase in the percentage of atretic follicles in ovaries of exposed animals. Dutta and Maxwell (2003) studied the effect of the pesticide, on the ovaries of bluegill (*Lepomis macrohirus*). He noticed adhesion of primary follicles, cytoplasmic retraction in oocyte II, cytoplasmic degeneration, increased atretic oocytes, damages to the oocyte IV, Partial destruction of the ovigerous lamellae and vitellogenic membrane, destruction of follicles, severe damage of the ovigerous lamellae, increased intrafollicular spaces, vacuolated cytoplasm, extrusion of karyoplasm and necrosis in the cytoplasm.

Khillare (1992), Sukumar and Karpagaganpathy (1992) observed that the exposed ovary to pesticide resulted in degenerative changes, liquification of perinuclear cytoplasm and condensation of nucleus, disappearance of nuclear membrane, cytoplasmic clumping, degenerated granulosa layer, and degenerated ovarian wall and wrinkled oocytes. Prominent inter-follicular spaces were observed in the ovary which was probably formed due to shrinkage of the oocytes. Based on observations of the ovarian anatomical changes with following given exposure times, it becomes evident that the sublethal doses of CPF can and will alter the microscopic anatomy of the fish ovary. Therefore this investigation demonstrates a relation among pesticidal stress, behavioral and morphological disorders, survival and mortality rates and histopathological changes in the ovary which further are a reflection of the disturbance in the endocrine/hormonal imbalance.

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CHAPTER – II

Effect of organophosphorous pesticide, chlorpyrifos on oocyte maturation and follicular steroids in freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)

ABSTRACT

The objective of the present study was to investigate *in vitro* effect of organophosphorous pesticide, chlorpyrifos (CPF) on hCG-induced oocyte maturation (OM) in the freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). In this observation, interplay of different steroids involved in oocyte maturation was also measured. For this, the post-vitellogenic follicles were incubated with human chorionic gonadotropin (hCG) alone to induce oocyte maturation in a concentration and duration-dependent manner and with CPF on hCG induced OM post-vitellogenic follicles. The result showed that CPF caused an inhibitory effect on hCG induced GVBD in co-incubation experiments at the dose of 0.26 pM/ml. Pre- and post-incubation of follicles with CPF resulted a significant inhibition in hCG induced oocyte maturation in all durations and maximum inhibition 87.67% and 76% was recorded at 12 hr incubation duration ($p < 0.05$, Newman-Keuls test). Effective dose of hCG (1.7 mg/ml, 8 hr) induced OM coincided with a significant increase in follicular pregnenolone, progesterone, deoxycorticosterone (DOC) and dihydroxyprogesterone (DHP) as well as significant decrease in estradiol-17β (E_2) as compared to control group of post-vitellogenic follicles. Under CPF exposure follicles elicited a sharp significant decrease in pregnenolone, progesterone and DHP. However, DOC and E_2 were increased significantly. Co-incubation study revealed that CPF

suppressed hCG induced concentrations of DHP, pregnenolone and progesterone significantly but side by side support DOC increase and maintain high E_2. Thus, the present results suggested that the CPF inhibited hCG induced oocyte meiotic maturation due to suppression of maturation inducing steroids within follicle.

Keywords: CPF, Pregnenolone, Progesterone, 17,20βP, Estradiol-17β, DOC, Oocyte maturation.

1. Introduction

In vertebrates, meiotic maturation of prophase-I arrested oocytes is pre-requisite for ovulation and subsequent fertilization to occur in vertebrates (Nagahama and Yamashita, 2008). There are several studies revealed that oocyte maturation in teleosts are three step induction process which involving gonadotropins (GtHs), maturation-inducing hormone (MIH) and maturation-promoting factor (MPF) (Nagahama and Yamashita, 2008). Developmental events in the ovaries are regulated by steroids synthesized under the direct influence of two gonadotropins; GtH1, GtH2 (Patino et al., 2001; Rahman et al., 2001; Mishra and Joy, 2006a, b, c; Nagahama and Yamashita, 2008; Skoblina, 2009). Gonadotropin plays an important role in the induction of final oocyte maturation by MIS synthesis in follicle cells (Kobayashi et al., 1988; Zhu et al., 1989; Nagahama, 1997; Patino et al., 2001; Senthilkumaran et al., 2004). Human chorionic gonadotropin (hCG) found in mammals is a structurally related variant of fish gonadotropin, which acts as an effective inducer of oocyte maturation in several teleost (Skoblina, 2009). It stimulates the *in vitro* steroidogenesis of granulosa cell and releases the MIS in the incubation medium (King et al., 1995; Sorbera et al., 1999). The follicular meiotic maturation in fish include germinal vesicle migration (GVM), a switch in follicular secretion from C_{18} to C_{21} steroids and the resumption of the meiotic process which are under the direct control of gonadotropin (Patino and Thomas, 1990; Nagahama et al., 1995; Patino et al., 2001; Mishra and Joy, 2006a; Nagahama and Yamashita, 2008; Skoblina, 2009). Meiosis is commenced with the surge in gonadotropin-releasing hormone (GnRH), followed by a rise in circulating luteinizing hormone (LH). Upon

binding of LH with the granulosa cell receptors, the production of maturation-inducing steroid (MIS) begins. MIH synthesis occurs in the ovarian follicle layers under the stimulation of GTHs, pre-ovulatory luteinizing hormone (LH) surge being the stimulus for final oocyte maturation (Nagahama and Yamashita, 2008). Different classes of steroid hormones (C_{18} to C_{21}) have shown to induce oocyte maturation in vertebrates including teleosts (Goswami and Sundararaj, 1974; Nagahama and Adachi, 1985; Trant and Thomas, 1989; Lutz et al., 2001; Senthilkumaran and Joy, 2001) apart from classical MIH, 17α-20β-dihydroxy-4-pregnen-3-one (1720βP) (Nagahama, 1997). The C_{21} steroids include pregnenolone (P_5: 5-pregnan,3β-ol-20-one), deoxycorticosterone (DOC: 4-pregnen-21-ol-3,20-dione), progesterone (P_4: pregn-4-ene-3,20-dione) and 17,20βP (DHP: 17α-20β-dihydroxypreg-4-ene-3-dione). Among these P_4, 17,20βP and DOC have been shown to be potent MIS or inducers of GVBD *in vitro* in teleosts (Goswami and Sundararaj, 1974; Sundararaj and Goswami, 1977; Young et al., 1982; Goetz, 1983; Nagahama 1983; Jalabert, 1976; Jalabert et al., 1991). MIS directly acts through the plasma membrane receptor on the oocyte surface and induces follicular oocyte maturation (Nagahama, 1997). 17,20βP is the most effective maturation-inducing steroid that induces resumption of meiosis in most of the fish species (Goetz, 1983; Sundararaj et al., 1985; Scott and Canario, 1987; Jalabert et al., 1991; Thomas, 1994; Nagahama, 1997; Mishra and Joy, 2006a). The estradiol-17β (E_2: C_{18} steroid), is the major regulator of vitellogenesis, a complex and elaborate process responsible for the growth of follicles and yolk, so deposited are used during early embryonic development (Nagahama, 1997).

A complex series of enzymes are responsible for the biosynthesis of gonadal steroid in fish. The pathway initiates with the synthesis of the C_{21} steroid precursor pregnenolone via side-chain cleavage of cholesterol by cholesterol side-chain cleavage cytochrome P450 (P450scc) enzyme. Pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme. Progesterone is then converted to 17α-hydroxyprogesterone by 17α-hydroxylase activity of cytochrome P450c17 enzyme which is followed by the production of 17,20βP by 20β-hydroxysteroid dehydrogenase (20β-HSD) enzyme, the key enzyme is induced by GtH (LH) in granulosa cells of post-vitellogenic follicles immediately prior to oocyte maturation (Nagahama et al., 1985) and

estradiol-17β by 17β-HSD enzyme and P450aromatase enzyme (P450arom). Progesterone is also converted in to deoxycorticosterone by 21-hydroxylase enzyme which further produced corticosterone by 11β-hydroxylase (Kagawa et al., 1982; Young et al., 1986; Nagahama, 1987; Nagahama and Yamashita, 2008).

Oocyte maturation (OM) has been widely studied in teleosts (Goetz, 1983; Scott and Canario, 1987; Jalabert et al., 1991; Nagahama et al., 1995; Patino et al., 2001; Mishra and Joy, 2006b). In the catfish *Mystus vittatus* and *C. batrachus*, 17,20βP has also been reported to be the most potent steroid for inducing OM (Upadhyaya and Haider, 1986; Haider and Rao, 1992). There have been several recent reports suggesting that MIS also plays an important role in ovulation. 17,20βP could induce ovulation *in vitro* in two catfish species, *Heteropneustes fossilis* (Tripathi and Singh, 1995) and *Pseudobagrus fulvidraco* (Lim et. al., 1997). In addition, a progestin was found to be an effective method for inducing OM and ovulation in *C. gariepinus (*Richter et al., 1985). Several studies have determined the effectiveness of various gonadotropin and pituitary preparations to induce oocyte maturation *in vivo* (Haniffa et al., 2000; Chuda et al., 2002; Mishra and Joy, 2006a) and *in vitro* (Kagawa et al., 1994; Sorbera et al., 1999; Rahman et al., 2001; Skoblina, 2009) in many species.

Chlorpyrifos [*O, O*-diethyl-*O*-(3, 5, 6-trichloro-2-pyridinyl)-phosphorothioate] (CPF), one of the broad-spectrum organophosphorous pesticide used to control the pest in agriculture areas. The recent increase in the agricultural use of organophosphorous pesticides may threaten the reproductive health of humans, wildlife and aquatic life. CPF is bio-activated by cytochrome P450 (CYP)-mediated mono-oxygenases enzyme through desulfuration reaction or dearylation reaction to a more potent cholinesterase inhibitor, chlorpyrifos-oxon (CPO) (Chambers, 1992). Organophosphates may act as neuroendocrine disruptors via inhibition of AChE activity and increase of acetylcholine level in the brain (Herken and Neubert, 1953; Whitney et al., 1995). The inhibition of acetylcholine esterase (AChE) enzyme affects pituitary and hypothalamus functions and the release of gonadotropins (Sarkar et al., 2001). Cholinesterase inhibitors were known to modify the pituitary–thyroid or pituitary–adrenal axes and to alter progesterone levels

(Smallridge et al., 1991; Prakash et al., 1992). It notably targets the cell signaling cascades governing neuronal and hormonal signals that are essential for cell differentiation and homeostatic regulation (Pope, 1999; Schuh et al., 2002; Slotkin, 2004). CPF was described as a potent inhibitor (Hodgson and Rose, 2007) of human liver cytochrome P450-dependent (CYP450) metabolism of testosterone (Usmani et al., 2003) and oestradiol (Usmani et al., 2006). Viswanath et al., (2010) reported chlorpyrifos as a most potent anti-androgenic compound. CPF significantly decreased the expression of cytochrome P450scc, 2β-HSD and 17β-HSD, and decreased luteinizing hormone receptor stimulated cAMP production (Viswanath et al., 2010). Chlorpyrifos is an endocrine disruptor (Oruc, 2010).

There are various studies reporting that organophosphates act like suppressor of gene expression related to gonadotropin synthesis (LH and FSH level) or steroidogenesis (Walsh et al., 2000; Gore, 2001; Kitamura et al., 2003). Importantly, this compound blocks both adrenocorticotropin and cAMP-mediated steroidogenesis but not pregnenolone-driven steroid production, indicating that they target the steroidogenic pathway between the formation of cAMP and the production of pregnenolone (Civen and Brown, 1974; Civen et al., 1977a, b).

Heteropneustes fossilis has been used, since 1960s, as a model for oocyte maturation studies due to best suitability for laboratory studies (Sundararaj and Goswami, 1977). The objective of the present study was to explore the impact of CPF in gonadotropin induced oocyte maturation of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). To see direct involvement of gonadotropin and CPF in oocyte maturation, *in vitro* study and analysis of maturation inducing steroid profile of post-vitellogenic follicles were performed.

2. Materials and Methods

2.1. Chemicals

The standard 5-pregnan,3β-ol-20one (Pregnenolone: P_5), 17β-dihydroxy-1,3,5(10)-estratriene (Estradiol-17β: E_2), 4-pregnen-21-ol-3,20-dione (deoxycorticosterone: DOC), pregn-4-ene-3,20-dione (Progesterone: P_4) and 17α-20β-dihydroxypreg-4-ene-3-dione (17,20βP: DHP) were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). The Ovidac® (human chorionic gonadotropin: hCG, Zydus Pharma Pvt. Ltd.) and Hilban® (20% EC CPF: chlorpyrifos, Hindustan insecticide Ltd.) were purchased from local medical stores. All other chemicals and reagents used were of analytical grade and were bought from HiMedia Laboratory Pvt. Ltd. India.

2.2. Animal collection and acclimatization

The experiments were performed in accordance with the local/national guidelines for animal experimentation and all care was taken to prevent any kind of cruelty to them.

The gravid female freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) (180±10 g) were collected from an aquatic dealer of local fish market during pre-spawning season (June) of annual reproductive cycle. They were maintained in laboratory condition with external aquarium filters of constant photoperiod (12:12 hr; L:D) and water temperature (24±1^{o}C) for two weeks. Fish were fed a diet of dead shrimp daily up to experiments. During the acclimatization, few fish were sacrificed to locate germinal vesicle (GV) migration under a dissecting microscope.

2.3. Preparation of oocyte incubation medium and clearing medium

The incubation medium was prepared (Mishra and Joy, 2006c) as follows: NaCl: 3.74, KCl: 0.32, $CaCl_2$: 0.16, $NaH_2PO_4.2H_2O$: 0.10, $MgSO_4.7H_2O$: 0.16, glucose: 0.40 and phenol red: 0.008 (in g) were dissolved in 1 L triple distilled water. The pH was adjusted to 7.5 with 1 N sodium bicarbonate and autoclaved. Penicillin (2,00,000 U) and streptomycin sulfate (200 mg) were added and filtered. The medium was stored at 4^{o}C

and prepared fresh every week. The clearing solution to see the germinal vesicle within gravid oocyte was prepared as follows: ethanol/acetic acid/formalin; 6:1:3.

2.4. Collection and preparation of post-vitellogenic follicles

The acclimatized fish were sacrificed by decapitation and their ovaries were transferred to a petri-dish containing fresh cooled incubating medium. The rounded dark green colored post-vitellogenic follicles were separated from each other with the help of the fine brush and forceps without causing any mechanical injury to them. Each experimental and control group consisted of a triplicate set of incubation of about 30-40 post-vitellogenic follicles from three fish. During incubation experiment, if incubation medium showed change in colour, immediately replaced with fresh medium (colour change in incubation medium reflects a pH change of medium).

2.5. Experiments

2.5.1. Effect of hCG on oocyte maturation: Concentration and Time response study

About 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were incubated with different concentrations (0.5, 1.0, 1.2, 1.5, 1.7, 2.0, 2.2, 2.5, 3.0, 3.5 mg/ml) of hCG for 24 hr. Same number of post-vitellogenic follicles were incubated in plain incubation medium as a control. After 24 hr of incubation, the follicles were removed from the medium, cleared in clearing solution and examined under a stereo-binocular microscope for determining germinal vesicle breakdown (GVBD) percentage as an index for oocyte maturation.

For time response study, about 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were incubated in effective concentration (1.7 mg/ml, as per the result of above experiment) of hCG for 2, 4, 6, 8, 12, 16, 24 hr interval. Control was used as described above. At the end of each interval, the incubation medium was removed with the fresh plain incubation medium and incubation maintained further to complete 24 hr.

After completion of experiment duration, follicles were processed for calculating the GVBD percentage as discussed earlier.

2.5.2. Effect of CPF on hCG induced oocyte maturation: Concentration and Time response study

About 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were incubated in medium containing different concentrations of CPF (0.0026, 0.026, 0.26, 2.6, 13, 26 pM/ml) with effective dose and duration of hCG (1.7 mg/ml, 8 hr; based on the result of above experiment). After 8 hr of duration of hCG, medium was replaced with incubation medium having only CPF concentration to complete 24 hr of the total incubation period. Control groups were maintained side by side. After 24 hr, the follicles were cleared in clearing solution and examined for calculating the GVBD percentage.

For time response study, about 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were co-incubated in medium containing an effective concentration of CPF (0.26 pM/ml; as examined earlier) for 4, 8, 12, 16, 24 hr interval with hCG (1.7 mg/ml, 8 hr). Control groups were maintained concurrently. After completion of each interval, the incubation medium was replaced by medium having desired drug to complete 24 hr. In brief, at the end of 4 hr CPF, the incubation medium was removed with the effective concentration of hCG for remaining of effective duration. After the completion of total 8 hr of incubation of hCG (1.7 mg/ml), the incubation medium was replaced with the fresh plain medium for remaining period to complete 24 hr. At the end of 8 hr CPF, the incubation medium was replaced by plain medium to complete 24 hr. In remaining 12 and 16 hr CPF group, medium was first replaced at 8 hr with medium having CPF only for desired duration then in plain medium to complete total 24 hr. In 24 hr CPF effect, one change was done after 8 hr with plain incubation medium having CPF. After 24 hr of total incubation, follicles were processed for calculating the GVBD percentage.

2.5.3. Pre-incubation study

About 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were first incubated with the medium containing CPF (0.26 pM/ml) for 4, 8, 12 hr time periods. After the respective time intervals, the follicles were rinsed in fresh incubation medium and then transferred into the incubation medium containing hCG (1.7 mg/ml). After 8 hr of hCG, they were rinsed in fresh incubation medium and maintained in the plain incubation medium to complete of total incubation duration 24 hr. Control group was maintained side by side. At the end of 24 hr, follicles were processed for scoring the GVBD percentage.

2.5.4. Post-incubation study

About 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were first incubated with the medium containing hCG (1.7 mg/ml) for 8 hr. Then the follicles were rinsed in fresh incubation medium and transferred into the incubation medium containing CPF (0.26 pM/ml) for 4, 8, 12 hr. After each interval, the follicles were rinsed in fresh incubation medium and maintained in the plain incubation medium for up to 24 hr. Control incubation set was maintained side by side. At the end of incubation follicles were processed for scoring the GVBD percentage.

2.5.5. In vitro effects of hCG and CPF on steroidal hormone levels in post-vitellogenic follicles

About 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were co-incubated in medium containing effective concentration and duration of hCG (1.7 mg/ml, 8 hr) CPF (0.26 pM/ml, 12 hr). After each interval, the follicles were replaced with fresh incubation medium with desired drug and further maintained in plain incubation medium upto 24 hr. Positive and negative control incubation sets were maintained side by side as described above. At the end of incubation, follicles were pooled for each animal and kept in -20^{o}C for further steroid (P_5, P_4, DOC, E_2 and DHP) extraction and estimation by HPLC.

2.6. Study parameters

2.6.1. Germinal vesicle breakdown

At the end of each experiment, the follicles were cleared in a clearing solution (ethanol: formalin: acetic acid; 6: 3: 1; Trant and Thomas, 1988) for 3-4 minutes to visualize germinal vesicle and examined at 4X magnification under Bright field microscope (Olympus CX41) using micropublisher 3.3 RTV camera for determining the GVBD. The translucent follicles without germinal vesicle (GV) and opaque follicles containing GV were captured separately. The percentage of GVBD was determined as number of translucent follicles divided by the total number of the follicles incubated multiplication of hundred.

2.6.2. Extraction of steroids from the sample

The extraction was performed as per Mishra and Joy (2006 b). In brief, the pooled post-vitellogenic follicles form different experiments and control was homogenized separately in 4 volumes of cold PBS (0.02 M, phosphate buffered-saline pH 7.4) with a homogenizer at 0^{o}C. The homogenate was centrifuged at 20,000 g for 20 min. at 4^{o}C. The supernatant was extracted with two volumes of hexane, thrice and three volumes of diethyl ether, three times. The ether phase was collected, pooled, evaporated and dried under N_2 gas and stored at -20^{o}C until chromatography. The incubation medium was directly extracted with diethyl ether and stored at -20^{o}C.

2.6.3. Chromatography

The chromatographic analysis of the steroids was conducted with a HPLC system (Waters, United States) consisting with two pumps (515 HPLC Pump) and a digital ultraviolet detector (2489 UV/ visible detector) with a variable wavelength (190-700 nm) based on Fastie-Ebert monochromator. The system was operated with Empower Pro-software (version: 6.00.00.00 copyright 2005 Waters corporation). The analysis was

performed with a reversed phase C_{18} column (4.6 x 25. Mm; Waters Spherisorb$^{(R)}$ 5 µm ODS2). The isocratic mobile phase was 60% methanol in water. The flow rate was 1.5 ml/min. and the total run time was 30 min. (Nagahama and Adachi, 1985). The mobile phase and samples were degassed just before use. Before injecting the standard/sample, the column was activated with 100% methanol, followed by conditioning with the mobile phase. The analysis was monitored at 240 nm wavelength.

2.6.4. Preparation of standards and retention time determination

The steroids (P_5, P_4, DOC, E_2 and DHP) were dissolved in methanol separately to prepare stock solutions. From the stock solutions serial dilutions were made with methanol. The diluted solutions were filtered (0.2 µm) and injected into 20 µl loop of HPLC system with the help of microlitre syringe (Hamilton). The standards were tested individually at different concentration to record retention time and peak area under isocratic condition. This analysis was repeated three times with each standard.

2.6.5. Validation of the assay

2.6.5.1. Response-linearity

Different concentrations of standards in triplicate were injected into the column to set up concentration vs peak area curve. The response was linear with the concentration ranges used (E_2: 16 ng/ml-10 µg/ml; P_4: 0.67-80 µg/ml; P_5: 0.04-40 µg/ml; DOC: 0.4-80 µg/ml; and DHP: 12 ng/ml-20 µg/ml). The minimum detection limit for the assay was 0.24 ng per injection.

2.6.5.2. Recovery and sensitivity

Known concentrations of the standards in different dilutions were processed in the same manner as tissue samples (described above) and were injected into the column, after filtration. This study was repeated three times for each dilution. Percentage recovery was

calculated from the concentrations of the standards injected directly and measured after extraction. The percentage recovery was 90-92% for E_2, 96-98% for P_4, 93-95% for P_5, 97-99% for DOC and 94-98% for DHP. The values were not corrected for loss.

2.6.5.3. Inter- and intra-assay variations

Inter- and intra-assay variations were determined from five chromatograms each, using the same set or different sets of diluted standards. The inter- and intra-assay variations were, respectively, 14 and 5% for E_2, 18 and 5% for P_4, 12 and 8% for P_5, 20 and 12% for DOC and 12 and 6% for DHP.

2.6.6 Sample analysis

The -20^{o}C stored dried samples of follicle were pooled (30-40 follicles in triplicate from three fish) and reconstituted in 1ml methanol. The reconstituted samples were filtered (0.2 µm) and 20 µl each of sample was injected into the column and eluted for 30 minutes. The samples were also co-eluted with known concentrations of the standards and compared with the elution of the respective standards in the mixture for identification and quantification of the steroids. Each sample was analyzed in triplicate in this manner. Chromatograms for blank were run with the vehicle (methanol and the mobile phase) to check any interference in the elution of the steroids. The blank had eluted before the steroids peaks appeared. The differences in the peak area between the standard and the standard with the sample in the chromatograms were recorded with the help of Empower Pro-software and the concentrations of steroids in samples were calculated.

2.7. Statistical analysis

The data were expressed as mean±SEM. The data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test ($p < 0.05$) for multiple group comparisons. The data of HPLC steroid quantification in different experimental

condition were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$) for comparisons with control group.

3. Results

3.1. Morphological changes in post-vitellogenic follicles

In the control group, the oocytes were generally opaque in size at the beginning of migration of germinal vesicle. The germinal vesicle was located at the center or near the center of the oocyte. The hCG (human chorionic gonadotropin) incubated follicles showed migrated germinal vesicle (GV) from the center to the animal pole and finally the nuclear membrane underwent dissolution or GVBD (Figure 13). The follicles showing GVBD response were translucent as against the opaque ones in the control group.

3.2. Effect of hCG on percentage of GVBD: Concentration and time response study

The hCG induced a significant elicited effect on percentage of GVBD during the 24 hr incubation period ($F = 8769.42$; $p < 0.001$, one-way ANOVA; Figure 14A). The GVBD response was increased significantly over the concentration and gave maximum response (96.33%±0.87) at 1.7 mg/ml as compared to control group (2.33%±0.32) ($p < 0.05$, Newman-Keuls test; Figure 14A). This was used as effective concentration in forthcoming experiments.

A pulse study was also performed to know the effective minimum duration of hCG (1.7 mg/ml) to elicit GVBD response. The GVBD response was significantly high from 2 hr onwards but maximum at 8-24 hr as compared to control ($p < 0.05$, Newman-Keuls test). At 8 hr interval, hCG produced a significant increase in % GVBD (97.19%±0.87) ($F = 17631.07$; $p < 0.001$, one-way ANOVA) (Figure 14B). Therefore, the minimum effective duration of hCG that produce maximum maturation was 8 hr. All the incubation sets recorded nil mortality of follicles.

3.3. Effect of CPF on hCG induced GVBD response: Concentration and time response study

CPF showed an inhibitory effect on hCG induced oocyte maturation in a concentration dependent manner as compared to alone gonadotropin (hCG) incubated follicles (F = 6255.47; $p < 0.001$, one-way ANOVA; Figure 15A). The inhibition was significantly higher with 0.26-26 pM/ml concentrations where it had shown 90% inhibition as compared with a hCG group ($p < 0.05$, Newman-Keuls test). Based on this result, the minimum concentration of CPF that inhibited maximum hCG induced GVBD was taken 0.26 pM/ml in next experiments.

A time response study of CPF (0.26 pM/ml) was conducted to know the effective minimum duration to inhibit hCG induced GVBD. The results showed that GVBD % was significantly inhibited 90%±1.2 from 12 hr onwards as compared to hCG group ($p < 0.05$, Newman-Keuls test). Although the CPF inhibition was significant at all intervals compared with the hCG groups (F = 1782.81; $p < 0.001$, one-way ANOVA; Figure 15B). Therefore, in successive experiments 4, 8, 12 hr treatment duration was selected to record CPF response. All the set showed nil mortality of follicles.

3.4. Pre-incubation and Post-incubation study

Both pre- and post-incubation study showed significant inhibition by CPF in hCG induced GVBD in all tested durations (4, 8, 12 hr). The percentage of CPF inhibition in hCG induced oocytes was increased with duration of CPF treatment in both pre- and post-incubation treatment in a duration dependent manner (pre-incubation inhibition (%): 52±0.06, 75.67±0.13, 87.67±0.14; post-incubation inhibition (%): 18±0.87, 44±1.15, 76±0.57; for 4, 8, 12 hr). However, the level of CPF inhibition in hCG induced GVBD was more in pre-incubation experiments as compared to post-incubation (Figure 16).

3.5. Steroid separation and retention time

With the chromatographic assistance and its adoptive condition, P_5 was eluted first, followed by E_2, DOC, P_4 and DHP. The retention times were 4.7, 5.9, 6.5, 11.6 and 21.3 min. respectively. However, in case of samples elution, the retention time of identified steroids showed minor shifts (Figure 17, 18). The authentication of steroids elution in sample was performed by co-running with known concentration of the standard in the sample mixture and compared with the peak of standard alone.

3.6. In vitro effects of hCG and CPF on steroid hormone levels in post-vitellogenic follicles

Human chorionic gonadotropin (hCG) enhanced the steroid synthesizing capability of oocyte and stimulated the meiotic resumption promoting steroids (P_5, P_4, DOC and DHP) significantly as compared to control group ($p < 0.001$, two-way ANOVA; Figure 18). However, the E_2 level was decreased significantly ($p < 0.05$, Tukey's test) (Figure 19). The co-exposure of CPF and hCG incubated follicles showed significant decreased in concentration of P_5, P_4 and DHP as compared to control group and hCG group ($p < 0.05$). However, the DOC and E_2 levels were increased in the CPF alone and in co-incubation with hCG exposed follicles as compared to control group ($p < 0.05$) (Figure 18). In control group, all hormones were detected, maximum level was of E_2. Other maturation inducing steroids were in their lower concentration in control group.

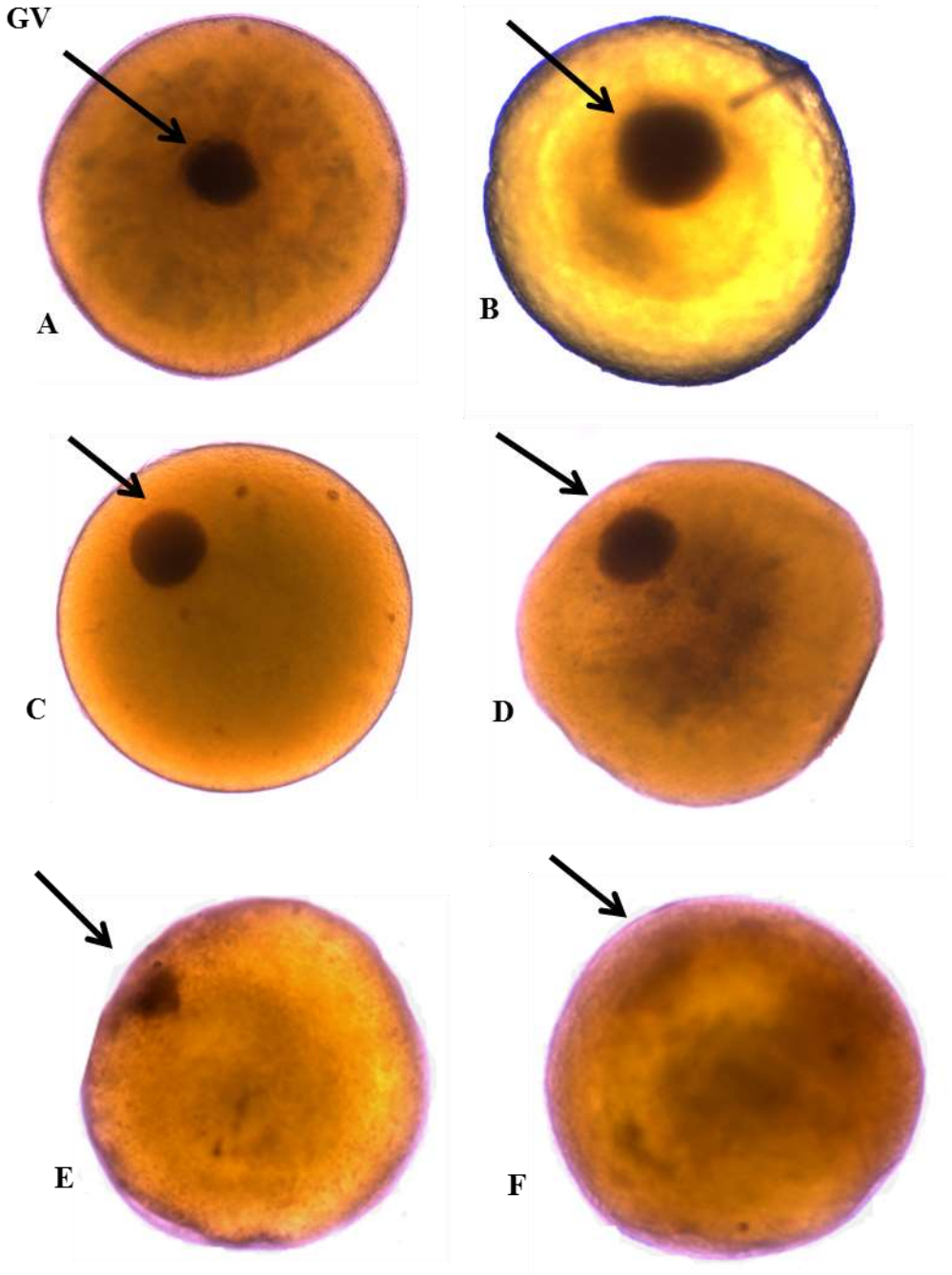

Figure 13: Process of oocyte maturation activity of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) after the hCG treatment. A-E: showed visible movement of germinal vesicle (GV) and F: showed the germinal vesicle breakdown (GVBD).

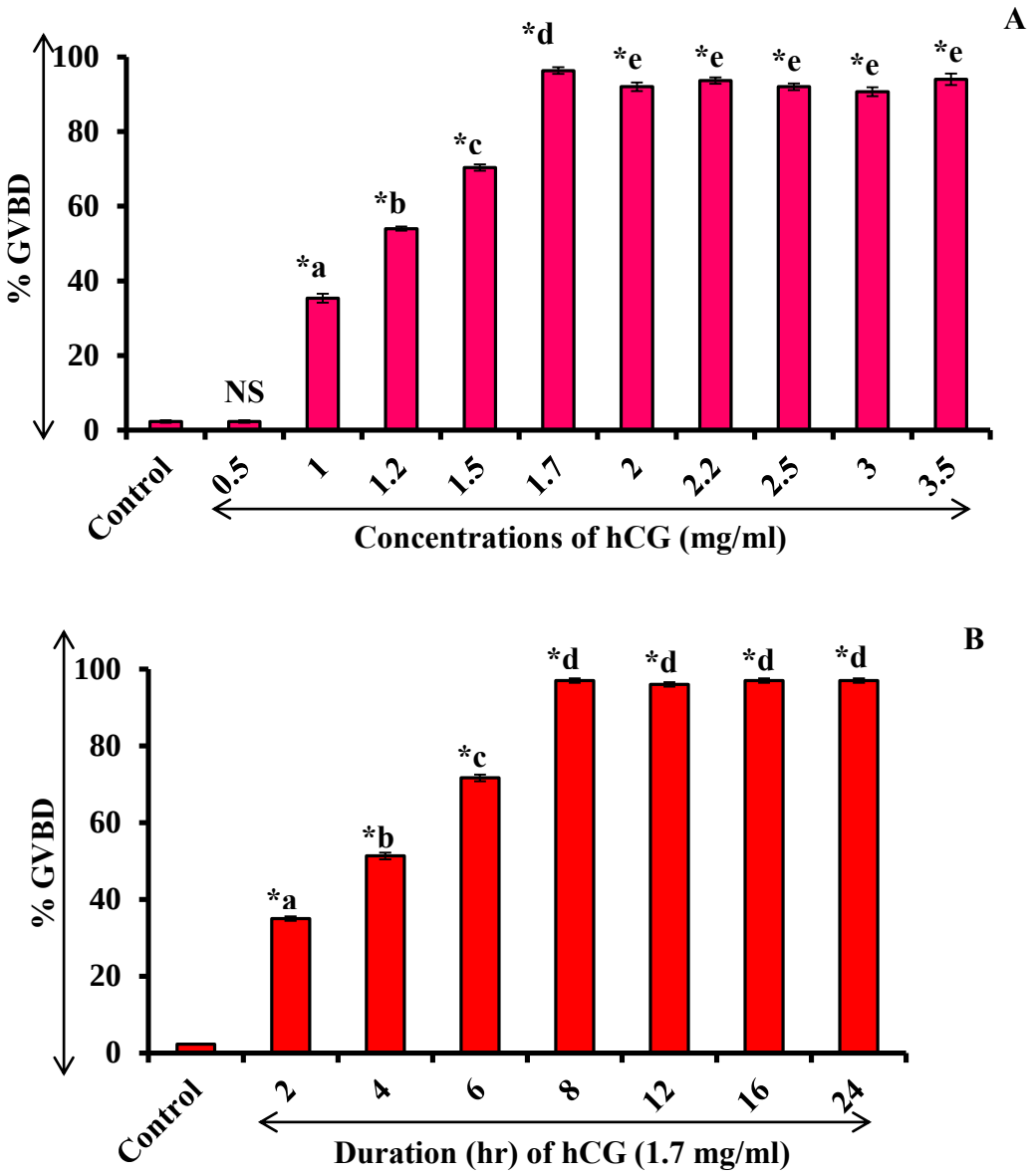

Figure 14: (A) Effect of different concentrations of hCG on *in vitro* oocyte maturation of gravid freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) post-vitellogenic follicles. (B) Pulse study of effective dose (1.7 mg/ml) of hCG on *in vitro* oocyte maturation. Values were mean±SEM of percentage GVBD of 30-40 follicles incubated in triplicate (n=3) for 24 hr. Data were analyzed by one way ANOVA ($p < 0.001$), followed by Newman-Keuls test ($p < 0.05$) for comparison within the groups. Asterisk (*) showed significant different from the control group at $p < 0.05$. NS showed non-significant from the control group. Group superscripted with different letter shows significant difference ($p < 0.05$) whereas same letter showed non-significance.

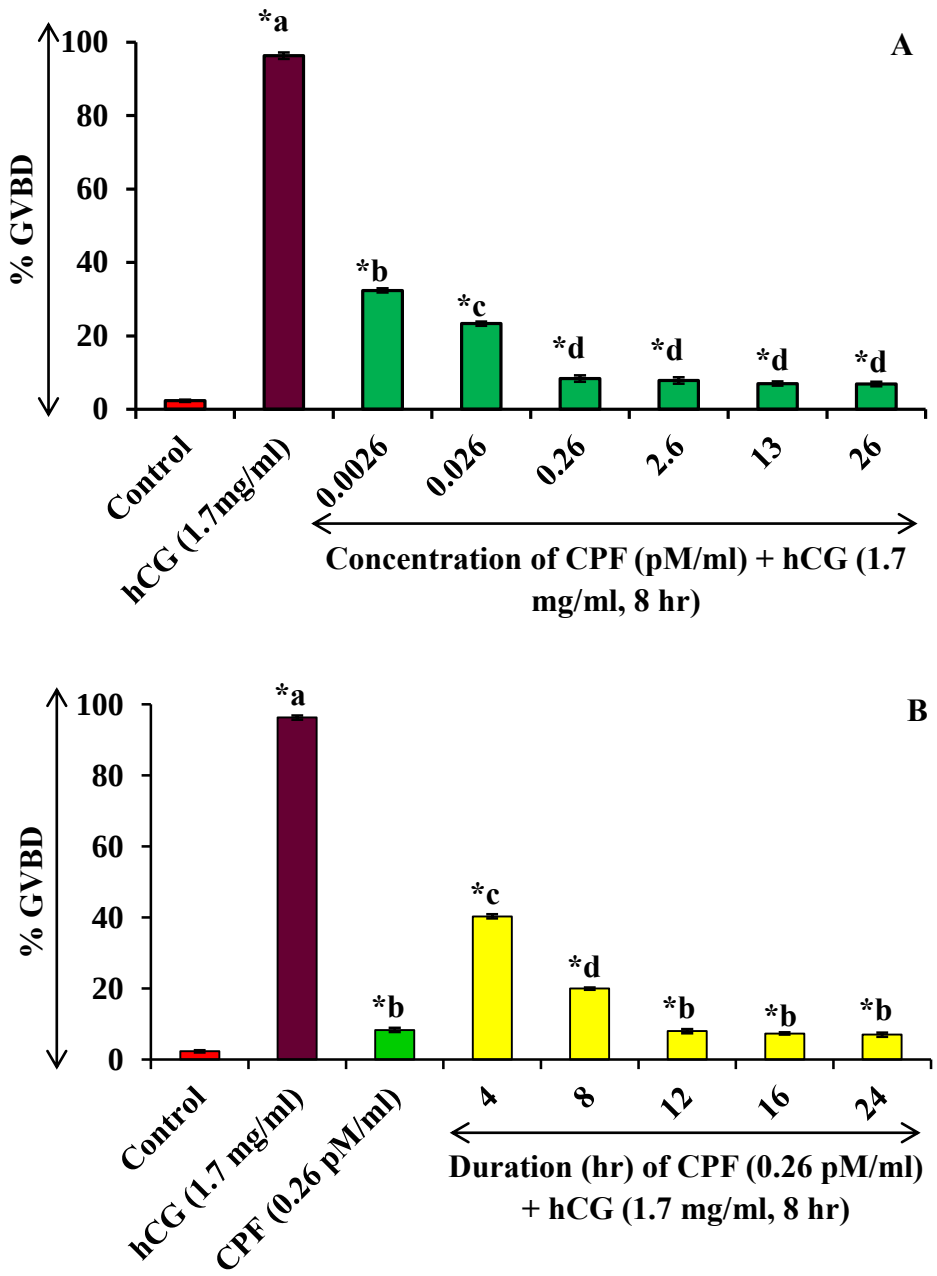

Figure 15: (A) Effect of different concentrations of CPF on hCG induced *in vitro* oocyte maturation. (B) Duration effect of CPF (0.26 pM/ml) on hCG (effective dose and duration: 1.7 mg/ml, 8 hr) induced *in vitro* oocyte maturation of gravid freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) post-vitellogenic follicles for 24 hr. Values were mean±SEM of percentage GVBD of 30-40 follicles incubated in triplicate (n=3) for 24 hr. Data were analyzed by one way ANOVA ($p < 0.001$), followed by Newman-Keuls test ($p < 0.05$) for comparison within the groups. Asterisk (*) showed significant different from the control group at $p < 0.05$. Group superscripted with different letters showed significant difference ($p < 0.05$) whereas same letter showed non-significance.

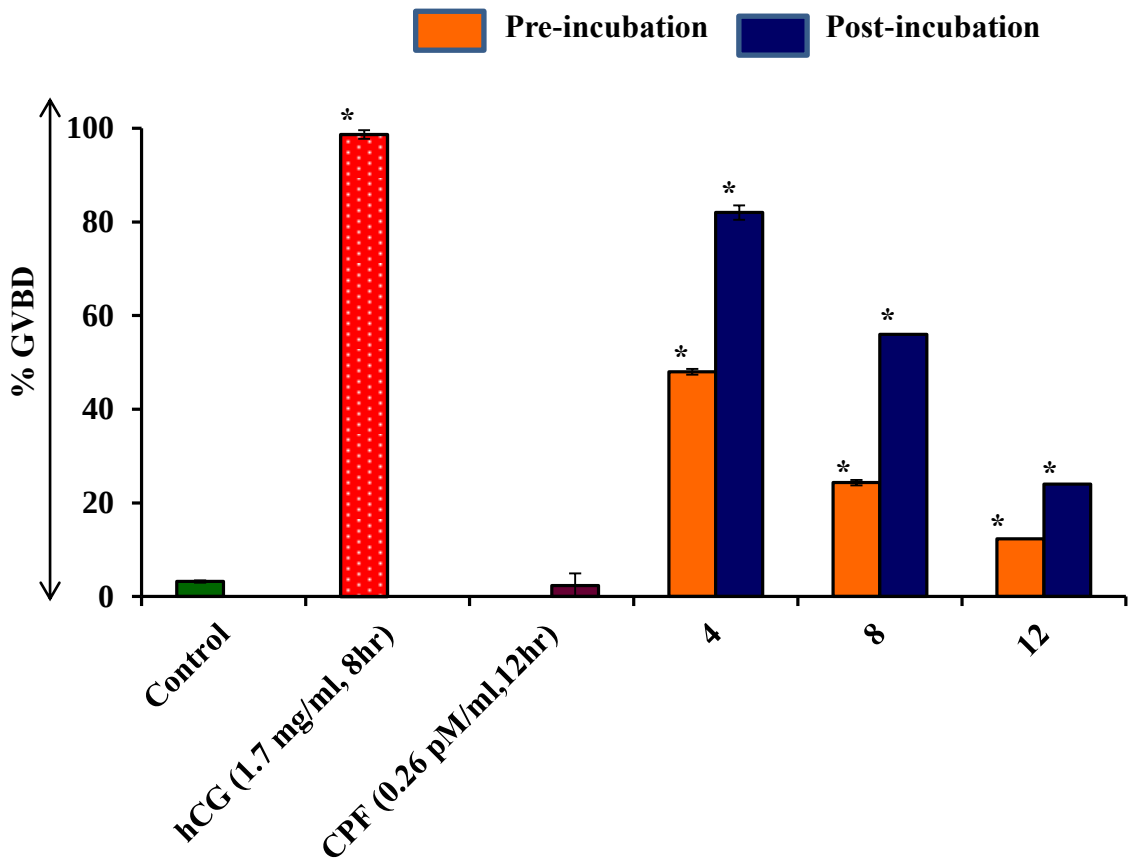

Figure 16: *In vitro* effects of pre- and post-incubation of post-vitellogenic follicles of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) with CPF (0.26 pM/ml) for 4, 8 or 12 hr on hCG induced GVBD. Values were mean±SEM of percentage of germinal vesicle breakdown (GVBD) of 30-40 follicles each incubated in triplicate (n=3). Data were analyzed by one way ANOVA ($p < 0.001$), followed by Newman keul's test ($p < 0.05$) for comparison within the groups. Asterisk (*) showed significant different from the control group at $p < 0.05$.

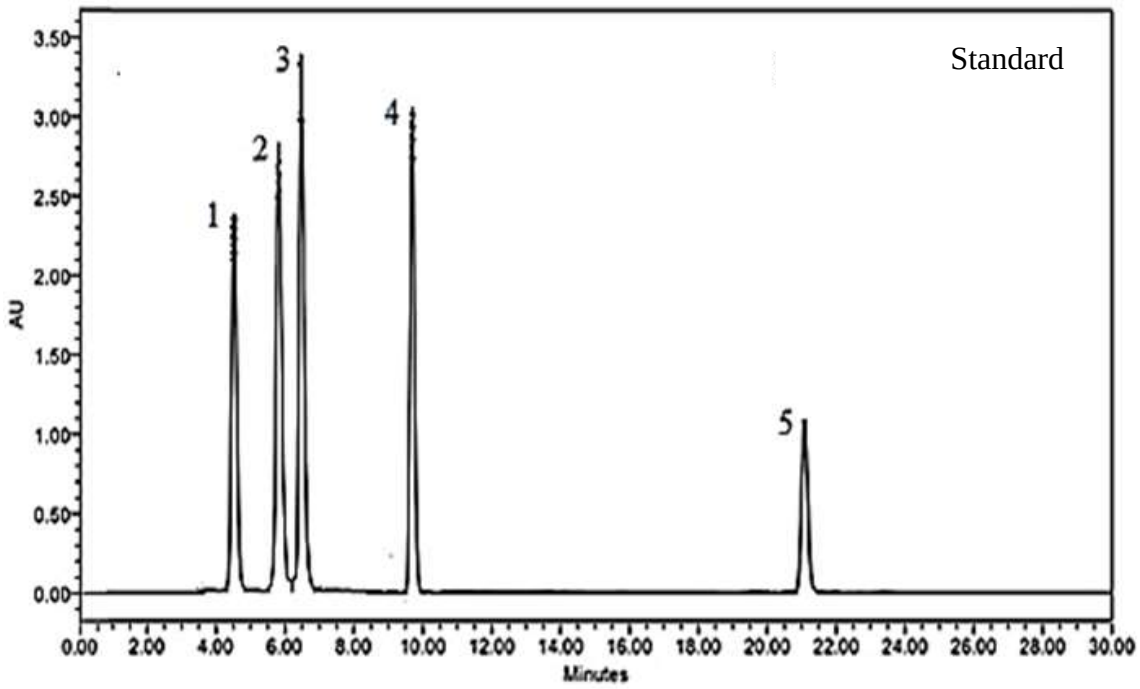

Figure 17: HPLC chromatogram showing separation profile of different steroids examined in the current study. Peak 1- P_5: 5-pregnan,3β-ol-20one (retention time = 4.7 min.; 0.15 µg/µl concentration); peak 2- E_2: 17β-dihydroxy-1,3,5(10)-estratriene (retention time = 5.9 min; 0.2 µg/µl concentration); peak 3- deoxycorticosretone: 4-pregnen-21-ol-3,20-dione (retention time = 6.5 min; 0.15 µg/µl concentration); peak 4- P_4: Pregn-4-ene-3,20-dione (retention time = 11.6 min; 0.15 µg/µl concentration) and peak 5- 17,20βP: 17α-20β-dihydroxypregn-4-ene-3-dione (retention time = 21.3 min; 0.2 µg/µl concentration).

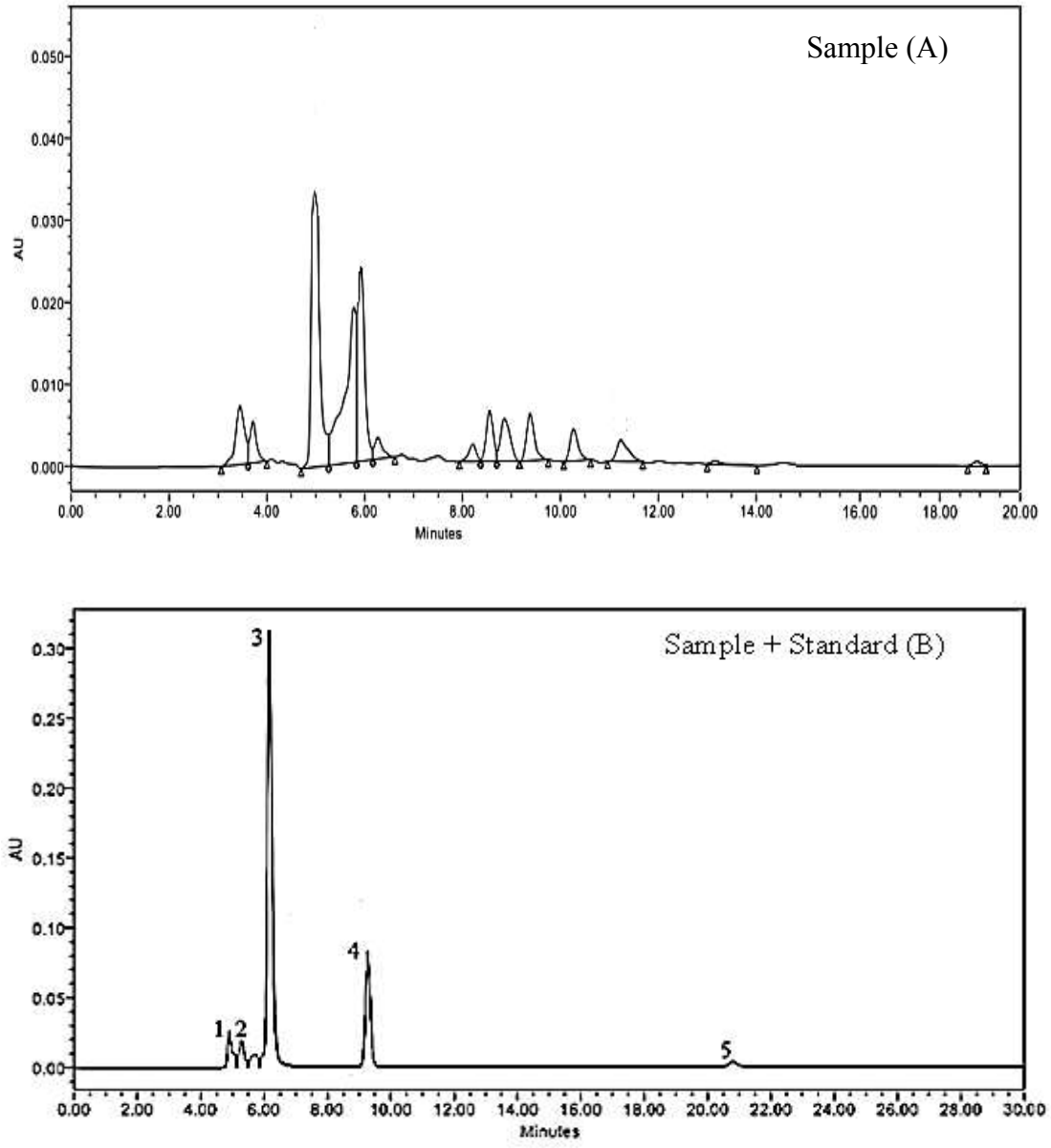

Figure 18: (A) HPLC chromatograms showing elution profile of sample: induced hCG (1.7 mg/ml) post-vitellogenic follicles of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) exposed to CPF (0.26 pM/ml) for 24 hr and (B) sample in the presence of known concentration of standards (Peak 1- P_5: 5-pregnan,3β-ol-20one, peak 2- E_2: 17β-dihydroxy-1,3,5(10)-estratriene, peak 3- deoxycorticosretone: 4-pregnen-21-ol-3,20-dione, peak 4- P_4: Pregn-4-ene-3,20-dione and peak 5- 17,20βP: 17α-20β-dihydroxypregn-4-ene-3-dione).

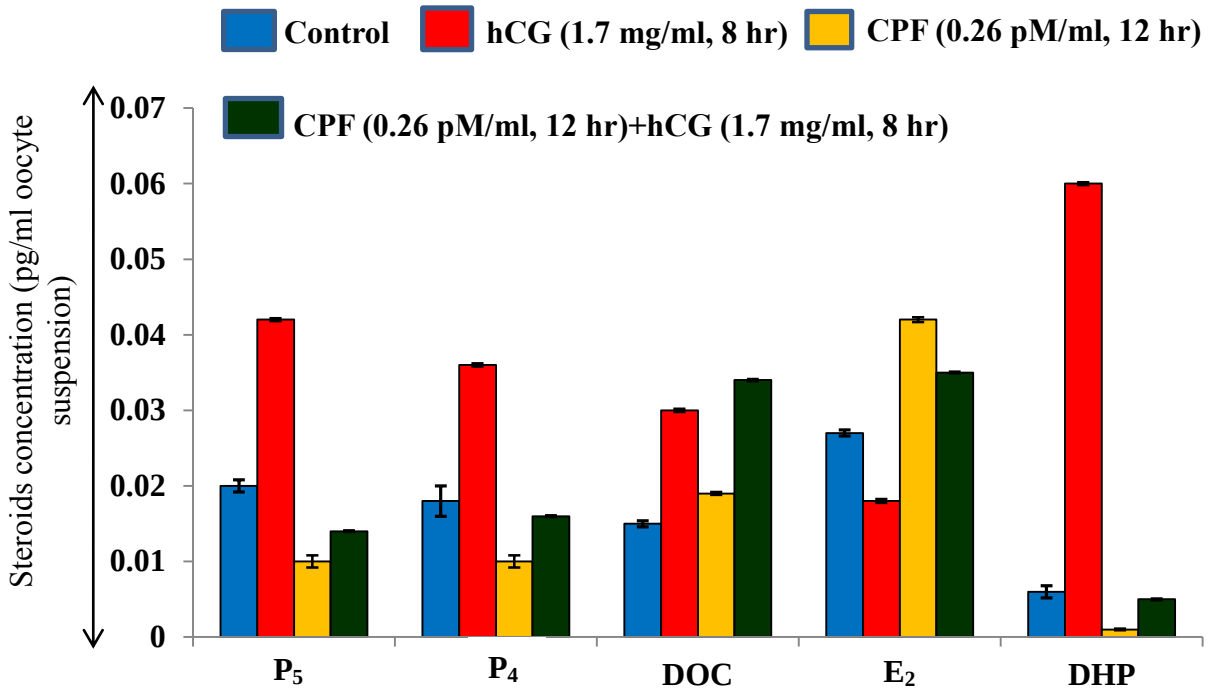

Figure 19: A comparative steroid profile of co-incubated post-vitellogenic follicles of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in hCG (1.7 mg/ml, 8 hr) and CPF (0.26 pM/ml, 12 hr) in comparison with different positive and negative control groups. After the respective drugs incubation duration, the experiment was continued up to 24 hr with plain incubation medium.. Values were mean±SEM (n=3). Data were analyzed by two way ANOVA ($p < 0.001$), followed by Tukey's test ($p < 0.05$). Asterisk (*) showed significant difference from the control group ($p < 0.05$). Note: P_5: Pregnenolone; P_4: Progesterone; DOC: deoxycorticosterone; E_2: Estradiol-17β; DHP: 17,20βP.

4. Discussion

The migration and breakdown of germinal vesicle are the first two morphological signs of oocyte maturation. GVBD which involves undulation, folding, fragmentation and disintegration of nuclear membrane at or before metaphase I (Masui and Clarke, 1979) has been the commonly used bioassay system of oocyte maturation. The oocytes were ready for resumption of meiosis after completion of the growth phase. Post-vitellogenic oocytes possess a large nucleus (germinal vesicle: GV) in the center in meiotic prophase I (Nagahama, 1983). Following the migration of germinal vesicle, the GV membrane disappears known as Germinal vesicle breakdown (GVBD), which indicates the end of prophase I. In teleost fishes, final oocyte maturation consists of the migration and breakdown of GVBD, chromosome condensation and formation of the first polar body (Goetz, 1983; Thomas, 1994; Mishra and Joy, 2006c).

The present result support that hCG (human chorionic gonadotropin) was capable of stimulating *in vitro* oocyte maturation in post-vitellogenic follicles of catfish, *H. fossilis* in a concentration and duration dependent manner. The hCG incubated follicles showed GV migration from the center to animal pole and finally the nuclear membrane underwent dissolution or germinal vesicle breakdown (GVBD). The follicles showing GVBD was translucent as against the opaque ones in the control follicles (Sundararaj et al., 1985). The response is similar to the earlier reported studies in different fish species *in vitro* and *in vivo* (Scott and Canario, 1990; York et al., 1993; Kagawa et al., 1994; Joy et al., 1998; Sorbera et al., 1999; Matsuyama et al., 2001; Lubzens et al., 2010). This species variation reflects with difference in the effective dose and duration of hCG (Sorbera et al., 1999; Zuberi et al., 2002).

The organophosphorous pesticide, chlorpyrifos (CPF) showed an inhibitory effect on hCG induced oocyte maturation in a concentration and duration manner. The earlier investigations also supported CPF exposure significantly knocked down the gonadotropin induced GVBD (Haider and Upadhyaya, 1986; Haider and Inbaraj, 1988). Haider and Upadhyaya, (1986) also reported that organophosphates significantly reduced the rate of

GVBD in oocytes of the catfish, *M. vittatus*, *in vitro* even in the presence of gonadotropin. The inhibition of hCG induced oocyte maturation was more in pre-incubation set as compare to post-incubation set. This suggest that CPF inhibit gonadotropin induced GVBD in a receptor dependent mechanism (Gore, 2001).

Gonadal steroids are the terminal or subterminal regulators of gametogenesis, oocyte maturation and ovulation. In teleosts, E_2 is the major regulator of vitellogenesis, a complex and elaborate process responsible for the growth of the follicle and the yolk so deposited are used during early embryonic development. 17,20βP is the maturation-inducing steroid that induces resumption of meiosis (Nagahama, 1997). In post-vitellogenic ovary, the C_{18}-C_{19} steroidogenic pathway is down regulated and the progestin pathway is up regulated by the LH surge to induce oocyte final maturation and ovulation. *In vitro* gonadotropins do not act directly on the oocyte to induce resumption of meiosis, but instead initiates maturation of the oocyte by synthesis of a MIS by the ovarian follicles (Senthilkumaran et al., 2004).

Control group follicles registered the presence of all measured hormones but high E_2 level was sufficient to keep follicles in their meiotic arrest form (Sundararaj and Goswami, 1977; Goetz, 1983; Jalabert et al., 1991; Mishra and Joy, 2006c). The result registered in a sharp decrease in E_2 level which may be due to conversion of E_2 in its metabolites (hydroxyl-metabolites) that enhanced the maturation process (Fostier et al., 1983; Mishra and Joy, 2006c). This increase and decreased level of the steroids may interpret their conversion in another or may adopt another pathway. Among the measured steroids DHP, P_4 and DOC associated with the maturational activity and ovulation. *In vitro* oocyte maturation by hCG resulted in the stimulation of progestin pathway (C_{21}) and inhibition of estrogen pathway (C_{18}) (Kobayashi et al., 1988; Zhu et al., 1989; Pinter and Thomas, 1999; Senthilkumaran et al., 2004). The *in vitro* treatment of hCG triggered the level of these steroids in post-ovulatory follicles as compared to control. The pregnenolone (P_5) level was also increased about two fold with hCG stimulus. The rise in P_4 and DHP could be correlated with its MIS activity within follicles (Goetz 1983; Jalabert et al., 1991; Nagahama, 1997; Matsuyama et al., 2001; Rahman et al., 2001;

Garcia-Alonso et al., 2004; Semenkova et al., 2006). Many researchers found that the increased production of DHP was associated with oocyte germinal vesicle migration and GVBD (Sundararaj et al., 1985; Zhao and Wright, 1985; King et al., 1995; Amiri et al., 2001; Garcia-Alonso et al., 2004). DOC is a known maturation inducing steroid in *H. fossilis* (Sundararaj and Goswami, 1977; Sundararaj et al., 1979). The result stated that hCG act as a possible maturation inducing agent *in vitro* which was supported by the findings of York et al. (1993), Joy et al. (1998), Sorbera et al. (1999), Matsuyama et al. (2002). Apparently increased production of these steroids in response to hCG is correlated with stimulation of P450c17 and 20β-hydroxysteroid dehydrogenase (20β-HSD) (Senthilkumaran et al., 2004; Nagahama and Yamashita, 2008).

However, CPF exposure caused suppression of maturation inducing steroids (P_4, P_5 and DHP) in post-vitellogenic follicles of *H. fossilis*. CPF inhibited oocyte maturation via manipulating hormone level that was triggered by hCG within follicle. In CPF alone, E_2 level was too high that maintains arrest. The inhibition of DHP by CPF seems to be the plausible mechanism to explain the adverse effect of this known endocrine disruptor (Sonnenschein and Soto, 1998). The E_2 and DOC concentrations were significantly increased in co-incubation with CPF groups as compared to hCG alone group. However, the increased concentration of E_2 was more pronounced than DOC. Presence of corticosteroids in *in vitro* follicles study was also reported earlier (Sundararaj et al., 1979; Mishra and Joy, 20006c; Chaube et al., 2010). Control group shown the presence of DOC that was increased in all treated groups, hCG, CPF and co-incubation group. Though level of increase, when compared among the groups, was more in combination group of hCG and CPF followed by alone hCG. It may be due to gonadotropin influence synthesis of P_4. In the presence of CPF, hCG induced progestin pathway was decreasing. The increased level of DOC due to CPF alone and in co-incubation with hCG group suggested an additional mineral operated channel, that may linked with hydration of oocyte (Bry, 1985; Milla et al., 2006; Chaube et al., 2010). On the basis of current result it may concluded that CPF inhibits DHP by keeping estradiol in higher level and induced DOC. The catfish ovary has reported DOC importance in oocyte maturation, oocyte hydration and ovulation (Sunderaraj and Goswami 1977; Bry 1985; Chaube et al., 2010). Increase

in DOC concentration occurs in the peripheral blood during the initiation of spawning indicates its possible importance in fish ovulation (Colombo et al., 1978; Milla et al., 2006). This supports an idea that CPF may involve in pre-mature hydration or ovulation process.

The steroid profile of P_5, P_4 and DHP hormone after the exposure of CPF on hCG induced post-vitellogenic follicles resulted into three to tenfold decrease. Since P_4 and P_5 are precursors, of DHP, therefore their reduction reflected may be due to their conversions via another pathway (21 hydroxylase, P450 with 17-20 Lyase activity) in to estradiol and deoxycorticosterone. In alone group, CPF increased both E_2 and DOC. This increase in E_2 maintains oocyte meiotic arrest (Jalabert et al., 1991; Mishra and Joy, 2006c). This might be due to the fact that CPF might have differentially altered the key enzymes involved in P_4, DHP and E_2 production. CPF are known to inhibit cytochrome P450-dependent enzymatic activities (Walsh et al., 2000), and thus might disrupt steroidogenesis and, consequently, DHP biosynthesis (Pope, 1999; Schuh et al., 2002; Gupta, 2004; Slotkin, 2004).

Thus, organophosphorous could have directly targeted the enzyme's active site or affected the availability and/or activity of cofactors and reducing equivalents (Walsh et al., 2000). The P450 side chain cleavage (P450scc) enzyme is part of the cholesterol side chain cleavage enzyme system (CSCC) (Simpson, 1979). CPF decreased the activity of P450scc enzyme. These pesticides disturb steroid secretion, either by impairing their synthesis or release, resulting in reproductive deficiencies in fish (Singh and Singh, 1980a, b). The pesticides interfere with the production of free cholesterol, the sex hormone precursor, and hence reduce steroid production (Lal and Singh, 1987).

CHAPTER – III

Effect of organophosphorous pesticide, chlorpyrifos on embryogenesis of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)

ABSTRACT

The present investigation was aimed to study the effect of chlorpyrifos on hatchlings of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). For this, *in vitro* and *in vivo* experiments were performed. In *in vitro* experiment, the fertilized eggs stripped from normal control catfish were exposed to different concentrations of chlorpyrifos viz., 0.52, 0.26, 0.052, 0.026, 0.013, 0.006, 0.004 µM/l. For *in vivo* study, the induced female catfish with synthetic hormone (hCG) was exposed to same concentrations of chlorpyrifos. The experiment was done in triplicates along with the control group. The result showed that both, fertilization and hatching rate was decreased significantly in exposed group in comparison to control. *In vivo* exposure showed significantly low embryo mortality (27.2±0.76%) ($F=893.67$; $p < 0.05$) and high hatching rate (90.5±0.5%) ($F=271.87$; $p < 0.05$) as compared to *in vitro* study (35.2±0.81% and 85.4±1.25%) ($F=1070.28$, $F=267.23$; $p < 0.05$) in lower concentration of CPF (0.004 µM/l). The percentage of malformed hatchlings was higher in *in vitro* experiment as compared to *in-vivo*. The occurrence of all studied parameters was observed in a concentration dependent manner, as the concentrations of CPF were increased, the percentage of embryo mortality and malformed hatchlings was decreased. However, the hatching rate was increased. The result showed that exposed group of hatchlings were affected by many morphological and notochordal deformities viz., ventral or lateral tail

flexure, abnormal spinal bending, irregular head shape and size, loss of eye, reduced barbel, pericardial edema, yolk sac edema, notochordal defect, tail fin flexure, reduction of brain development and reduction of pigmentation. The result suggested that OPs play an important role in inducing the muscular dystrophy and morphological deformities during embryonic development and organogenesis.

Keywords: Chlorpyrifos, fertilization rate, embryo mortality, hatching rate, morphological deformities

1. Introduction

The study of fish embryogenesis is an important aspect to know the developmental processes of any fish spp. and also to understand species-specific adaptations and their ecological value in the course of speciation (Meijide and Guerrero, 2000). Fish are largely used for the assessment of the quality of aquatic environment and as such can serve as bio-indicator species of environmental pollution (Lopes et al., 2001; Whitefield and Elliott, 2002; Dautremepuits et al., 2004). However, to understand the effects of environmental contaminants on fish, their action should be studied during the main life cycle, especially during the first stages of development. Measures of toxicity derived from these early life stage tests can provide a strong indication of the potential range of biological effects of toxicant action (U.S. EPA 2002). The slight alterations or modifications in the external environment, may lead to developmental malformations. These changes produced as a result of metabolism of teratogens and interacting factors. Embryonic responses to teratogens occur at various developmental stages and at different levels of organogenesis that may lead to modification of normal development.

The hazardous effects of rapidly degraded organophosphorous pesticides (OPs) are less evident. Organophosphorous pesticides may disrupt normal patterns of neuronal connectivity in the developing nervous system (Howard et al., 2005). The role of cholinesterase during organogenesis (Moody and Stein, 1988) and anti-ChE activity of organophosphorous compounds should not be ignored. The history of OP

compounds and anti-ChE activity is more than a century old but the study on this topic seems to be highly neglected. The scanty literature on the subject and consistent reports of harmful effects on vertebrates and invertebrates (Rull et al., 2006; Peiris-Johna and Wickremasinghe, 2008) is contradictory. The importance of AChE in the function of the nervous system has been recognized for a long time, yet its role in development remains mysterious (Brimijoin and Koenigsberger, 1999). AChE is transiently expressed during discrete periods of neural development of the thalamocortical pathways, and transient AChE activity correlates with the specific growth of thalamic axons into the cortex and synaptogenesis with cortical neurons (Robertson and Yu, 1993). In addition, significant sequence similarity exists between AChE and cell adhesion proteins that function in morphogenic phenomena. These observations have led to the hypothesis that AChE may play key roles in neural development.

Chlorpyrifos (CPF) an organophosphorous pesticide is a most potent neuro-toxicant. AChE inhibition is the primary manifestation of toxicity during OP exposure (Thomson et al., 1991; Sultatos, 1994; Calumpang et al., 1997; Fulton and Key, 2001). CPF neurotoxicity mediated by the phosphorylation and subsequent inhibition of acetylcholinesterase (AChE) (Oehmichen and Besserer, 1982; Finkelstein et al., 1988), whose primary function is to terminate nerve impulse transmission at cholinergic synapses by hydrolyzing the neurotransmitter acetylcholine (ACh) (Kennedy, 1991; Murty and Ramani, 1992). This inactivation of AChE results to nerve exhaustion, nervous system failure and ultimately to death. Most chemicals in this group require oxidative desulfuration to achieve their greatest cholinesterase-inhibiting potencies.

Developmental exposure to CPF elicits long-lasting alterations in cell-signalling cascades. Further, they are shared by various neurotransmitter and hormonal inputs. Thus resulting in a abnormalities of synaptic communication and affect the corresponding behaviors (Meyer et al., 2004b). There are several studies which were controversial regarding fetal and embryo toxicity of OPCs. For example some organophosphorous pesticide like: parathion, diazinon, malathion and dichlorvos induced maternal toxicity but there is no evidence of teratogenicity (Vogin et al., 1971; Talens

and Wooley, 1973; Spyker and Avery, 1977; Baksi, 1978). However another OP, dipterex has shown to cause teratogenic effect at high concentration (Baksi, 1978) and acephate was found to cause developmental toxicity at maternal toxic dose to mice (Staples et al., 1976). Chung et al. (2002) also reported that flupyrazofos, a new OP, causes fetal growth retardation at maternal toxic doses in rats. Similarly in Swiss albino mice, Ambali et al. (2010) reported that chlorpyrifos affected conception and pre-implantation losses in dose dependent manner. Eskenazi et al. (1999) reviewed that there are numerous animal studies have shown that in utero or early exposure to OP pesticides affect neurodevelopment. There are growing data showing that they are teratogenic on the grounds of experimental concentrations in non-mammal developing embryos, such as amphibians (Richards and Kendall, 2002) and birds (Meiniel, 1981). Exposure to CPF can affect both the fertility and reproductive outcomes because of its ability to interact with steroid hormones receptors (Peiris-John and Wickremasinghe, 2008). CPF target the early stage of development of the cerebral and cerebellum cortice (D'Arcangelo et al., 1995; Eksloglu et al., 1996; Gleeson et al., 1998; Keller and Persico, 2003). Its *in vivo* administration has been associated with deficits in neurons and cholinergic functions (Jameson et al., 2006).

Organophosphorous compounds are able to induce muscular damage (Gupta et al., 1987; Karalliedde and Henry, 1993; De Bleecker et al., 1994; John et al., 2003). The teratogenic effects may be observed as morphological abnormalities or functional defects that may only occur later in life. Developmental toxicity also involves non-permanent manifestations such as growth retardation and edema. Thus, the present work was dealt with the teratogenic potential of chlorpyrifos (CPF) in freshwater catfish, *Heteropneustes foosilis* hatchlings in relation to the fertilization rate, hatching rate, embryo mortality and percentage of malformations during development.

2. Materials and Methods

2.1. Chemicals

The organophosphorous pesticide, chlorpyrifos (CPF) was used which was procured from the local market of Lucknow, Uttar Pradesh, India, under the trade name Hilban® (20% EC CPF), supplied by Hindustan insecticide limited. The Ovidac® (human chorionic gonadotropin: hCG, Zydus Pharma Pvt. Ltd.) was purchased from local medical stores. All other chemicals and reagents used were of analytical grade and were bought from HiMedia Laboratory Pvt. Ltd. India.

2.2. Animal collection and their acclimatization

Experiment was performed in accordance with local/ national guidelines of ethical committee for experimentation in animals to avoid any type of cruelty.

The healthy brooders of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) of relatively same size (17±2 cm) and weight (185±20 gm) were collected from commercial fisherman of Lucknow, Uttar Pradesh, India. Brooders were brought to the laboratory in wide mouthed large plastic containers in natural water avoiding stresses and injuries as possible. Later, they were acclimatized in 120 l glass aquaria containing water having a pH of ≈ 7.5, dissolved oxygen 5-6 mg/l and a temperature of 24±1^{o}C for one week. The photoperiod was 12h: 12h (light: dark). Water was renewed daily to remove faecal matter and waste metabolite of fish during acclimatization. During this period, Fish were fed a diet of dead shrimp.

2.3. Teratogenic assay

2.3.1. In vitro experimental setup

To study the *in vitro* effect of chlorpyrifos, male and female fish were selected based on the external morphological features. The female fish were artificially induced by synthetic hormone (hCG hormone: 6.95 IU/g dose of body mass) intramuscularly. Experiment was conducted in five replicates. Approximately 12 to 14 h after the injection, eggs were obtained by stripping method into fertilization tray and fertilized by sperm suspension that was previously obtained by mincing of adult male testes in 0.4%

fish saline. Successful fertilization was achieved when the eggs were oriented with the red cap animal pole side up. Screening of unfertilized eggs was performed. After obtaining fertilized eggs, one fifty eggs were kept in different concentrations of chlorpyrifos viz., 0.52, 0.26, 0.052, 0.026, 0.013, 0.006 and 0.004 µM/l along with control set of fertilized eggs in five replicates (n=3). These concentrations were selected on the basis of sub-lethal toxicity experiment of CPF in pre-spawning phase as discussed earlier in Chapter 1. Dead eggs become white due to coagulation and precipitation of protein. Dead eggs were counted and removed daily. At the end of incubation period, the hatched eggs were counted. The teratogenic changes were recorded after 24 hr of hatching. Water was changed daily with same concentration of pesticide.

2.3.2. In vivo experimental setup

For *in vivo* study, a set of injected female brooders with hCG (6.95 IU/g dose of body mass) were transferred in different concentrations of chlorpyrifos viz., 0.52, 0.26, 0.052, 0.026, 0.013, 0.006 and 0.004 µM/l along with control set upto their desired ovulation time. Approximately after 10 to 12 hr of latency period, the eggs were obtained by applying gentle pressure on the female's abdomens were put into rectangular plastic fertilization tray and then artificially inseminated with a milt of sperm suspension previously obtained by mincing adult male testes in 0.4 % fish saline. The fertilized eggs were identified by the appearance of red cap at animal pole side up. Thereafter, within a one minute the screening of unfertilized eggs was performed. The obtained fertilized eggs were transferred into normal tap water for analysis of further observations.

2.4. Data collection

The fertilization and hatching rate were calculated by formula as given below:

Fertilization rate (%) = (No. of fertilized eggs/ Total no. of eggs) × 100

Hatching rate (%) = (No. of eggs hatched/ Total no. of fertilized eggs) × 100

The data were expressed as mean±SEM. The data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test ($p < 0.05$) for multiple group comparisons. For the morphological examination of surviving hatchlings, the images were captured under Bright field microscope (Olympus CX41) using micropublisher 3.3 RTV camera per 24 h interval.

3. Results

The purpose of the present investigation was to study the effect of chlorpyrifos (CPF) on the fertilization rate, hatching rate and malformations of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). All the observed parameters were concentration dependent of chlorpyrifos.

3.1. Fertilization rate and embryo mortality

The result showed that chlorpyrifos (CPF) exposure caused lower fertilization rate as compared to control (98.7±0.5%). The fertilization rate was depending on the concentrations of CPF. At higher concentration of chlorpyrifos (0.52 µM/l), the fertilization rate was significantly lower i.e., 42.7±0.94% and at lower concentration of CPF (0.004 µM/l), rate was significantly higher 80.37±0.76% ($F=289.23$; $p < 0.05$) (Figure 20A).

The organophosphorous pesticide, chlorpyrifos caused severe embryo mortality. The present findings represented that in *in vitro* exposed group, the percentage of embryo mortality was higher as compared to *in vivo* exposure. The severity of toxicity was occurred in a concentration dependent manner. The control group had negligible mortality (0.2±0.12%) as compared to exposed group of CPF. *In vitro* exposure of CPF caused significant high embryo mortality (90.0±0.42%) at high concentration of CPF (0.52 µM/l) whereas at low concentration of CPF (0.004 µM/l), 35.2±0.81% embryo mortality was found ($F=1070.28$; $p < 0.05$). However *in vivo* exposure of CPF showed significantly low embryo mortality (27.2±0.76%) at low concentration of CPF (0.004

µM/l) and high mortality (79.7±0.94) was found at high concentration of CPF (0.52 µM/l) (F=893.67; $p < 0.05$) (Figure 20B).

3.2. Hatching rate and malformed hatchlings percentage

The hatching rate was also depending on different concentrations of CPF. *In vitro* exposure caused decreased hatching rate as compared to *in vivo* exposure of CPF. In *in vitro* exposure, the significant low hatching rate (33.83±2.12%) was noticed in higher concentration of CPF (0.52 µM/l) as compared to lower concentration of CPF (0.004 µM/l), it was 85.42±1.25% (F=267.23, $p < 0.05$). The control group had highest hatching rate 98.3±2.5%. In case of *in vivo* exposure, at 0.004 µM/l concentration of CPF, the rate of hatching was 90.5±0.5%, which was significantly higher than higher dose of CPF i.e., 0.52 µM/l, rate was 40.8±1.91% (F=271.87, $p < 0.05$) (Figure 21A).

The percentages of malformed hatchlings were also following a concentration dependent manner of CPF. These were increased in *in vitro* exposure as compared to *in vivo* exposure. At higher concentration of CPF (0.52 µM/l), the percentage of malformed hatchlings was significantly increased in both the exposures (*in vitro* and *in vivo*) i.e., 86.6±0.87% *in vitro* and 68.9±0.94% *in vivo* (F=640.81, F=225.93; $p < 0.05$). As the concentration of CPF was decreased, the percentage of malformed hatchlings was also decreased in both the exposure of CPF. At lower concentration of CPF (0.04µM/l), the low malformed hatchlings were found significantly (37.5±1.07 and 20.4±1.11; *in vitro* and *in vivo*) (F=640.80, F=225.93; $p < 0.05$) (Figure 21B).

3.3. Embryonic development and malformations due to chlorpyrifos

The effect of organophosphorous pesticide, chlorpyrifos (CPF) on embryonic development of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) was observed in the present study. The result showed that *in vivo* exposure caused delayed embryonic development whereas *in vitro* exposure of CPF faster the embryonic development as shown in Table 10. The present study resulted that CPF produced a concentration

dependent inhibitory response in normal development of the hatchlings. There are many malformations noticed due to chlorpyrifos toxicity. These malformations were formation of eyes and barbel, brain development, pericardial edema, yolk sac edema, fin fold defect (tail fin flexure), reduction of pigmentation and different notochordal defects. In higher concentrations of chlorpyrifos (0.52 µM/l), the hatchlings were recorded with combinations of all these abnormalities (Figure 22B-23B).

Four major categories of gross morphological abnormalities (irregular head development, cardiac edema, yolk sac edema and notochordal defect) and three minor deformities (body shortening, tail fin flexure and loss of pigmentation) were observed in the present study (Figure 22-23). Among major categories, the chlorpyrifos treated hatchlings showed an irregular head shape. This abnormality was observed in CPF exposures i.e., *in vitro* and *in vivo* exposure (Figure 22-23: B, C). The malformed head beard swelling like protrusion on lateral side or the ventral (Figure 22-23: B, C). The malformation was often associated with a reduced barbel and reduced eye development (Figure 22-23). Another major malformation was cardiac and yolk sac edema. The cardiac edema was found as an enlargement in the pericardial sac or pericardial edema (Figure 22-23: B, C). Also yolk sac edema was often associated with irregular development of head and notochordal (spinal cord) curvature. The yolk sac and cardiac edema were observed in exposed group of hatchlings to different concentrations of chlorpyrifos both in *in vitro* and *in vivo* exposure. Malformed hatchlings were characterized by poorly developed mouth.

Further the most frequently observed gross morphological deformations in both of the exposures of CPF *in vitro* and *in vivo*, were bent body or a notochordal curvature. Different types of notochordal abnormalities were recorded (Figure 24): (1) C-shaped curvature (Figure 24A), (2) lordosis (dorso-ventral curvature) (Figure 24B), (3) Kyphosis (ventro-dorsal curvature) (Figure 24C), and (4) Scoliosis (lateral curvature) (Figure 24D). The presence of these defects was following the concentration manner of CPF.

Table 10: Effect of organophosphorous pesticide, chlorpyrifos on embryonic development of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)

Parameters (h:min)	**Control**	**Treatment**	
		In vitro	***In vivo***
Latency period	12±2	16±2	10±2
Fertilization	0	0	0
Blastodisc formation	0:10	0:05	0:15
2 celled stage	0:15	0:10	0:22
4 celled stage	0:30	0:25	0:40
8 celled stage	1:15	1:10	1:25
16 celled stage	1:25	1:20	1:30
32 celled stage	2:00	1:40	2:15
Morula	2:30	2:10	2:45
Blastula stage	3:30	3:10	3:55
Gastrula stage	6:30	6:10	6:45
Yolk plugged stage	7:00	6:45	7:15
Kidney shaped embryo	09:00-10:00	08:40	09:30-10:30
Twisting movement	20:00-21:00	19:00-19:30	20:30-21:30
Fully active embryo	22:00	21:30	23:00
Hatching	23:00-24:00	23:00-23:30	24:00

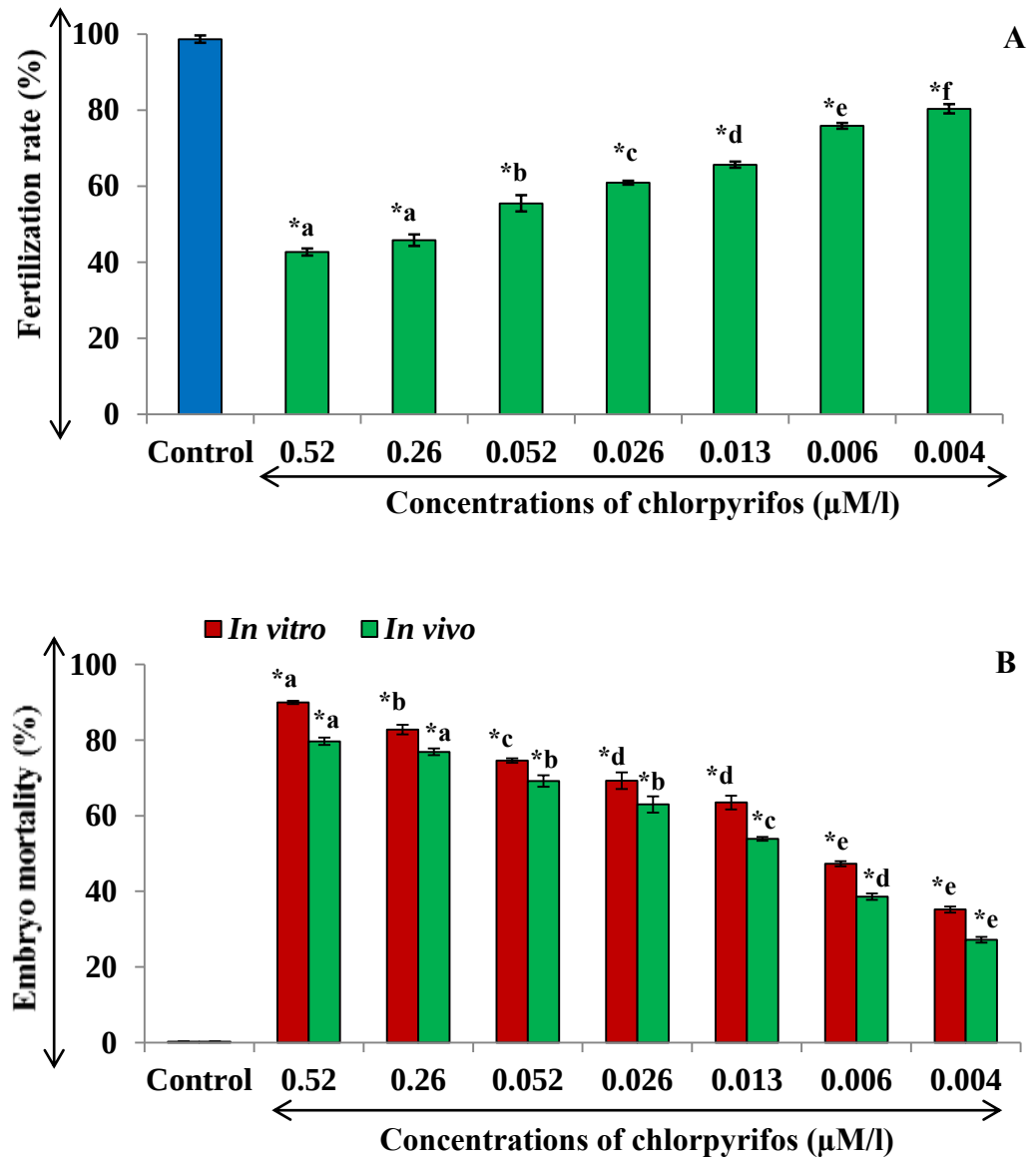

Figure 20: (A) Effect of *in vivo* exposure of different concentrations of chlorpyrifos on the fertilization rate (%). (B) *In vitro* and *In vivo* exposure of different concentrations of chlorpyrifos on embryo mortality (%) of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). Data were presented as mean±SEM. Values were analyzed by one way ANOVA ($p < 0.05$), followed by Newman-Keuls test ($p < 0.05$) for comparison within the groups. Asterisk (*) shows significant difference from the control group at $p < 0.05$. Group superscripted with different letter shows significant difference ($p < 0.05$) whereas same letter shows non-significance.

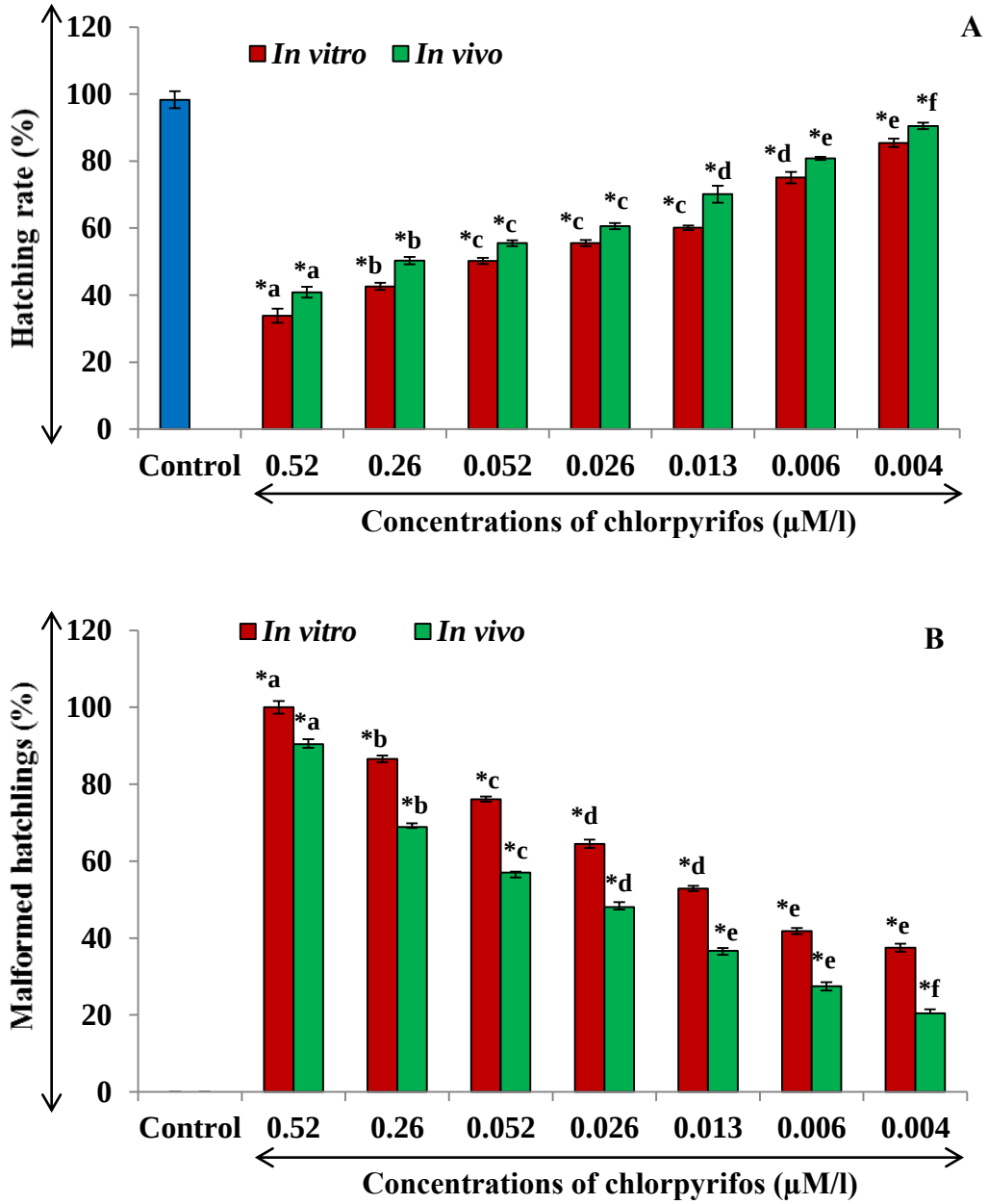

Figure 21: Effect of *in vitro* and *in vivo* exposure of different concentrations of chlorpyrifos on hatching rate (%) (A) and malformed hatchlings (%) (B), of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). Data were presented as mean±SEM. Values were analyzed by one way ANOVA ($p < 0.05$), followed by Newman-Keuls test ($p < 0.05$) for comparison within the groups. Asterisk (*) shows significant difference from the control group at $p < 0.05$. Group superscripted with different letter shows significant difference ($p < 0.05$) whereas same letter shows non-significance.

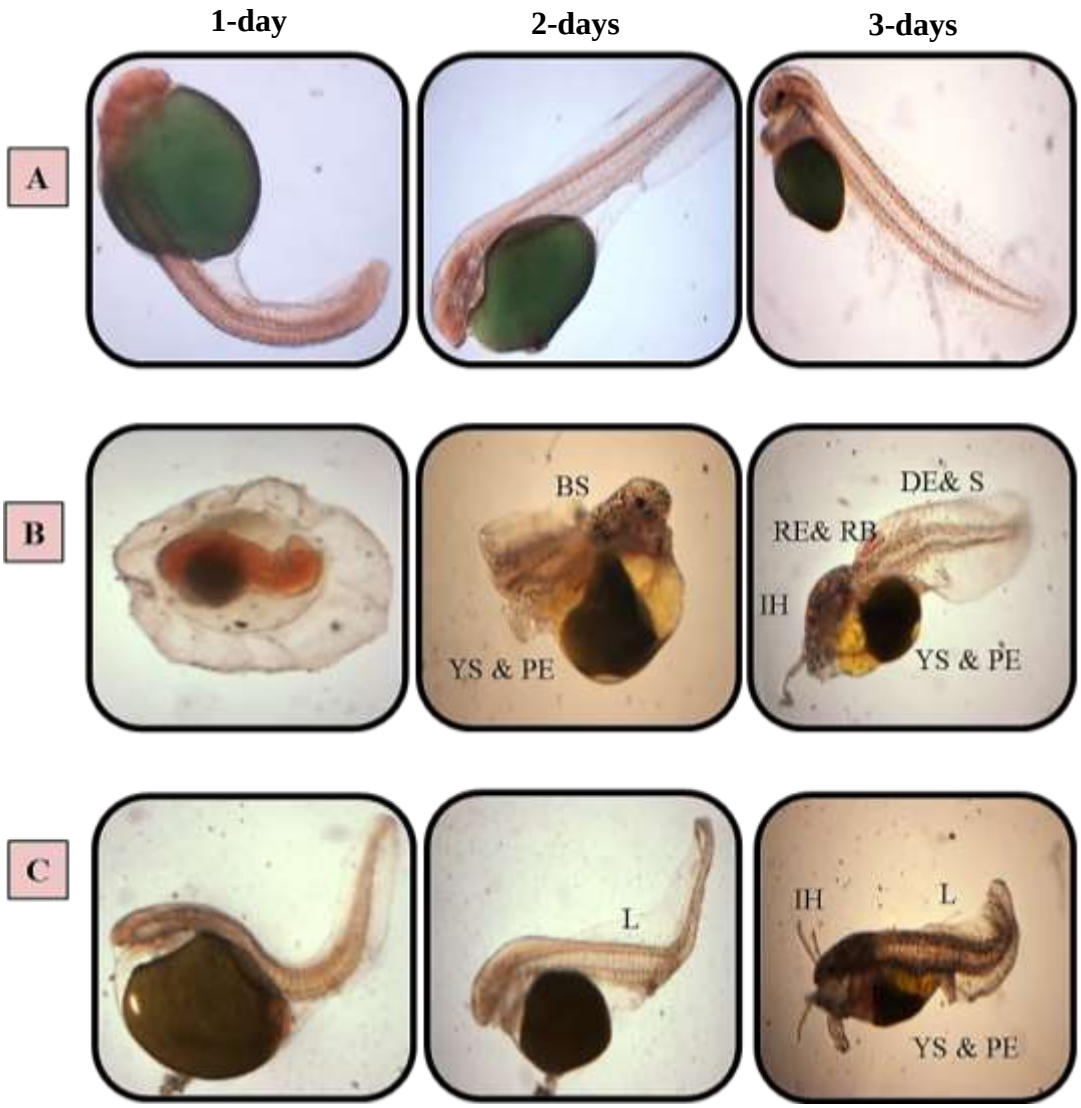

Figure 22: Photographs of 1-day, 2-days and 3-days hatchlings of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). A: Control group, B: *in vitro* exposure of maximum dose of chlorpyrifos i.e., 0.52 µM/l and C: *in vitro* exposure of minimum dose of chlorpyrifos i.e., 0.004 µM/l. Note showing: IH: Irregular head shape, BS: body shortening, YS & PE: yolk sac & pericardial edema, L: lordosis, RE & RB: reduced eye & reduced barbel, LP: loss of pigmentation, DE & S: deformed eye & skull.

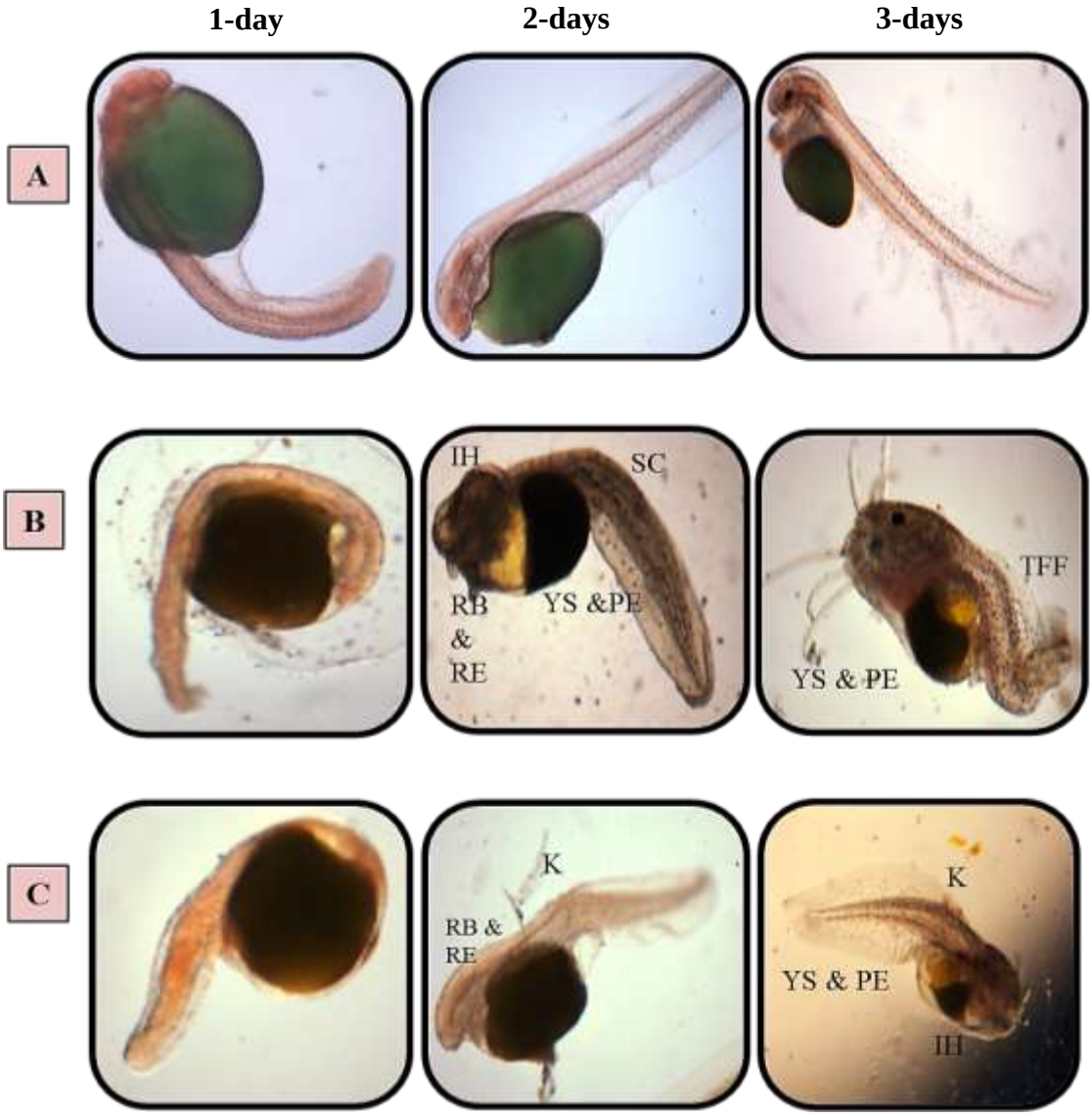

Figure 23: Photographs of 1-day, 2-days and 3-days hatchlings of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). A: Control group, B: *in vivo* exposure of maximum dose of chlorpyrifos i.e., 0.52 µM/l and C: *in vitro* exposure of minimum dose of chlorpyrifos i.e., 0.004 µM/l. Note showing: IH: Irregular head shape, BS: body shortening, YS & PE: yolk sac & pericardial edema, RE & RB: reduced eye & reduced barbel, TFF: tail fin flexure, SC: scoliosis, K: kyphosis

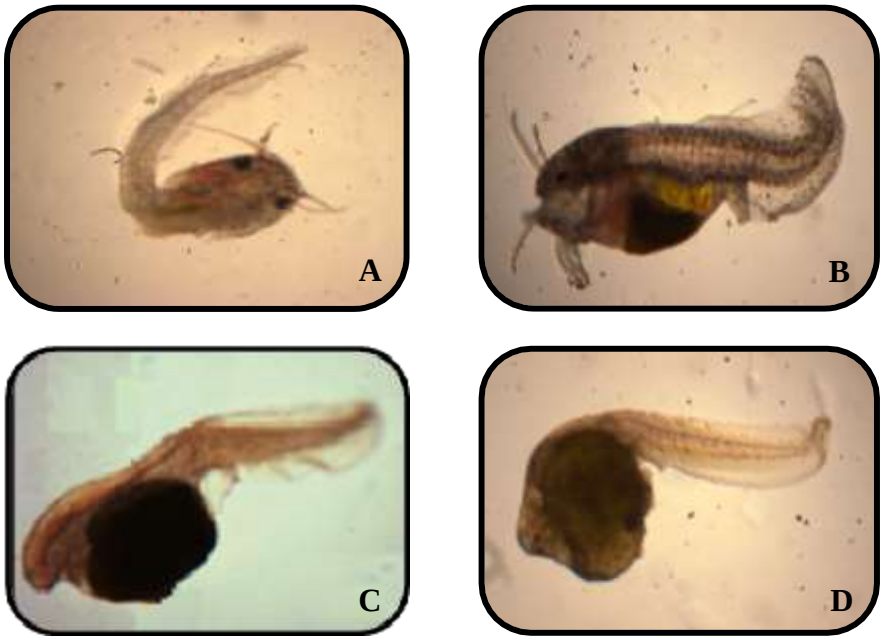

Figure 24: Photographs showing notochordal defects found in the chlorpyrifos exposed group of hatchlings of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). Note showing; A: C-shaped, B: Lordosis, C: Kyphosis, D: Scoliosis.

4. Discussion

The result of the present study showed that organophosphate pesticide, chlorpyrifos (CPF) not only affect embryo survival and hatching rate but it also influence period of embryonic development. The embryonic development was faster in *in vitro* exposure of CPF, as compared to control and *in vivo* exposure of CPF. CPF also caused teratogenic changes in term of morphological and notochordal defects. According to Dawson and Bantle (1987), CPF must be considered as a powerful teratogenic compound.

The fertilization rate was decreased in chlorpyrifos treated group of experimental fish as compared to control. The decreased embryo mortality was also observed in CPF exposed group of fish in a concentration manner as compared to control group. As comparison to *in vitro*, *in vivo* exposed fish had higher embryo mortality. Same observation was noticed by Rand and Petrocelli (1985) who reported that dimethoate, an organophosphorous pesticide caused a large increase in mortality associated with a small dose of pesticide. The hatching rate was low in *in vitro* exposed group as compared to *in vivo* exposed group of fish and control group. It has been observed that chlorpyrifos had significant effects on hatchability. Toor and Kaur (1974) also reported various adverse effects of pesticides on survival of developing eggs, hatchability and hatchlings of carp. Kaur and Dhawan (1993) found that at higher concentrations of pesticides (carbaryl, carbofuran, malathion and phosphamidon), egg development was arrested and heavy mortality (>50%) was occurred at this stage, indicating the greater sensitivity of early embryonic stages to pesticides. Many environmental pollutants (phosphor-organic pesticides, some herbi- and fungicides, heavy metals, PCBs, petroleum hydrocarbons) caused increased embryo mortality, decreased hatching rates and teratogenic effects (Hoffman and Sileo, 1984; Deli and Varnagy, 1985; Garrison and Wyttenbach, 1985; Indyk, 1989/1990a, b, c; Hoffman, 1990; Kumar and Devi, 1992; Lenselink et al., 1993). Kaur and Toor (1977) reported that chlorpyrifos was inhibited some hatching enzymes which resulted in lower hatching rate. According to Boone and Chambers, (1997), the main factor of high CPF toxicity was its property of AChE-target enzyme sensitivity.

There were not so much studies of pesticidal exposure towards the embryo toxicity, and the past investigations in this regards have indicated alarming situation in infertility, abnormal gamete, defective embryos, poor fertilization and birth defects (Fuortes et al., 1997; Smith et al., 1997; Greenlee et al., 2003; Tian and Yamauchi, 2003). In present study, a high tolerance of CPF was expressed in terms of survivability of hatchlings. The result found that at lower concentration of chlorpyrifos (0.004 µM/l), 37.5% of malformed hatchlings in *in vitro* and 20.4% in *in vivo* exposure was noticed. In the present observation, various types of malformations were observed in hatchlings of freshwater catfish, *H. fossilis* due to chlorpyrifos toxicity such as axial (lordosis or kyphosis) or lateral (scoliosis) notochordal curvature, deformed skull and eye, yolk sac and cardiac edema, shortened body, reduced barbels and tail fin flexure.

The result showed that the hatchlings of freshwater catfish, *Heteropneustes fossilis* showed gross alterations in tail flexure coupled with a decrease of neuromuscular activity. These results were well correlate with findings of other authors (Snawder and Chambers, 1989; Vismara et al., 1996; Richards and Kendall, 2002). The abnormal tail flexure observed in CPF could be the consequence of the cholinergic phase, where the AChE inhibition causes repetitive firing of muscle fibres leading to the axis tail folding and consequence of uncontrolled and continuous contraction of the tail musculature (Lien et al., 1997). This hypothesis was confirmed by Behra et al. (2002) who noticed that the first movement of zebrafish embryos homozygous for AChE gene mutation was characterized by tail twitching. Anti-ChE has an important role in neural development in function of nervous system. It is transiently expressed during discrete periods of neural development of the thalamo-cortical pathways, and transient AChE activity correlates with the specific growth of thalamic axons into the cortex and synaptogenesis with cortical neurons (Robertson and Yu J, 1993). There is a significant sequence similarity exists between AChE and cell adhesion proteins that function in morphogenic phenomena. Mack and Robitzki (2000) investigated a functional role of AChE in regulation of cell proliferation and the onset of differentiation during early neuronal development which was independent of its enzymatic activity. The most common and important malformation viz., Yolk sac edema, has been previously reported by a number

of author's viz., Dushkina (1973), Eaton (1974), Linden (1978), Helder (1980), Dominguez and Chapman (1984), Middaugh et al. (1988), Marty et al. (1990), Spitsbergen et al. (1991), Walker et al. (1991, 1992), Prince and Cooper (1995).

The observed deformities in the present study were also reported in many fish by many researchers due to pesticidal exposure viz**.,** Mehrle and Mayer (1975) in *Pimephales promelas*, Weis and Weis (1976) in *Cyprinodon variegatus*, Lien et al. (1997), in *Clarias gariepinus,* Solomon (1977) in *Oryzias latipes,* Kumar and Ansari (1984) in *Brachydanio rerio*, Srivastava and Srivastava (1990) in *H. fossilis*, Jezierska et al. (2000) in carp, Sanchez-Bayo and Gaka (2005) in *Oryzias latipes* and Mochida et al. (2008) in *Fundulus heteroclitus*. Many researchers also reported that pesticides such as toluene, toxaphene, malathion, kepone, trifularin and pyrithione can cause morphological and skeleton abnormalities in many freshwater fish (Weis and Weis, 1987; Lien et al., 1997; Mochida et al., 2008). The occurrence of these notochordal deformities could arise due to decreasing the collagen amount in spinal column, changing amino acid composition (Mehrle and Mayer, 1975), deficiencies of vitamin C (Kumar and Ansari, 1984), neuromuscular spasms (Meiniel, 1981; Couch et al., 1977) and absence of a functional swim bladder (Chatain, 1994). The observed morphological and muscular damages may be related to inhibition of acetylcholinesterase activity that showed a possible role played by organophosphorous on induction of muscular dystrophy and morphological deformities during development. Abu-Qare et al. (2001a, b) found that diazinon and methyl parathion exposure caused inhibition of cholinesterase (ChE) enzymes during maternal and fetal tissues assessment. The ChE enzyme plays a significant role during organogenesis (Moody and Stein, 1988). Behra et al. (2002) stated that AChE is required for embryo muscular development. In mammals, OP compounds produce typical signs of anti-cholinesterase toxicity such as complex posturing movements, notochordal morphogenesis and skeletal muscle fasciculation associated with muscle fibre damages (Meiniel, 1981; Gupta et al., 1987; Karalliedde and Henry, 1993; De Bleecker et al., 1994; Snawder and Chambers, 1993).

Consolidated summary and conclusion

Organophosphorous pesticide (OP) has been widely used in agricultural practices. It is second largest selling organophosphorous pesticide and found to be highly toxic to aquatic animals. Chlorpyrifos is an organophosphate insecticide, acaricide and nematicide. Its direct application to soil, vegetation and animals can result in exposure to non-target organisms or aquatic organisms.

The present research included the acute toxicity of chlorpyrifos (CPF) to freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). Fish were exposed to varied range of concentration of CPF (0.002 - 2.28 mM/l) for 96 h in three respective reproductive phases viz, resting, preparatory and pre-spawning phase. The finney's probit analysis (1971) was used to determine median lethal concentration (LC_{50}). The safe concentration of the studied pesticide was calculated as per Hart et al. (1945) and Henderson et al. (1959) in all respective seasons. The obtained LC_{50} were found to 1.547, 0.678, 0.299 and 0.174 mM/l in resting phase, 0.332, 0.193, 0.152 and 0.123 mM/l in preparatory phase and 0.296, 0.107, 0.044 and 0.026 mM/l in pre-spawning phase for 24, 48, 72 and 96 hr respectively. The safe concentrations were 0.039 mM/l in resting phase, 0.19 mM/l in preparatory phase and 0.004 mM/l in pre-spawning phase. Susceptibility of catfish, *H. fossilis* to CPF was found to be duration and concentration dependent. Their tolerance to CPF was increasing with inactiveness of gonadal activity (resting season). At higher concentration of CPF, fish showed alterations in morphological and behavioral responses, especially erratic and jerky swimming, frequent surfacing and ingulping, mucus secretion, an increase in opercular movement and copious secretion of mucus all over the body. The histological assessment resulted that CPF caused reduction in size of oocytes, vacuolation in cytoplasm, damaged ovarian follicles, increase in inter-follicular space, breaking of ovarian wall, occurrence of necrosis, damaged structure of ovarian follicles, formation of fragmented ova, atretic oocytes and nucleolar extrusion. It is concluded that CPF is highly toxic to catfish and severely affect their physiology and behavior.

To investigate *in vitro* effect of chlorpyrifos (CPF) on hCG-induced oocyte maturation and different steroids involved in oocyte maturation of post-vitellogenic follicles of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794), the follicles were incubated with human chorionic gonadotropin (hCG), CPF and in combination of hCG and CPF in a concentration and duration dependent manner. The result showed that CPF caused an inhibitory effect on hCG induced GVBD in co-incubation experiments at the dose of 0.26 pM/ml. Pre- and post-incubation of follicles with CPF resulted a significant inhibition in hCG induced oocyte maturation in all durations and maximum inhibition 87.67% and 76% was recorded at 12 hr incubation duration ($p < 0.05$, Newman-Keuls test). Effective dose of hCG (1.7 mg/ml, 8 hr) induced OM coincided with a significant increase in follicular pregnenolone, progesterone, deoxycorticosterone (DOC) and dihydroxyprogesterone (DHP) as well as significant decrease in estradiol-17β (E_2) as compared to control group of post-vitellogenic follicles. Under CPF exposure follicles elicited a sharp significant decrease in pregnenolone, progesterone and DHP. However, DOC and E_2 were increased significantly. Co-incubation study revealed that CPF suppressed hCG induced concentrations of DHP, pregnenolone and progesterone significantly but side by side support DOC increase and maintain high $E_{2.}$

To observe the effect of CPF on developmental embryo of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794), *in vitro* and *in vivo* experiments were performed. For this, *in vitro* and *in vivo* experiments were performed. In *in vitro* experiment, the fertilized eggs stripped from normal control catfish were exposed to different concentrations of chlorpyrifos viz., 0.52, 0.26, 0.052, 0.026, 0.013, 0.006, 0.004 µM/l. For *in vivo* study, the induced female catfish with synthetic hormone (hCG) was exposed to same concentrations of chlorpyrifos. The experiment was done in triplicates along with the control group. The result showed that both, fertilization and hatching rate was decreased significantly in exposed group in comparison to control. *In vivo* exposure showed significantly low embryo mortality (27.2±0.76%) ($F=893.67$; $p < 0.05$) and high hatching rate (90.5±0.5%) ($F=271.87$; $p < 0.05$) as compared to *in vitro* study (35.2±0.81% and 85.4±1.25%) ($F=1070.28$, $F= 267.23$; $p < 0.05$) in lower concentration of CPF (0.004 µM/l). The percentage of malformed hatchlings was higher in *in vitro*

experiment as compared to *in-vivo*. The occurrence of all studied parameters was observed in a concentration dependent manner, as the concentrations of CPF were increased, the percentage of embryo mortality and malformed hatchlings was decreased. However, the hatching rate was increased. The result showed that exposed group of hatchlings were affected by many morphological and notochordal deformities viz., ventral or lateral tail flexure, abnormal spinal bending, irregular head shape and size, loss of eye, reduced barbel, pericardial edema, yolk sac edema, notochordal defect, tail fin flexure, reduction of brain development and reduction of pigmentation.

From the present study it can be concluded that CPF caused a significant impact on behavioral, morphological, histo-pathological, endocrinological and developmental parameters of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). CPF acts as a potent endocrine disruptor affecting ovarian steroidogenesis and ovulation. It inhibits the meiotic oocyte maturation involved reducing enzymatic activity as well as MIS inhibition which may lead to adverse effect on fish reproduction. It also plays an important role in inducing the muscular dystrophy and morphological deformities during embryonic development and organogenesis. The findings of this research proposed that CPF may be teratogenic for catfish. The present study of chlorpyrifos toxicity of air-breathing freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) concluded that this fish is very sensitive to low level of organophosphorous pesticide toxicity. Therefore, these pesticides should be used with great caution and in a sustainable way.

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General Introduction

Agriculture remains the backbone of India. It is most important sector in the economy upon which the Indian population depends. To increase the agriculture crops, pesticides are widely used which constitute an important component in agriculture development and protection of public health. Pesticides are being used by the human beings for their benefits like control of insect vectors and increased crop yield (Prakasam et al., 2001). It makes a significant contribution to maintaining world food production. However, the extensive use of pesticides has caused severe environmental and health hazards to many organisms also (Abdollahi et al., 2004;Tuzmen et al., 2008).

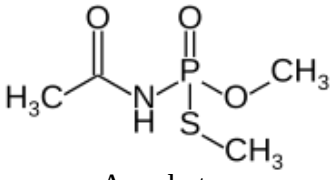
1. Pesticide classification

Pesticides can be divided into two main group viz., inorganic and organic group. Inorganic pesticides are derived from natural resources and don't contain carbon for example arsenic, cyanide, mercury, thallium etc. (Hassall, 1990). However organic pesticides are synthetic compounds either alphatic or aromatic hydrocarbons. They are further classified on the basis of chemical structure and their physical state viz., pyrethroids, organophosphorous, organochlorine, carbamate and sulfonylurea pesticides (Wasswa, 2009). The organophosphorous pesticide further classified in two major categories which include: organophosphate and organothiophosphate.

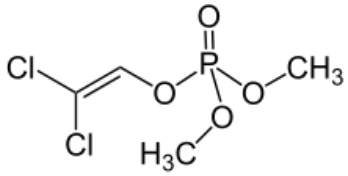
2. Organophosphorous pesticide

Organophosphorous pesticides (OPs) are ubiquitous in the environment, commonly used as agricultural and vector control. OPs are phosphate esters derived from phosphoric acid comprising of a central phosphate atom and three organic side chains (R), two of which are usually alkyl substitutions and third group is more labile to hydrolysis (termed leaving group). The P=S bond in OPs require metabolic activation by oxidative desulfuration to their corresponding oxon (P=O) metabolite. They are not very stable either chemically or biochemically, but they are readily hydrolysed in soil, sediment or surface water (Walker, 2001). This group of pesticides has virtually replaced

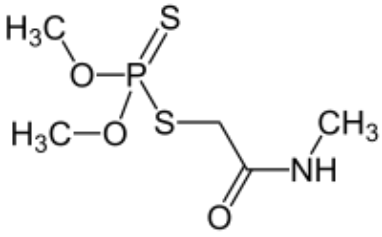
the persistent organochlorine compounds (Briggs, 1992). It may contaminate the environment and accumulate in food chains there by posing hazardous effect to human and aquatic animal health (Pesticides Action Network, 2001; Blasco et al., 2003; Khogali et al., 2005; Ali et al., 2009).The application of pesticides to flooded areas, along with accidental spillage, spray drift, leaching, runoff or drainage from treated agricultural lands has the potential to expose the non-target organisms especially the fish. Nearly 90% of pesticides were dispersed through air, soil and water (Moses et al., 1993). OPs are highly toxic in acute poisoning than other classes of pesticides (Mansour, 2004; Collins, 2006). There are several pesticides belong to organophosphorous category such as, acephate, dichlorvos, dimethoate, ethion, malathion, mevinphos, chlorfenvinphos, parathion, chlorpyrifos and diazinon. The chemical structures of OPs compounds are given below.



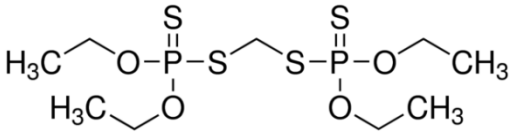
Acephate



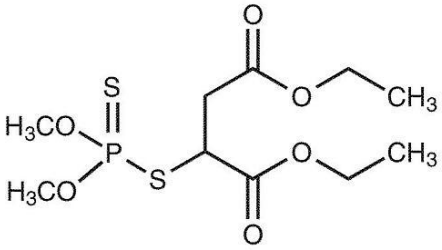
Dichlorvos



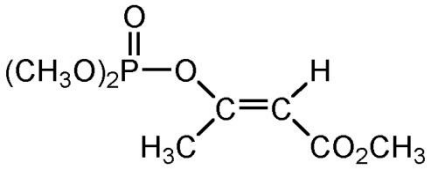
Dimethoate



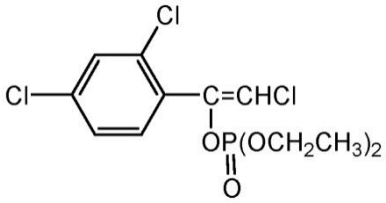
Ethion



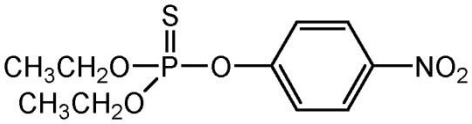
Malathion



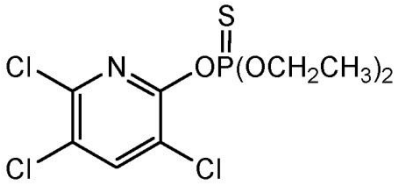
Mevinphos



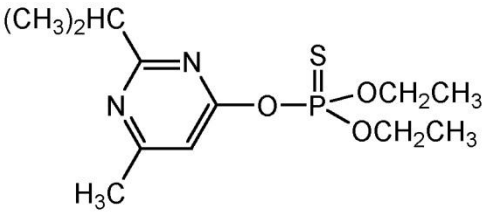
Chlorfenvinphos



Parathion



Chlorpyrifos



Diazinon

Chemical structure of organophosphorous compounds

The primary mechanism of action of OPs is as neurotoxic agents. They showed their toxic activity by inhibiting acetylcholinesterase enzyme (AChE) through the interaction of nucleophilic active site serine of the enzyme with the OP to form a phosphorylated enzyme derivative. The phosphorylated derivative is much more resistant to subsequent hydrolysis than the normal acetylated derivative. AChE is responsible for hydrolysis of acetylcholine, a neurotransmitter that conducts nerve impulses across neuromuscular junction in the nervous system. Acetylcholine acts as a neurotropic factor in brain development, promotes cell differentiation, in cholinergic targets sites (Hohmann et al., 1988; Navarro et al., 1989; McFarland et al., 1991). Inhibition of AChE causes accumulation of neurotransmitter acetylcholine in sympathetic and parasympathetic fibres, neuromuscular junction and some synapses within the vertebrate central nervous system (Koelle, 1970) leading to generalized cholinergic action and muscles. This inhibition eventually leads to paralysis, respiratory failure and death (Marrs, 1996; Guilermino et al., 2004; Podolska and Napierska, 2006). Acute exposure of OP caused acute toxicity which includes dizziness, headache, sweating, fatigue, numbness, vomiting neurological effects, coma or death (Turgut, 2007).Chronic exposure to low doses of pesticide through air, water and food may lead to chronic toxicity via accumulation of residues in the body over a long period of time which resulted in chronic pesticide toxicity including cancer, congenital malfunction, neurological disorders, infertility, impotence, immunological disorders, liver and kidney damage, skin alterations and worsening of existing health conditions (Jobling etal., 1995).

The present thesis was dealt with widely used compound i.e., chlorpyrifos (CPF) which comes under classification of organothiophosphate under the pyridine category which comes under heterocyclic organothiophosphate. Pesticides are extensively used to protect agriculture crops against the damages caused by pests. However these chemicals may reach other ecological compartments as lakes and rivers through rain and wind affecting other organisms away from primary target only 0.10%reaches the specific target. The significant increase of chemical emissions of the water resources had led to deleterious effect for aquatic organism (Livingstone, 2001; Matsumoto et al., 2006). Their widespread use not only brought adverse effect on agro ecosystem but also caused

alteration in physiological processes of non-target organisms that inhabit natural environment close to agricultural fields(Werimo et al., 2009).

2.1. Chlorpyrifos (CPF)

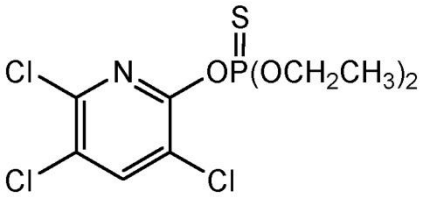
Chlorpyrifos (O,O-diethyl-O-3,5,6-trichlor-2-pyridyl) phosphorothioate: CPF) is a emulsifiable broad spectrum organophosphorous insecticide (OP), that has been widely used all around the world. It has been ranked third among all pesticides in use for household and commercial applications (Aspelin, 1994; Richardson, 1995). It was first introduced into the marketplace in 1965 by DowElanco, formerly by Dow Chemical Company. It is currently produced by Dow Chemical Company, India Medical Corp. It is the second largest selling OP and found to be more toxic to aquatic animals than other organochlorine and organophosphorus pesticide compounds (Tilak et al., 2001). CPF has an average half-life of 30 days in soils and two months in less alkaline soils (Howard, 1991).Chlorpyrifos enhanced the half-life of soil about in water (Racke, 1993; Wauchope etal., 1992).The principle metabolite of chlorpyrifos, TCP (3,5,6-trichloro-2-pyridinol), absorbs weekly to soil particle and persistent in soils. It also can persist indoors for weeks to months (Arcury et al., 2007). According to world health organization (WHO) pesticide classification system, CPF is ranked in classII and regarded as only moderately hazardous (WHO, 2004). The indoor residential use of CPF was terminated by United States Environmental Protection Agency (USEPA) since 2000, however, it is widely use in agriculture field in US (USEPA 2011a, b). Its use for agricultural purposes is one of the most anxious issues (Chen et al. 2009; Sun and Chen, 2008).

2.1.1. Common name

An organophosphorus pesticide, chlorpyrifos (CPF) is registered for use in over 30 products under its several names viz., Brodan, Detmol UA, Dowco 179, Dursban, Hilban, Empire, Eradex, Lorsban, Paqeant, Piridane, Stipend (Eisler, 2000; EXTOXNET, 1996).

2.1.2. Physical and Chemical properties of CPF

Chemical structure of chlorpyrifos:



CAS Registry number:	2921-88-2
Empirical formula:	$C_9H_{11}Cl_3NO_3PS$
IUPAC name:	O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)-phosphorothioate
Molecular weight:	350.6
Colour:	white
Nature:	granular, crystalline solid
Melting point:	41-42.5^{o}C
Vapour pressure:	3.35×10^{-3}Pa at 25^{o}C (EFSA, 2005), 2.54×10^{-3}Pa at 25^{o}C (WHO, 2009)
Odour:	Mercapton (thiol) similar to sulphur compounds
Solubility:	Moderately soluble in water (Kidd and James, 1991; Mackey et al., 1999) and readily soluble in acetone, benzene, iso-octane and methanol (Tomlin, 1997)

Log k_{ow} (Octanol-water partition coefficient): 4.7 at 20^{o}C (EFSA, 2005; Tomlin, 2006), 5.0 at 25^{o}C (WHO, 2009)

Soil sorption coefficient, K_{oc}: 652-30,381 L/kg (Gebremarian et al., 2012)

Aquatic sediment sorption, K_{oc}: 3,000-25,656 L/kg (higher affinity for aquatic sediment than soil; Gebremarian et al., 2012)

2.1.3. Toxicologically relevant metabolites

- 3,5,6-trichloro-2-pyridinol (TCP) (EFSA, 2005)
- 3,5,6-trichloro-2-methoxypyridine (TMP) (EFSA, 2005)
- O-ethyl-O-(3,5,6-trichloro-2-pyridoyl) phosphorothioic acid (phosphorothioate) (EFSA, 2005)

- Chlorpyrifos oxon (US EPA, 2009)

2.1.4. Uses of chlorpyrifos

It is used to control many types of insects and pests in a wide range of crops, ornamentals and also for indoor purposes (EPA, 1997; PMRA, 2000). In agricultural settings, it is being used to protect a number of important agricultural crops such as corn, citrus, alfalfa and peanuts from pest insect attack and food crops viz., cranberries, strawberries, apples, figs, pears, nectarines, cherries, peaches, plums, grapes, almonds, pecans, walnuts, onions, peppers, kale, broccoli, Brussels sprouts, cabbage, cauliflower, collards, cucurbits, asparagus, roots/tubers, tomatoes, lentils, beans, peas, sorghum, tobacco, wheat, soybeans, sunflower, cotton, sugar beets, mint, and bananas (Whitmore et al.,1992; Reinecke and Reinecke, 2007). It is also being used to control over 250 non-agricultural insects and arthropod pests, including subterranean termites, cockroaches, fleas, ants, and others, that are found in and around household items, on lawns, trees and shrubs. CPF uses have changed drastically over the last ten years. The agricultural applications have been reduced and commercial applications around residential locality have increased.

2.1.5. Mechanism of CPF metabolism

The primary mechanism of chlorpyrifos (CPF) toxicity is cholinesterase (ChE) inhibition. The inhibition results in the buildup of acetylcholine (ACh) at choline receptors, causing continual nerve stimulation (Giesy et al, 1999). CPF is transformed inside animals to chlorpyrifos-oxon which is about 3000 times as potent against nervous system as chlorpyrifos itself (Sultatos, 1991; Chambers and Car, 1993; El-Merhibi et al., 2004). CPF pesticide is bio activated by the microsomal cytochrome P450 (CYP)-mediated monooxygenase system to their more potent cholinesterase inhibitor, chlorpyrifos-oxon (CPO). Thus toxicity is initiated by the formation of chlorpyrifos-oxon by oxidative desulfuration (Giesy et al., 1999; Eisler, 2000). When CPF was exposed to UV light or to sunlight, it underwenthydrolysis in the presence of water to liberate3,5,6-trichloro-2-pyridinol, which underwent further decomposition todiols and triols and ultimately cleavage of the ring to fragmentary products (Smith, 1968). Hydrolysis in

water occurs least readily atabout pH 6 and very readily above pH 8. Chlorpyrifos and chlorpyrifos-axon kill insects and other animals, including humans (Chambers and Car, 1993). These two OPs pesticide inhibit acetylcholinesterase (AChE), which plays an important role in neurotransmission at cholinergic synapses by rapid hydrolysis of neurotransmitter acetylcholine to choline and acetate (Kwong, 2002). The inhibition of AChE activity caused by chlorpyrifos is more persistent than that caused by other organophosphates (Sakai, 1990; Pope, 1992; Bushnell and Padilla, 1993).Its inhibitory effect depend on its binding capacity to the enzyme active site and by its rate of phosphorylation in relation to the behavior and age of spp. (Richmonds and Dutta, 1992; Dutta et al., 1995) and is widely used for rapid detection to predict early warning of pesticide toxicity (Dutta and Arends, 2003). Most bio-activation takes place in the liver, while detoxification takes place in the liver and plasma.

2.1.6. CPF toxicity

CPF is highly toxic to varied vertebrates and invertebrates. CPF inhibits the acetycholinesterase (AChE) enzyme which caused accumulation of acetylcholine resulting in acute toxicity (Nolan et al., 1984; Pope et al., 1991, 1992; Pope and Chakraborti, 1992; Chakraborti et al., 1993; Chambers and Carr, 1993), delayed neuropathy (Kaplan et al., 1993; Richardson et al., 1993) and ultimately death (Cremlyn, 1991) of an organism. Symptoms of delayed neuropathy include cramps, weakness, tingling and numbness of extremities, a high stepping gate, paralysis (Fikes, 1992; Lotti, 1992). The CPF toxicity was influenced by number of factors include metabolic rate, number of target sites available for CPF metabolism (Chambers and Carr, 1995), organism surface area, and life stage (El-Merhibi et al., 2004). Chlorpyrifos affects the activity of ATPase, an enzyme important in cellular respiration (Sakai, 1990) thus impending cellular respiration. Its metabolite, chlorpyrifos-oxon inhibits the enzyme cholesterol-ester-hydrolase thus eliminating normal reactions to stress (Civen et al., 1977). Researchers believed that this is because chlorpyrifos is lipophilic, which causes changes in behavior of animal along with AChE inhibition. Reduction in working memory and a slowing of motor activity were documented two to three weeks following exposure. Weekly injections of smaller amount of chlorpyrifos caused similar effect (Bushnell et al., 1993). Intoxication of CPF caused a significant decrease in the reduced

glutathione (GSH), catalase (CAT) and glutathione S-transferase (GST) activities (Goel et al., 2005).

Human exposures to organophosphates are broadly classified into two categories: occupational and environmental (Racke, 1993). Occupational exposures occur among agricultural and industrial workers and pest control exterminators. Environmental exposure affects a large population. It includes residential exposures, dietary and accidental exposures via breathing contact with CPF treated soil, CPF exposed farms area, and exposures in chemical warfare (Jaga and Dharmani, 2003). CPF has been associated with chronic effects in humans, including chronic neurobehavioral effects and multiple chemical sensitivities. The neurobehavioral effects included persistent headaches, blurred vision, unusual fatigue or muscle weakness and problems with mental function including memory, concentration, depression, and irritability (Jobling et al., 1995). It can cause acute poisoning and well known symptoms include myosis, increased urination, diarrhoea, diaphoresis, lacrimation and salivation in rats (Samsum et al., 2005; Turgut, 2007). Several studies reported the CPF involvement in multiple mechanisms like causing hepatic dysfunction (Poet et al., 2003), genotoxicity (Mehta et al., 2008), neurobehavioral and neurochemical changes (Slotkin et al., 2005). Earlier study concluded that CPF was reported to be activated by dermal contact, air inhalation, ingestion of contaminated food or water, and vapour action, causing convulsions and paralysis.

CPF also affect male reproductive system in animals. Exposure to a chlorpyrifos product causes death of cells in male testes and a decrease in sperm production in cattle (Everett, 1982). This chemical has been detected in cervical mucous, semen and human milk in various studies conducted by German scientists (Wagner et al., 1994). Immune system abnormalities have been reported in patients exposed to chlorpyrifos. Many individuals reported developing sensitivities to a broad array of substances following chlorpyrifos exposure (Thrasher et al., 1993). Chlorpyrifos has been responsible for causing genetic damage in lymph and blood cells, mice spleen cells and hamster bone marrow cells (Amer and Aly, 1992). The developmental effects cannot always be visible at birth or even in later life. The disturbances of nervous system and brain may be expressed in terms of how an individual functions and behaves throughout life

with considerable variation from birth through adulthood. Functional deficiencies range from very mild to very severe to totally debilitating, consequently, it is difficult to quantify neuro-developmental impairment.

CPF spread through air drift or surface runoff into surrounding waters and gets accumulated in different aquatic organisms, particularly fish, and adversely affecting them via several sources (Varo et al., 2002). Fish are the non-target organism of pesticidal exposure as they play an important in food chain. Recent evidences indicate that fish, an extremely valuable resource, are quickly becoming scares. One consequence of this scarcity is the increasing concern for fish survival and growing interest in identifying the level of various chemical pollutants. Earlier investigation reported that the incident of fish killing was highest in chlorpyrifos toxicity (AbdelHalim et al., 2006). Fish are exposed to aquatic toxicants via the extensive and delicate respiratory surface of the gills and inhaling sea water (Wendelaar, 1997). Hence, for the study of toxicological impacts, fish serves as an excellent bio assay animal and have been widely used for this purpose (Ruggieri, 1975; Swarup et al., 1977; Blaxter and Hallers, 1992; Espelid et al., 1996; Wendellar, 1997; Hollis et al., 1999; Pratap, 1999; Kumar et al., 2007). There are number of studies reporting the pathological changes in fish exposed to different organochlorine, organophosphorous, carbamate and pyrethroid pesticides. The extent of severity of tissue damage of a particular compound as toxicant depends on its toxic potentiality in the tissue of organism (Murty, 1986; Veer, 2005). Several study reported the acute toxicity of CPF in response to mortality, LC_{50} and behavioral study in several fish (Barron and Woodburn, 1995; Kavithaa and Venkateswara, 2008; Oruc, 2010; Sharbidre et al., 2011; Xing et al., 2011). CPF caused developmental dysfunctions (Levin et al., 2003; Levin et al., 2004; Richendrfer et al., 2012) and also produce neurological effects (Braquenier et al., 2010; Eddins et al., 2010; Middlemore-Risher et al., 2010; Sledge et al., 2011). CPF is popular for its AChE inhibitory nature in fish (Straus and Chambers, 1995; Taylor and Brown, 1999; Kavithaa and Venkateswara, 2008; Leticia and Derardo, 2008). AChE activity in fish is essential for normal behavior and muscular function (Kirby et al., 2000). It also interferes with hormone synthesis (Hontela et al., 1997; Oruc, 2010).

3. Animal model

For the investigation, the freshwater catfish,*Heteropneustes fossilis* (Bloch, 1794) was selected. The freshwater catfish is one of the important groups of fish in our country and is getting increasingly popular showing a promising future for commercial culture (Barua, 1989). *Heteropneustes fossilis* (Bloch, 1794) locally known as "Shinghi" is an important air breathing catfish, due to presence of accessory respiratory organ which extends through the dorsal muscle of the body on either side of the vertebral column. This air-breathing adaptation enables it to survive in water of low oxygen content and also out of water on wet ground. This species found in ponds, pools, swamps and rivers of India, Sri Lanka, Burma, Laos, Thailand and Vietnam (Day, 1989; Dutta-Munshi and Hughes, 1992). This catfish is very hardy and can be cultured in swamps and derelict ponds of our country where carp culture is not possible. This species is ideal for wastewater aquaculture.

3.1. Systematic position

Group: Pisces
Phylum: Chordata
Subphylum: Vertebrata
Superclass: Gnathostomata
Class: Actinopterygii
Division: Teleostei
Superorder: Ostariophysi
Order: Siluriformes
Family: Heteropneustidae
Genus: *Heteropneustes*
Species: *fossilis* (Bloch, 1794)

H. fossilis is good in taste, highly nutritious and medicinal quality, and rich in protein and minerals (Islam, 1989). The chemical composition of the fish is 72% water, 19% protein, 8% fat, 0.15% calcium, 0.25% phosphorus and 0.10% vitamin A, B, C and D (Sahidullah, 1964). The muscles of the fish have been reported to have very high content of iron (226mg/l0gm) and fairly high content of calcium (Saha and Guha, 1939).

3.2. Fish Synonyms

As listed by IUCN (The International Union for Conservation of Nature), there are many synonyms reported for freshwater catfish, *Heteropneustes fossilis*:

- *Clarisilurus kemratensis* (non Fowler, 1937)
- *Heteropneustes microps* (non Gunther, 1864)
- *Heteropneustes microps* (Gunther, 1864)
- *Saccobranchus fossilis* (Bloch, 1794)
- *Saccobranchus microcephalus* (Gunther, 1864)
- *Saccobranchus singio* (Hamilton, 1822)
- *Silurus biserratus* (Swainson, 1839)
- *Silurus fossilis* (Bloch, 1794)
- *Silurus singio* (Hamilton, 1822)

3.3.

4. Objectives

On the basis of previously investigated information about chlorpyrifos toxicity, the present work was focused on the chlorpyrifos toxicity on reproductive potential of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). The main objectives of the presented work were as followed:

1) Effect of organophosphorous pesticide, chlorpyrifos on mortality, behavior, morphology and ovarian histology of freshwater catfish,*Heteropneustes fossilis* (Bloch, 1794)
2) Effect of organophosphorous pesticide, chlorpyrifos on oocyte maturation and follicular steroids in freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)
3) Effect of organophosphorous pesticide, chlorpyrifos on embryogenesis offreshwater catfish,*Heteropneustes fossilis* (Bloch, 1794).

The thesis is divided into 3 chapters, besides a general introduction and consolidated summary. The chapters were written in research manuscript form and referencesof all chapters were compiled at the end of the thesis to avoid repetition.

CHAPTER – I

Effect of organophosphorous pesticide, chlorpyrifos on mortality, behavior, morphology and ovarian histology of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)

ABSTRACT

The present investigation was undertaken to explore the toxic effect of organophosphorous pesticide, chlorpyrifos (CPF) to assess the mortality, behavior, morphology and ovarian histology of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). This study was conducted by exposing the fish to different concentrations of CPF (0.002-2.28 mM/l) for 96 hr experimental duration along with control group of fish in different reproductive phases (resting, preparatory and pre-spawning phase). **The finney's probit analysis (1971) was used to determine median lethal concentration (LC_{50}). The safe concentration of the studied pesticide was calculated as per Hart et al. (1945) and Henderson et al. (1959) in all respective seasons. The obtained LC_{50} were found to 1.547, 0.678, 0.299 and 0.174 mM/l in resting phase, 0.332, 0.193, 0.152 and 0.123 mM/l in preparatory phase and 0.296, 0.107, 0.044 and 0.026 mM/l in pre-spawning phase for** 24, 48, 72 and 96 hr respectively. The safe concentrations were 0.039 mM**/l** in resting phase, 0.19 mM**/l** in preparatory phase and 0.004 mM**/l** in pre-spawning phase. Susceptibility of catfish, *H. fossilis* to CPF was found to be duration and concentration dependent. Their tolerance to CPF was increasing with inactiveness of gonadal activity (resting season). At higher concentration of CPF, fish showed alterations in morphological and behavioral responses, especially erratic and jerky swimming, frequent surfacing and ingulping, mucus secretion, an increase in opercular movement and copious secretion of mucus all over the body. The histological assessment resulted

that CPF caused reduction in size of oocytes, vacuolation in cytoplasm, damaged ovarian follicles, increase in inter-follicular space, breaking of ovarian wall, occurrence of necrosis, damaged structure of ovarian follicles, formation of fragmented ova, atretic oocytes and nucleolar extrusion.It is concluded that CPF is highly toxic to catfish and severely affect their physiology and behavior.

Keywords: Chlorpyrifos, LC_{50}, mortality, *Heteropneustes fossilis*, ovarian histology.

1. Introduction

In the midst of most recent one decade, a colossal progress has been made in the improvement of new compounds with better toxicity, in this way, a great deal of work has been done on impact of pesticides on non-target aquatic organisms (Battaglin and Fairchild, 2002**;** Prasanth et al., 2005). A vast scale mortality of fish in various water bodies has been recorded and the reason behind was the huge application of pesticides and insecticides in agricultural practices which further contaminate the aquatic environment (Stephen, 2004).

Aquatic contamination of pesticides cause acute and chronic poisoning in fish and other organisms directly or indirectly via food chain (Heger et al., 1995; Omitoyin et al., 2006; Velmurugan et al., 2007). The reactions of fish towards the lethal chemicals are wide gone relying upon the toxicant type, exposure duration, water quality and the species (Fisher, 1991; Richmonds and Dutta, 1992; Venkateswara, 2004).

Toxicity tests are a vital segment in creating awareness and evaluating the potential effect of chemicals on aquatic communities since they illustrate the toxic impacts of chemicals in living beings by controlling their morphology, behavior and survival rate (Adedeji et al., 2008; Onyedineke et al., 2010). It may be interpret as an estimation of the concentration of biologically active substances by the level of their impact on living beings (Chapman and Long, 1983). Acute toxicity of different pesticides is influenced by the age, sex, genetic properties, body size of fish, water quality and its physico-chemical parameters, and purity or formulation of insecticides. The frequency or intensity of injury or abnormalities to fish in pesticide toxicity may be more or even less depends on exposure duration.

In addition to mortality test, study of behavioral markers to assess the toxicant affects are the most promising and sensitive indicator of ecotoxicology (Drummond and Russom, 1990; Scherrer, 1992; Cohn and MacPhail, 1996). The behavioral study is becoming prominent in toxicity assessment in an unicellular organism (Tadehl and Hader, 2001), insects (Martin, 2003; Venkateswara et al., 2004), fish (Hansen et al., 1999; Rao et al., 2005) and rodents (Dell'Omo et al., 1997). It is a unique perspective of an organism that produced a link between the physiology and environmental ecology (Little and Brewer, 2001). It serves as significant tool in toxicology to perceive and assess impacts of natural stressors that cause variations in the responses and range of sensitiveness towards contaminants. However, these variations are recognized as an adaptive mechanism which permits fish to cope with genuine or perceived stressors so that the typical homeostatic state could be kept up maintained (Barton, 2002).

Organophosphate compounds (OPs) are powerful neurotoxins, since they inhibit AChE activity (Coppage et al., 1975; Klaverkamp and Hobden, 1980) results in overwhelming of post-synaptic acetylcholine receptors and hyper-stimulation, which leads to physiologic aberrations ranging from behavioural impairment to death (Garcia et al., 2002; Howard et al., 2005; Eaton et al., 2008). Several factors seem to be involved in affecting the AChE activity caused by OPs such as duration and concentration of exposed compounds (Adedeji, 2011). CPF directly inhibits acetyl cholinesterase enzyme activity in fish and invertebrates (Fulton and key 2001; Rao et al., 2005) which may lead to decreased mobility of fish (Bretaud et al., 2000). Therefore, it primarily works as nerve poison which is reflected in uncoordinated abnormal fish behavior after the toxicant application (Scott and Sloman, 2004; Krian and Jha, 2009; David et al., 2010; Ilavazhahan et al., 2010; Nwani et al., 2010; Nagaraju et al., 2011; Prashanth et al., 2011; Satyavardhan, 2013; Rani and Kumaraguru, 2014; Ullah et al., 2014).

The main clinical internal sing of fish includes neurological disorder, disruption of nerve functions, respiratory dysfunction and suffocation (Banaee et al., 2011) that lead to death of fish. It has been found that different fish species, even from the same family, show differences in the sensitivity to high concentrations of insecticides in water.

Fish are used as an indicator of environmental suitability. So that any change in fish behavior including change in swimming (Brewer et al., 1999) or respiratory pattern, indicated the water quality deterioration (Olla et al., 1983; Byrne and O'Halloran, 2001). Swimming pattern of fish is frequently assessed as a response of toxicity because altered locomotive activity can indicate pesticidal effect on nervous system. The different behavioral manifestations are reported by many researchers (Brewer et al., 1999; Ayuba and Ofojekwu, 2002; Pandey et al., 2011). The pesticides also caused changes in respiratory functions, frequency of opercular movement (Shivakumar and David, 2004; Lawrence and Temiotan, 2010), surfacing (Katja et al., 2005) and gulping of air (Shedd et al., 2001; Scott and Sloman, 2004).

The assessment of morphological deformities is another method to study the effects of contamination on fish (Thiyagarajah et al., 1996; Sun et al., 1998; Tricklebank, 2001; Lemos et al., 2005; Liao et al., 2005; Linde et al., 2008; Hart et al., 2008; Yap et al., 2009). Different studies reported that fish exposed to a wide range of pesticides exhibited abnormal behavioral and morphological alterations (Devi and Mishra 2013). There are different types of morphological alterations reported in fish of contaminated waters, including fin necrosis (Sindermann, 1979; Reash and Berra, 1989; Sun et al., 1998), skull deformation (Lindesjoo and Thulin, 1992), opercular deformity (Handwerker and Douglas, 1994; Sun et al., 1998) and fin splitting (Sun et al., 1998; Almeida, 2008). Various studies were reported on the toxicity of different organophosphate pesticides on fish (Dikshith and Raizada, 1981; Verma et al., 1982; Srivastava and Singh, 2001; Gul, 2005; Pandey et al., 2005).

Fish are very sensitive to a wide variety of toxicants in water (Herger et al., 1995). Due to accumulation of pesticides in tissues, it produces many physiological, histological and biochemical changes in the fish and freshwater fauna by influencing the activities of several enzymes and metabolites (Nagarathnamma and Ramamurthi, 1982). The histopathology proves to be a sensitive and effective biomarker to study lesions or abnormalities on a cellular level and hence reflect the health of the entire aquatic ecosystem (Mumford, 2004; Camargo and Martinez, 2007; Datta et al., 2007; Monsefi et al., 2010; Nikalge et al., 2012). It constitutes a useful tool for assessing the degree of pollution, particularly for sublethal and chronic effects (Bernet et al., 1999; Pieterse,

2004). It is a mechanism which includes study of diseased or injured cell after death of the aquatic animal may serves to identify the cause of death of an aquatic animal (Pieterse, 2004).

The earlier histopathological observations of fish exposed to pollutants revealed that fish organs are an efficient indicators of water quality (Cardoso et al., 1996; Cengiz et al., 2001). Therefore, it is necessary to study the histopathological alterations in different organs of fish to assess the extent of damage (Palmes, 1976; Singh and Sahai, 1985; Rastogi and Kulshrestha, 1990; Mumford, 2004; Pieterse, 2004; Deshmukh and Kulkarni, 2005; Camargo and Martinez, 2007; Verma and Srivastava, 2008; Pugazhvendan et al., 2009; Monsefi et al., 2010). Many Literatures have been reported on the impact of pesticides on various fish tissues but studies of reproductive organ in fish are not as many. The pesticide cause several damages to the reproductive organ as observed earlier by Boyd (1964), Burdick et al. (1972), Carlson (1972), Freeman and Idler (1975), Saxena and Garg (1978), Singh and Singh (1980), and Dey and Bhattacharya (1989).

The selection of organism for toxicity test is mainly based on certain criteria like its ecological status, position within the food chain, suitability for laboratory studies, genetically stable, uniform population and adequate background data of the organism (Buikema at al., 1982). The species selected for current study viz. freshwater catfish, *Heteropneustes fossilis* satisfied most of the above criteria. The objective of the present study was to examine acute toxicity level of chlorpyrifos with special emphasis on behavioral, morphological responses andhistopathological changes in the ovary of a freshwater catfish, *Heteropneustes fossilis* in resting, preparatory and pre-spawning phases of annual reproductive cycle.

2. Materials and Methods

2.1. Chemicals and Instruments

An organophosphorous pesticide, chlorpyrifos (CPF) was procured from the local market of Lucknow, Uttar Pradesh, India, under the trade name Hilban® (20% EC CPF), supplied by Hindustan insecticide limited. All other chemicals used for this study was of

analytical grade of HiMedia Laboratory Pvt. Ltd., India company. The instruments were used in current analysis was rotary microtome (Weswox) and brightfield microscope Olympus equipped with CX41 camera.

2.2. Test Animal and their acclimatization

An experiment was performed in accordance with local/ national guidelines of ethical committee for experimentation in animals.

The live freshwater catfish *H. fossilis* of relatively same size and weight were collected from commercial fisherman of Lucknow, Uttar Pradesh, India in resting (December), preparatory (February), and pre-spawning (April) phase of annual reproductive cycle. Fish were brought to the laboratory in wide mouthed large plastic containers in natural water avoiding stresses and injuries as possible. Before introducing in the aquarium, fish were treated with 0.05% $KMnO_4$ solutions to remove any dermal infection (Figure 1). Later, they were acclimatized in 120 L glass aquaria containing water having constant physico-chemical characteristicas standardized by APHA (1998) techniques, for two weeks. Water was renewed daily to remove fecal matter and waste metabolite of fish during acclimatization. During this period, fish were fed regularly with commercial fish food pellets and goat liver. Diseased and dead individuals were removed immediately if any.

2.3. Experimental design

2.3.1. Seasonal median lethal concentration bioassay

The toxicity test was performed under natural photoperiod for 96 hr to determine LC_{50} values of CPF in resting, preparatory and pre-spawning phase of reproduction. For this bioassay, normal and healthy fish of uniform weight and length were selected. Fish were exposed in the batch of ten to varying concentrations (0.002, 0.005, 0.028, 0.057, 0.085, 0.114, 0.142, 0.171, 0.199, 0.228, 0.285, 0.313, 0.342, 0.37, 0.399, 0.456, 0.513, 0.57, 0.627, 0.684, 0.741, 0.798, 0.855, 1.14, 1.425, 1.71, 1.99 and 2.28 mM/l) of chlorpyrifos (CPF). The control group was also maintained concurrently in all the seasons. Acute toxicity test were carried out for period of 96 hr. Mortality data was

recorded after 24, 48, 72 and 96 hr to get LC_{50} of respective intervals. The concentrations of the test compound used were selected on the basis of our trial experiments that caused 0-100% mortality in respective reproductive phase. The experiment was set in five replicates having ten fish each in all reproductive phases. All solutions (control and test) were renewed daily and dead fish were removed immediately. Fish were not given any food during the experiment.

2.3.2. Estimation of safe concentration

The safe concentration of any compound was presumed harmless. It was calculated as per Hart et al. (1945) and Henderson et al. (1959). This calculation was based on the 24 and 48 hr LC_{50} values. The safe concentration was obtained from the following formula:

$$\mathbf{C = 48\ hours\ LC_{50} \times Application\ factor\ (A) \times S^2}$$

Where: C = Safe concentration

A = application factor i.e. 0.3 according to Henderson et al (1959)

S= 48 h LC_{50} ÷ 24 h LC_{50}

2.3.3. Observation of behavioral and morphological responses

The behavioral and morphological alterations of healthy and exposed fish to various concentrations of CPF were recorded up to 24 hr. These alterations were consistently monitored and recorded as Gupta and Dua (2010), Nimila and Nandan (2010), AL-Aker and Shamsi (2000). The frequency of occurrence of different behavior was counted for group of exposed fish.

2.3.4. Ovarian histology of freshwater catfish, Heteropneustes fossilis

2.3.4.1. Experimental design

To study the concentration effect of chlorpyrifos (CPF) on ovarian tissue of

freshwater catfish, *H. fossilis,* different concentrations of CPF were selected in different reproductive phase. In resting phase, seven different concentrations of CPF were selected that were 0.057, 0.085, 0.114, 0.171, 0.228, 0.285 and 0.57 mM/l. In preparatory phase, 0.028, 0.057, 0.085, 0.114, 0.142, 0.171 and 0.199 mM/l concentrations were selected. In pre-spawning phase, 0.002, 0.005, 0.028, 0.057, 0.085, 0.114 and 0.142 mM/l doses of CPf were selected. Those all concentrations were selected on the basis of their mortality response (Earlier experiment). A control group of fish was also maintained concurrently. Experiment was set in triplicate with ten numbers of fish each. The water was changed daily with the required amount of the CPF.

To study the chronic or duration effect of CPF, short term and long term experiments were designed. In short term assay, fish were exposed to $1/3^{rd}$ of LC_{50} of 96hr of CPF (0.058, 0.041 and 0.008 mM/l in resting, preparatory and pre-spawning phase) for 15 days exposure period. For long term exposure, $1/10^{th}$ of LC_{50} of 96 hr of CPF (0.017, 0.012 and 0.002 mM/l in resting, preparatory and pre-spawning phase) for 30 day exposure duration was selected. The LC_{50} of CPF for 96 hr were found 0.174 mM/l in resting period, 0.123 mM/l in preparatory period and 0.026 mM/l in pre-spawning period.The control set of fish was running side by side with experimental group. In each set of experiment ten fish were taken. During both of the period, dead fish were removed immediately.

At the end of concentration (acute) and duration (chronic) experiment, the desired tissue (ovary) was dissected out by using stainless steel dissecting kit to avoid metal contamination for histo-pathological process in different reproductive phases.

2.3.4.2. Process of histological assessment

Tissue Fixation and trimming

The ovary tissues were dissected out, washed in normal saline and fixed in Bouin's fixative. After 5 hr of fixation, the tissues were trimmed as in square pieces of about 3-5 mm. Then further for 19 hr, the tissues were fixed in same fixative. After 24 hr of completion, tissues were washed with tap water, distilled water, 30% alcohol, 50% alcohol and 70% alcohol.

Block preparation

The block preparation includes two processes: dehydration and embedding. The tissues were dehydrated in ascending order of different concentrations of alcohol i.e., 90% alcohol and 100% alcohol for 15 min. with two changes in each grade of alcohol. After dehydration, tissues were transferred in xylene+abs. alcohol (1:1) and xylene for 15 min. each. The embedding of tissues was performed in hot air oven at 60^0C temperature. In this process, the tisues were put in xylene+filtered wax (1:1) for 15 min. with two changes, and further processed in filtered wax_1, wax_2 and wax_3 (100%) for 30 min with two changes each. At the end of embedding, the tissues were taken out and placed in a metal L shaped angle kept ready by filling with wax and the air bubble arising were removed by using hot spatula.

Sectioning of block

The excess of wax was trimmed with the help of spatula and knife till the material is slightly visible and then it was fixed on the wooden block holder for sectioning. Before sectioning, the Mayer's egg albumin was spread on the slides and leaves it up to dry. Sectioning of the tissue was performed by rotary microtome (Weswox). The thickness of the sections was 7 µm and the sections (ribbon) were spread on the slides having albumin by putting on hot plate having approximately 30-35^0C temperature. All sections were allowed to cool at 37^0C overnight when they spread properly.

Slide preparation

The procedures of slide preparation include three steps viz., staining, dehydration and mounting. For tissue anatomical analysis, standard double staining (haematoxylin and eosin) technique was used. The procedure was shown in Figure 2.

2.4. Data analysis

The fish mortality was presented in percentage. It was defined as number of dead fish divided by total number of exposed fish in aquarium multiplied by hundred. It was recorded every 24 hr up to 96 hr study period. The data were analyzed statistically by

one-way analysis of variance (ANOVA) among different annual reproductive phases. The LC_{50} with 95% confidence limits of 24 hr, 48 hr, 72 hr and 96 hr was estimated by probit analysis (Finney, 1971) with the statistic software (IBM SPSS version 20). For tabulation of behavioral and morphological alterations, the frequency of occurrence was considered as behavior/min. and morphological alterations were observed in each experimenta set. These responses were recorded in different phase of annual reproductive cycle. The histological sections of ovarian tissues were examined at 10X magnification under Bright field microscope (Olympus CX41) using mcropublisher 3.3 RTV camera.

Figure 1

A: Acclimatization of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in laboratory condition.

B: Experimental control freshwater catfish *Heteropneustes fossilis* (Bloch, 1794).

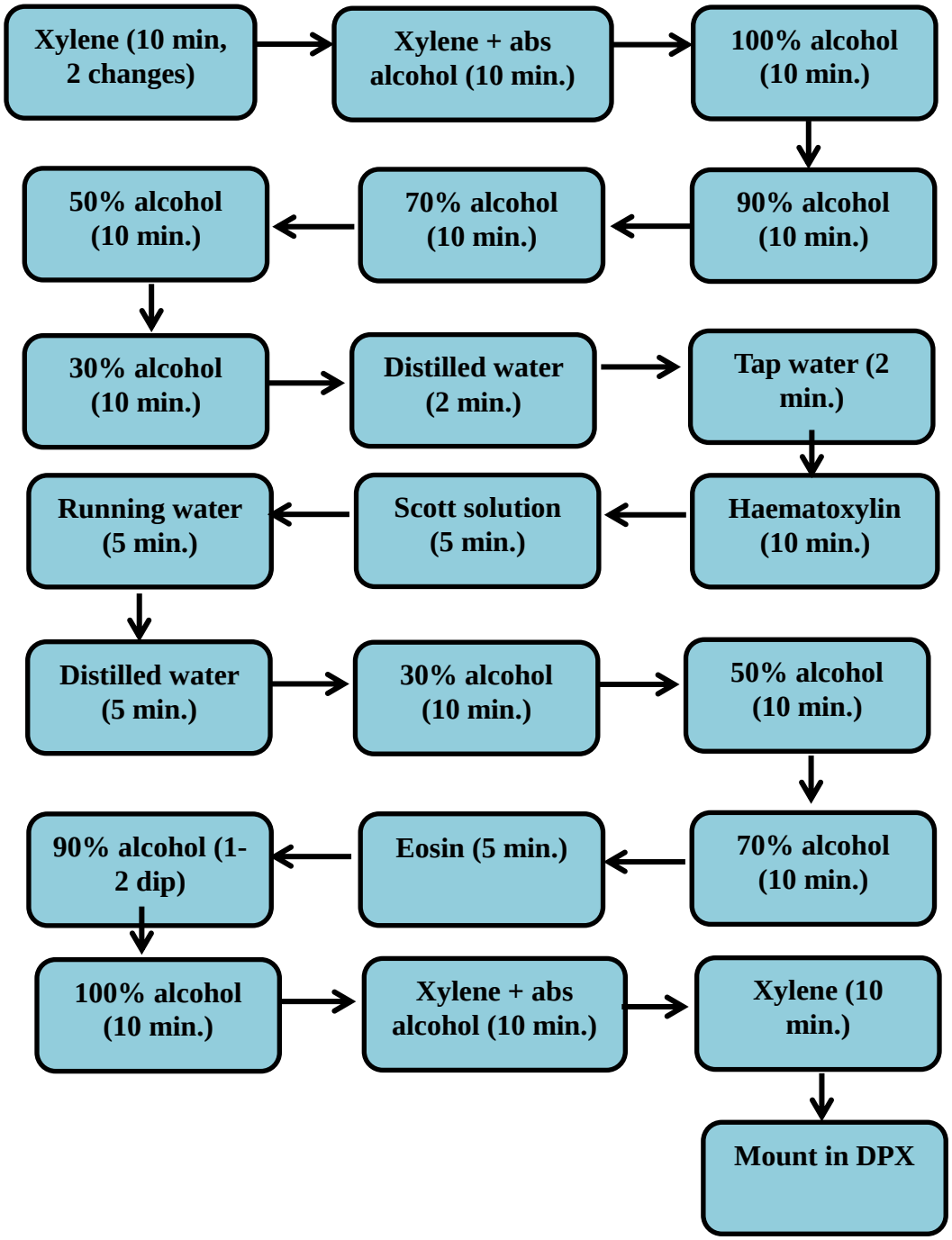


Figure 2: Detailed procedure of staining and slide preparation with its estimated duration

3. Result

3.1. Lethal toxicity test (LC_{50})

The result showed that CPF was highly toxic to fish. Exposure to freshwater catfish, *H. fossilis* with different concentrations of CPF showed varied degree of mortality in different reproductive season with a wide range of concentrations. The percentage of mortality was significantly increased with the increase in concentration of the toxicant as well as duration of the experiment (F=52.27 in pre-spawning phase, F=134.24 in the preparatory phase, F=211.35 in resting phase; $p< 0.05$; Figure 3-4). In lower concentration the adult (0.002 mM/l) showed 0% mortality while in highest concentration (0.142 mM/l), 100 % mortality was recorded in 96 hr duration in pre-spawning phase (Figure 4B). However, in the preparatory phase, 0% mortality was occurred at 0.005 mM/l and 100% mortality at 0.199 mM/l in 96 hr exposure (Figure 3B). In the resting phase, 0% mortality was found at 0.057 mM/l and at 0.57mM/l, 100% mortality was recorded in 96 hr toxicity duration (Figure 3A). On the basis of mortality, LC_{50} value was found higher in resting phase (1.547, 0.678, 0.299 and 0.174 mM/l), moderate in preparatory phase (0.332, 0.193, 0.152 and 0.123 mM/l), whereas minimum in pre-spawning phase (0.296, 0.107, 0.044 and 0.026 mM/l) for 24, 48, 72, and 96 hr respectively (Table 1). The observation of the safe concentration value study represented that in resting phase, the safe concentration was higher as compared to preparatory and pre-spawning phase of reproductive cycle (Table 2).

3.2. Behavioral alterations

The behavior responses (Table 3) are the most sensitive indicator of potential toxic effects. CPF influences the behavioral pattern of fish. The observation of behavioral changes was started within 30 min after treatment with CPF. The response time was decreased as the toxicant concentration was increased. The behavioral alterations were positively correlated with the concentration of toxicant in different seasons of reproduction (Table 4-6). These alterations were very clear in all the reproductive phases. But the occurrence of alterations in treatment groups were higher even in low concentrations during pre-spawning phase than preparatory and resting phase of reproductive cycle as compared to their control groups may be due to maturity aspects.

The control group showed a normal behavior and tends to move together. They came to the surface at intervals to gulp air. In low concentrations of CPF in all reproductive phase viz., in resting phase (0.057 and 0.085 mM/l), in preparatory phase (0.005, 0.028 mM/l) and in pre-spawning phase (0.002, 0.005 mM/l), fish showed similar behavior as of control group. The exposed fish showed a number of abnormalities in their swimming pattern. The swimming speed of fish was frequent and become erratic, and fish appeared hyper excitablewith jerky movements in higher concentration and this behavior was shown with immediate exposure of toxicant that slow down with the passage of treatment duration. The schooling behavior was also disturbed in comparison to control, followed by hyperactivity, pectoral fin forward, convulsions, ingulping, avoidance behavior, escaping tendency and loss of buoyancy by vertically hanging in the test aquaria after treatment and moreover progressively became sluggish, lethargic and finally lead to death as compared to control group.

Convulsion prior to death were most evident, the severity of which paralleled with the concentration of CPF. Fins drooping were also observed inexposed fish. On initial exposure at higher concentration, the fish exhibited characteristic avoidance behavior by rapid swimming, stretching half of their body out of the water surface and trying to jump out. The opercular movements or beats are major indicators of stress. The opercular beats increased to 130/min in CPF exposed fish group from 85-90/min obtained in the control group. When the oxygen was reduced in toxic medium of different concentration fish were showed surfacing and air ingulping.

3.3. Morphological alterations

The exposed fish to CPF showed various abnormal morphological features which include pale yellow body colour, discoloration of skin, lesion of skin, eye deformities (Pop eye and Anapthalmia), fin deformities and high mucus secretion and its coagulation all over the body as compared to control (Figure 5-6). Barbels were also losing their colour. These deformities were positively correlated with CPF concentrations and exposure duration among all phase of reproduction (Table 7-9).

3.4. Histological analysis

The histological observation of ovary of freshwater catfish, *H. fossilis* presented clear cut evidence of pesticidal toxicity. The changes were severe in all treated groups of chlorpyrifos, and both short term and long term study also when compared with control fish tissue in all reproductive phases' viz., resting, preparatory and pre-spawning phase.

In this study a variety of histo-pathological changes were observed in the ovary of *H. fossilis* after CPF treatment. In the control group of animal, the ovaries were surrounded by germinal cuboidal epithelium. There was a connective tissue called tunica albugina which in turn surrounded by ovarian stoma at which female cell was embedded. The ovary of control fish showed large number of mature and maturing oocytes in pre-spawning phase. The immature oocytes were transparent with nucleus and cytoplasm. Mature oocytes are round opaque and contain large and small yolk globules. The vitelline membrane and follicular layer become more prominent.

3.4.1. Ovarian histology of control catfish (H. fossilis)

The histological examination of control catfish, *H. fossilis* ovary of resting phase showed an oogonia or primary oocytes and chromatin nucleolus oocytes (Figure 7). The entire oocyte was covered by a layer of follicular cells. They were present along the ovigerous lamellae and characterized by a homogeneous basophilic cytoplasm with a large nucleus. The chromatin nucleolus oocytes were originated from oogonia. They had little cytoplasm large and stained nucleus and central nuclei.

The preparatory phase of reproduction was clearly characterized by the presence of perinucleolar and cortical alveoli or yolk vesicle oocytes- an indication of vitellogenesis in preparatory phase (Figure 9). These oocytes were larger oocyte in relation to earlier oocytes. The number of nucleoli had increased, and the nuclear membrane acquired an irregular outline. The cytoplasm now appeared dense and granular, and there was a slight reduction in its basophilia. These oocytes were covered by prominent follicular layer. The yolk vesicle oocytes were characterized by the presence of yolk in the cytoplasm. Initially these vesicles were deposited along the periphery in the form of minute granules which were interspersed with the vacuoles. As the diameter of the oocyte increased, these granules coalesced to form platelets which

impart the characteristic greenish-brown colour to the ovary. The fully formed primary oocyte was invested with an outer-most layer of thin and elongated thecal cells which abuts against an inner layer of granulosa cells. The yolk was separated from the granulosa layer by a thin vitelline membrane or chorion. The nucleus was smaller and compare to the earlier stages and chromatin threads still occurred in the nucleus, and the nuclear membrane began to degenerate. The zona radiate layer was very evident and follicle epithilium was more developed.

During pre-spawning phase, the ovaries were enlarged and various cytological changes were observed in the oocytes indicating rapid growth and maturation (Figure 11). This phase was observed with the presence of yolk granule and primary vitellogenic oocytes. The growth during this phase was mainly due to formation of yolk vesicles and deposition of yolk. The nuclear membrane was completely degenerated. The follicular layer and zona radiate were now well developed and distinct. In *Heteropneustes fossilis* during this phase, oocytes proliferated and all types of oocytes were visible except the matured ones.

3.4.2. Ovarian histology of CPF exposed H. fossilis: Concentration study

The experimental fish exposed to different concentrations of chlorpyrifos (CPF) and two sub-lethal concentrations of CPF, showed marked histological changes in the ovary. In acute exposure with different concentrations of CPF, ovary showed significant changes in all reproductive phases viz., resting, preparatory and pre-spawning phase. The changes observed in ovarian section of *H. fossilis* were dose-dependent. The histological changes were partial disruption of ovarian follicles, cytoplasmic vacuolization of germinal cells and reduction in number of maturing ovum and oocytes (Figure 7-12). The inter-follicular connective tissues were damaged. The cytomorphological structure of ovarian follicles got deformed and elongated, losing their typical configuration. Necrosis and fibrosis in connective tissue and damage to yolk vesicles of maturing oocytes were seen. Degenerative oocytes became phagocytic and formed atretic oocytes. The greater degree of histopathological changes was marked in the ovary exposed to higher concentration of CPF in all reproductive seasons. During resting phase, the cytoplasmic agglutination was noticed in increasing concentration of chlorpyrifos from 0.085 mM/l to

0.57 mM/l (Figure 7). The oocyte showed vacuole formation and disintegration of nucleus and more number of immature oocytes was noticed in higher concentration of CPF (Figure 7). At0.57 mM/l,ovary showed the degenerative oocyte and the presence of vacuolization in cytoplasm. During preparatory phase, the formation of inter follicular spaces was noticed in exposed fish to different concentrations of CPF (Figure 9). Large number of oogonia and perinucleolar oocytes were present with proliferation of ovigerous lamella and thecal and granulosa layer. Oocyte nucleous exhibited degenerative changes (liquefaction of the perinuclear cytoplasm) followed by the condensation of the nucleous. After dissolution of the nucleoli, the oocytes become atretic. There was disruption in follicular wall by CPF exposure during pre-spawning phase (Figure 11). These all manifestations were occurred according to the potentiality of toxicant in the environment. At higher concentration of CPF, inter-follicular spaces were larger and vacuolation in developing oocytes were also observed. In certain oocytes inversion of granulosa layer was also observed (Figure 9). The above result showed the retardation of oocytes, proliferation and increase in the number of atretic follicles, fibrosis and necrosis or degeneration in the ovary was more in higher concentration exposure. At lower concentration of CPF (0.057, 0.028 and 0.002 mM/lin resting, preparatory and pre-spawning phase), ovary showed no more histological changes as compared to higher concentrationsof CPF.

3.4.3. Duration study of chlorpyrifos

In short term study of CPF, fish were exposed to 0.058 mM/l, 0.041 mM/l and 0.008 mM/l concentrations of CPF ($1/3^{rd}$ of LC_{50} of 96 hr) in resting, preparatory and pre-spawning phase of reproduction for 15 days. CPF caused histological changes in ovary viz., primary follicles began to show adhesion and as well as cytoplasmic retraction in oocyte was also occurred. There was partial disruption of ovarian follicles and vacuolation in cytoplasm of germinal cells. The ovarian follicles were losing their typical configuration. The inter-follicular connective tissue was damaged. Number of atretic oocytes increased. Cytoplasmic retraction, degeneration and clumping were more visible in oocyte. Partial destruction of the ovigerous lamellae and vitellogenic membrane was noticed. Necrosis and fibrosis in connective tissue and damage to yolk vesicles of maturing oocytes was observed (Figure 8, 10, 12). In pre-spwaning phase, in maturing

oocytes, the granulosa layer got separated and complete or partial rupture was observed. Overall deformed ovarian tissue was observed in the section of the ovary of *Heteropneustes fossilis*.

In the long term study, the exposure duration was 30 days. The tested concentrations of chlorpyrifos (CPF) in different phase of reproduction were 0.017 mM/l, in resting phase, 0.012 mM/l in preparatory phase and 0.002 mM/l in pre-spawning phase. These all concentrations were $1/10^{th}$ of LC_{50} of 96 hr of CPF in their respective phases. The noticed changes were severe damage of the ovigerous lamellae, increased intrafollicular spaces, vacuolated cytoplasm, extrusion of karyoplasm and necrosis in the cytoplasm. The ovarian wall became frayed and broken. Additionally, a marked increase of atretic follicles, shrinkage, and embedded nucleoli into the surrounding cytoplasm in oocyte were observed (Figure 8, 10, 12). This study revealed that oocytes at their different stages of maturation get affected differently. Based on observations of the ovarian tissue, it becomes evident that consistent $1/10^{th}$ of sub-lethal doses of CPF can bring a considerable change in the ovarian histological structure of the fish *Heteropneustes fossilis*.

Table 1: LC_{50} values with its 95% confidence limit (upper and lower) at various exposure periods in different reproductive phases in freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) exposed with organophosphorous pesticide, chlorpyrifos through Finney Probit analysis.

Reproductive phase	**Test duration (hr)**	**LC_{50} (mM/l)**	**95% confidence limits**	
			Upper	**Lower**
Resting phase	24	1.547	2.564	1.103
	48	0.678	0.969	0.514
	72	0.299	0.347	0.261
	96	0.174	0.191	0.159
Preparatory phase	24	0.332	0.41	0.283
	48	0.193	0.21	0.179
	72	0.152	0.166	0.14
	96	0.123	0.135	0.113
Pre-spawning phase	24	0.296	0.356	0.243
	48	0.107	0.143	0.074
	72	0.044	0.066	0.023
	96	0.026	0.051	0.01

Table 2: Safe concentrations of organophosphorous pesticide, chlorpyrifos (CPF) exposed to freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in different reproductive (resting, preparatory and pre-spawning) phase, calculated by Hart et al., (1945) and Henderson et al., (1959).

	Resting phase	**Preparatory phase**	**Pre-spawning phase**
Safe Concentration of CPF (mM/l)	0.039	0.019	0.004

Table 3: Operational descriptions of observed behavioral and morphological responses caused by organophosphorous pesticide, chlorpyrifos during current investigation

Behavioral and morphological responses	**Descriptions**
Opercular movement	Opening and closing of operculum
Ingulping	fish suddenly came to the water surface and in gulp air and just back to the bottom to settled down
Surfacing	fish swims below 20 mm from the water surface
Avoidance behavior	defense mechanism by which fish removes themself from unpleasant situations
Pectoral fin forward	pectoral fins are held perpendicular or in a more forward posture towards the head and no movement of pectoral fins
Convulsions	no swimming; continuous ataxia with intermittent body spasms, violent shaking
Loss of buoyancy	fish roll over on side or back by vertically hanging
Discoloration of skin	depigmentation of colour of skin
Fins drooping/ necrosis	damage and deterioration in fins
Pop eye	Protruding eyes, eyes become white or cloudy
Anapthalmia	One side of eye absent or lost

Table 4: Effect of different concentrations of organophosphorous pesticide, chlorpyrifos (CPF) on the behavioral pattern of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in resting phase of annual reproductive cycle. The represented data was of five independent experiments and number of fish in each experiment was ten.

Behavioral alterations	**Control**	**Concentration of CPF (mM/l)**								
		0.057	**0.085**	**0.114**	**0.142**	**0.171**	**0.199**	**0.228**	**0.285**	**0.57**
Opercular movement	-	-	-	+	+	++	++	+++	+++	+++
Ingulping	-	-	-	+	+	++	++	+++	+++	+++
Surfacing	-	-	-	+	+	++	+++	+++	+++	+++
Frequent swimming	-	-	-	-	++	++	++	+++	+++	+++
Avoidance behavior	-	-	-	-	+	++	++	+++	+++	+++
Hyperactivity	-	-	-	+	+	++	++	+++	+++	+++
Pectoral fin forward	-	-	-	+	+	++	++	+++	+++	+++
Convulsions	-	-	-	+	+	++	+++	+++	+++	+++
Abrupt swimming	-	-	-	+	+	++	+++	+++	+++	+++
Escaping tendency	-	-	-	+	++	++	++	+++	+++	+++

Loss of buoyancy	-	-	-	+	++	++	++	+++	+++	+++

Normal (-), Mild (+), Moderate (++), Maximum behavior (+++)

Table 5: Effect of different concentrations of organophosphorous pesticide, chlorpyrifos (CPF) on the behavioral pattern of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in preparatory phase of annual reproductive cycle. The represented data was of five independent experiments and number of fish in each experiment was ten.

Behavioral alterations	**Control**	**Concentrations of CPF (mM/l)**							
		0.005	**0.028**	**0.057**	**0.085**	**0.114**	**0.142**	**0.171**	**0.199**
Opercular movement	-	-	-	+	+	++	+++	+++	+++
Ingulping	-	-	-	+	+	++	+++	+++	+++
Surfacing	-	-	-	+	+	++	+++	+++	+++
Frequent swimming	-	-	-	-	+	++	+++	+++	+++
Avoidance behavior	-	-	-	+	+	++	+++	+++	+++
Hyperactivity	-	-	-	+	+	++	+++	+++	+++
Pectoral fin forward	-	-	-	+	+	++	+++	+++	+++
Convulsions	-	-	-	+	+	++	+++	+++	+++

Abrupt swimming	-	-	-	+	+	++	+++	+++	+++
Escaping tendency	-	-	-	+	++	++	+++	+++	+++
Loss of buoyancy	-	-	-	+	++	++	+++	+++	+++

Normal (-), Mild (+), Moderate (++), Maximum behavior (+++)

Table 6: Effect of different concentrations of organophosphorous pesticide, chlorpyrifos (CPF) on the behavioral pattern of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) in pre-spawning phaseof annual reproductive cycle. The represented data was of five independent experiments and number of fish in each experiment was ten.

Behavioral alterations	**Control**	**Concentrations of CPF (mM/l)**						
		0.002	**0.005**	**0.028**	**0.057**	**0.085**	**0.114**	**0.142**
Opercular movement	-	-	-	+	++	++	+++	+++
Ingulping	-	-	-	+	+	++	+++	+++
Surfacing	-	-	-	+	+	++	+++	+++
Frequent swimming	-	-	-	-	++	++	+++	+++
Avoidance behavior	-	-	-	+	+	++	+++	+++
Hyperactivity	-	-	-	+	+	++	+++	+++

Pectoral fin forward	-	-	-	+	+	++	+++	+++
Convulsions	-	-	-	+	+	++	+++	+++
Abrupt swimming	-	-	-	+	+	++	+++	+++
Escaping tendency	-	-	-	+	++	++	+++	+++
Loss of buoyancy	-	-	-	+	++	++	+++	+++

Normal (-), Mild (+), Moderate (++), Maximum behavior (+++)

Table 7: Effect of different concentrations of organophosphorous pesticide, chlorpyrifos (CPF) on the morphological alterations of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) at 96 hr of exposure in resting phase of annual reproductive cycle. The represented data was of five independent experiments and number of fish in each experiment was ten.

Morphological observations	**Control**	**Concentrations of CPF (mM/l)**								
		0.057	**0.085**	**0.114**	**0.142**	**0.171**	**0.199**	**0.228**	**0.285**	**0.57**
Mucus secretion	-	-	-	-	++	++	+++	+++	+++	+++
Discoloration of skin	-	-	-	-	++	++	+++	+++	+++	+++
Fins drooping/ necrosis	-	-	-	+	+	++	+++	+++	+++	+++
Lesions of skin	-	-	-	+	++	+++	+++	+++	+++	+++
Pop eyes	-	-	-	-	-	+	++	++	+++	+++
Anapthalmia	-	-	-	-	+	+	++	++	+++	+++

Normal (-), Mild (+), Moderate (++), Maximum behavior (+++)

Table 8: Effect of different concentrations of organophosphorous pesticide, chlorpyrifos (CPF) on the morphological alterations of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) at 96 hr of exposure in preparatory phase of annual reproductive cycle. The represented data was of five independent experiments and number of fish in each experiment was ten.

Morphological observations	**Control**	**Concentrations of CPF (mM/l)**							
		0.005	**0.028**	**0.057**	**0.085**	**0.114**	**0.142**	**0.171**	**0.199**
Mucus secretion	-	-	-	+	++	++	++	+++	+++
Discoloration of skin	-	-	-	+	++	++	++	+++	+++
Fins drooping/ necrosis	-	-	-	++	++	++	+++	+++	+++
Lesions of skin	-	-	-	+	++	++	+++	+++	+++
Pop eyes	-	-	-	-	+	+	++	+++	+++
Anapthalmia	-	-	-	-	+	+	++	++	+++

Normal (-), Mild (+), Moderate (++), Maximum behavior (+++)

Table 9: Effect of different concentrations of organophosphorous pesticide, chlorpyrifos (CPF) on the morphological alterations of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) at 96 hr of exposure in pre-spawning phase of annual reproductive cycle. The represented data was of five independent experiments and number of fish in each experiment was ten.

Morphological observations	**Control**	**Concentrations of CPF (mM/l)**						
		0.002	**0.005**	**0.028**	**0.057**	**0.085**	**0.114**	**0.142**
Mucus secretion	-	-	-	+	++	++	+++	+++
Discoloration of skin	-	-	-	+	++	++	+++	+++
Fins drooping/ necrosis	-	-	-	+	+	++	+++	+++
Lesions of skin	-	-	-	+	++	+++	+++	+++
Pop eyes	-	-	-	-	-	+	++	+++
Anapthalmia	-	-	-	-	+	+	++	++

Normal (-), Mild (+), Moderate (++), Maximum responses (+++)

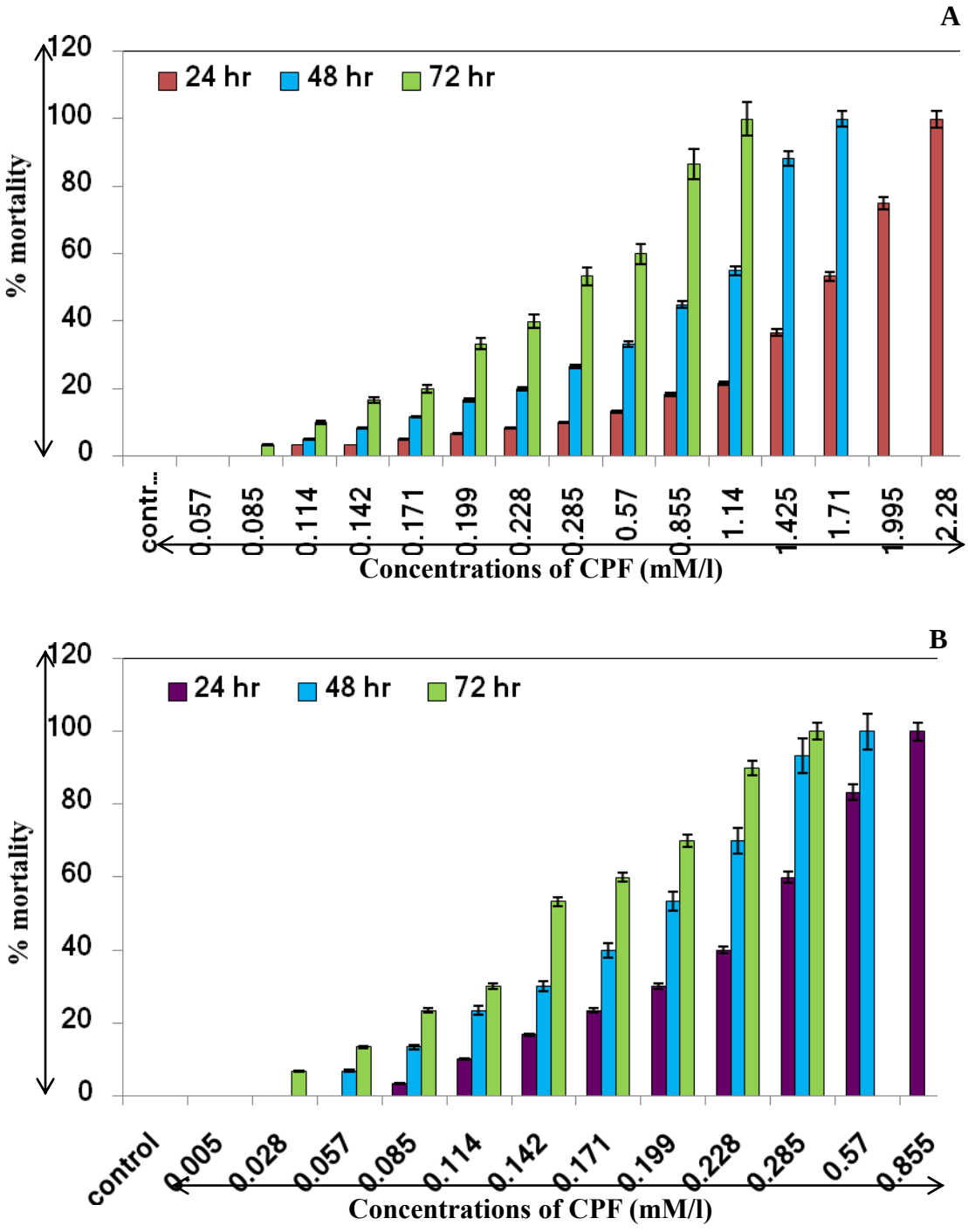


Figure 3: Effect of different concentrations of chlorpyrifos for 24 hr, 48 hr and 72 hr on % mortality of freshwater catfish, *Heteropneustes fossilis* in different phase of reproduction viz., A: *Resting phase* and B: *Preparatory phase*. Values were represented as means±SEM of percentage of mortality. Data were analyzed by one way ANOVA ($p < 0.001$).

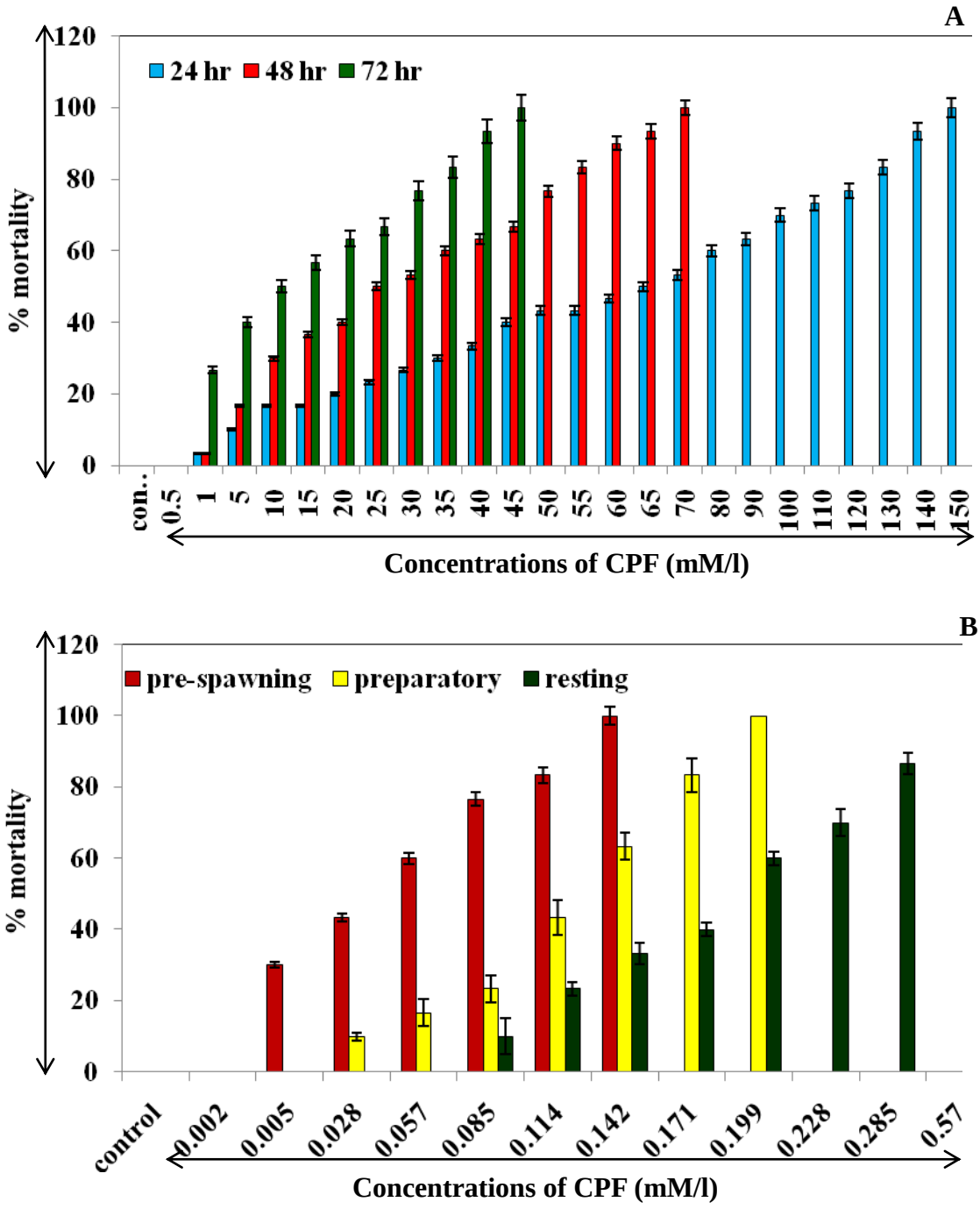


Figure 4:(A) Effect of different concentrations of chlorpyrifos (CPF) for 24 hr, 48 hr and 72 hr on % mortality of freshwater catfish, *Heteropneustes fossilis* in *pre-spawning phase* of reproduction. (B) Effect of different concentrations of CPF for 96 hr on % mortality of freshwater catfish, *Heteropneustes fossilis* in *pre-spawning, preparatory* and *resting phase.* Values were represented as means±SEM. Data were analyzed by one way ANOVA ($p < 0.001$), followed by Newman-Keuls test ($p < 0.05$) for comparison within the groups.

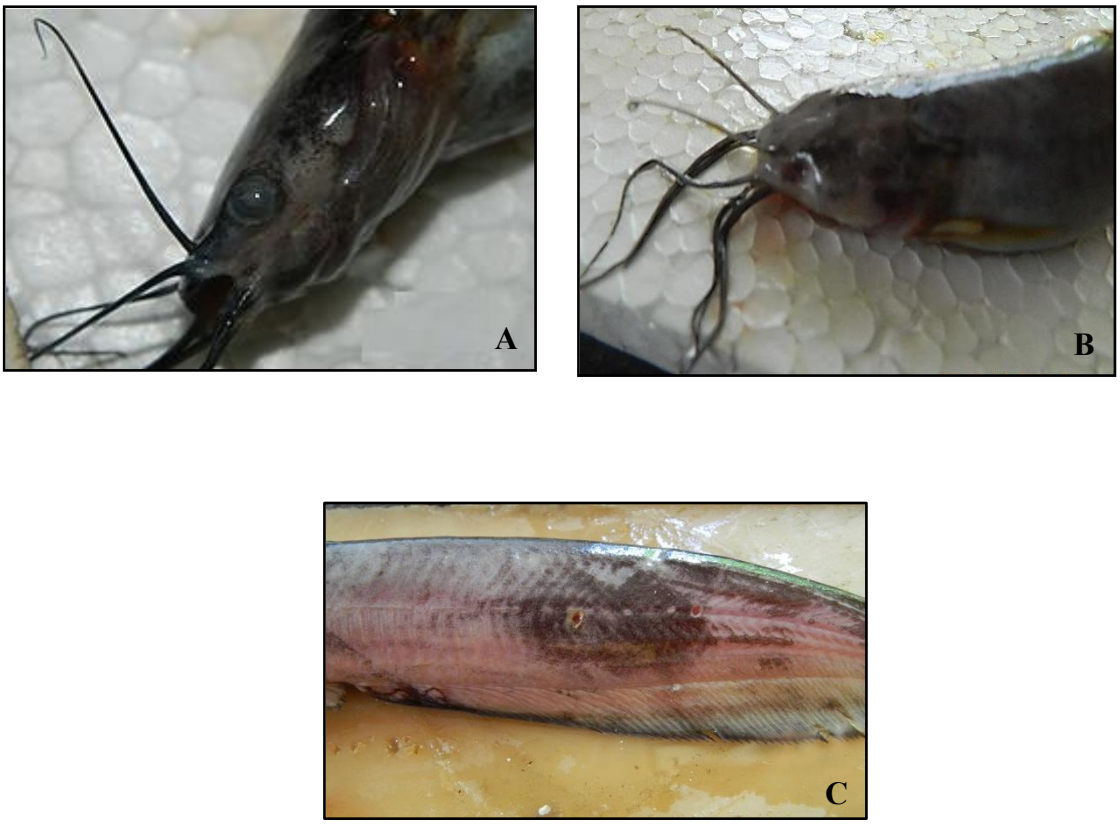


Figure 5: Effect of organophosphorous pesticide, chlorpyrifos (CPF) on the morphological appearance of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) up to 96 hr exposure period. Fish showed (A): Pop eye, (B): Anapthalmia, (C): skin lesions.

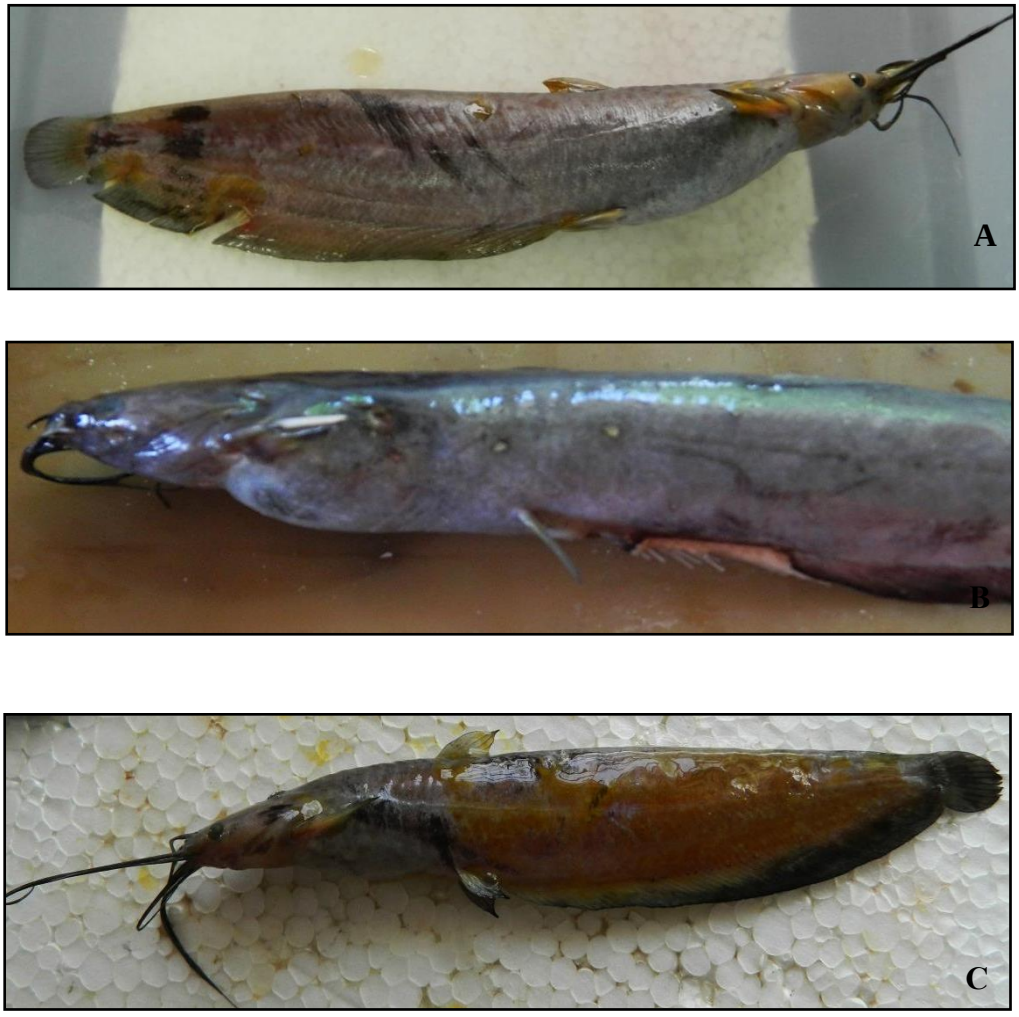


Figure 6: Effect oforganophosphorous pesticide, chlorpyrifos (CPF) on the morphological appearance of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794) up to 96 hr exposure period. Fish showed (A): fin splitting, (B): fin necrosis, skin discoloration and skin lesions, (C): heavy mucus secretion.

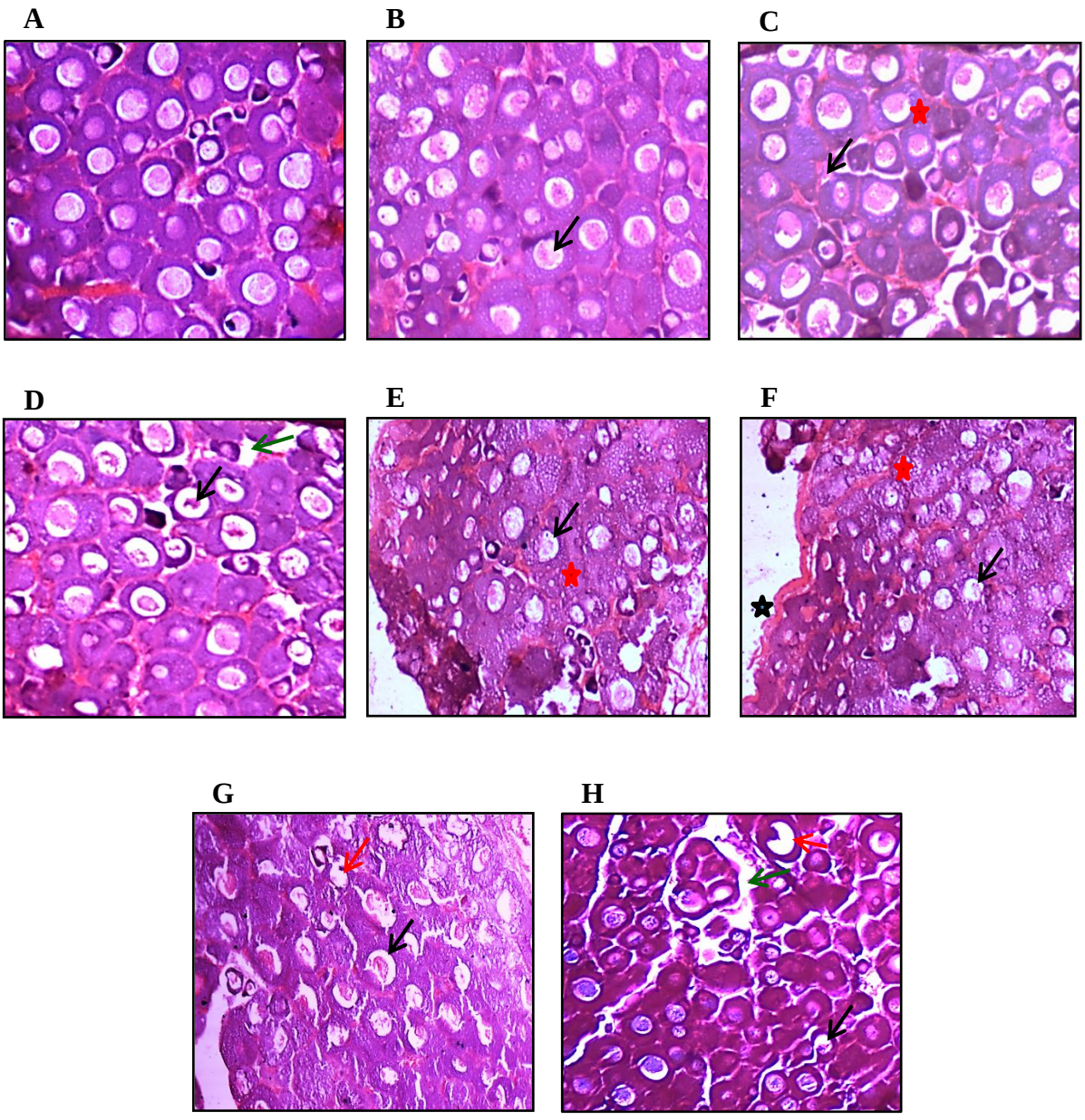
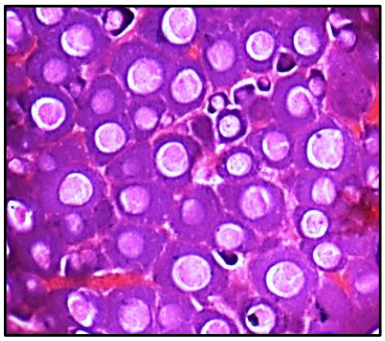
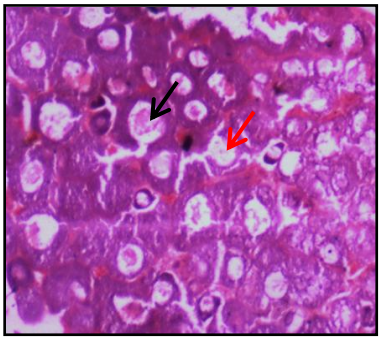


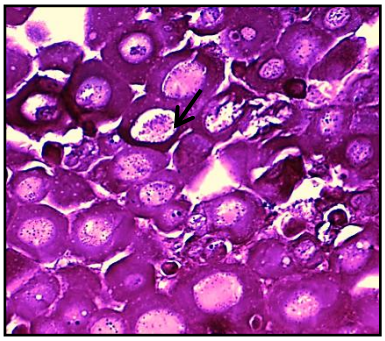
Figure 7: Transverse section of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)exposed to different concentrations of chlorpyrifos for 96 hr in *resting phase.* Histologicalphotographsshowed ruptured ovarian wall (star), cytoplasmic retraction of oocyte (black arrow), cytoplasmic liquefaction (red arrow), wrinkled oocyte (red star) and inter-follicular space (green arrow). A- Control, B- 0.057 mM/l, C- 0.085 mM/l, D- 0.114 mM/l, E- 0.171 mM/l, F- 0.228 mM/l, G- 0.285 mM/l, H- 0.57 mM/l. Images were captured at 10X magnification.



Control fish



Short term (1/3rd of LC_{50})



Long term (1/10th of LC_{50})

Figure 8: Transverse section of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)exposed to 1/3rd and 1/10th of LC_{50} of 96 hr of CPF (0.058 mM/l and 0.0174 mM/l) in *resting phase* for 15 and 30 days of exposure duration. Histologicalphotographsshowed cytoplasmic retraction of oocyte (black arrow) and cytoplasmic liquefaction (red arrow). Images were captured at 10X magnification.

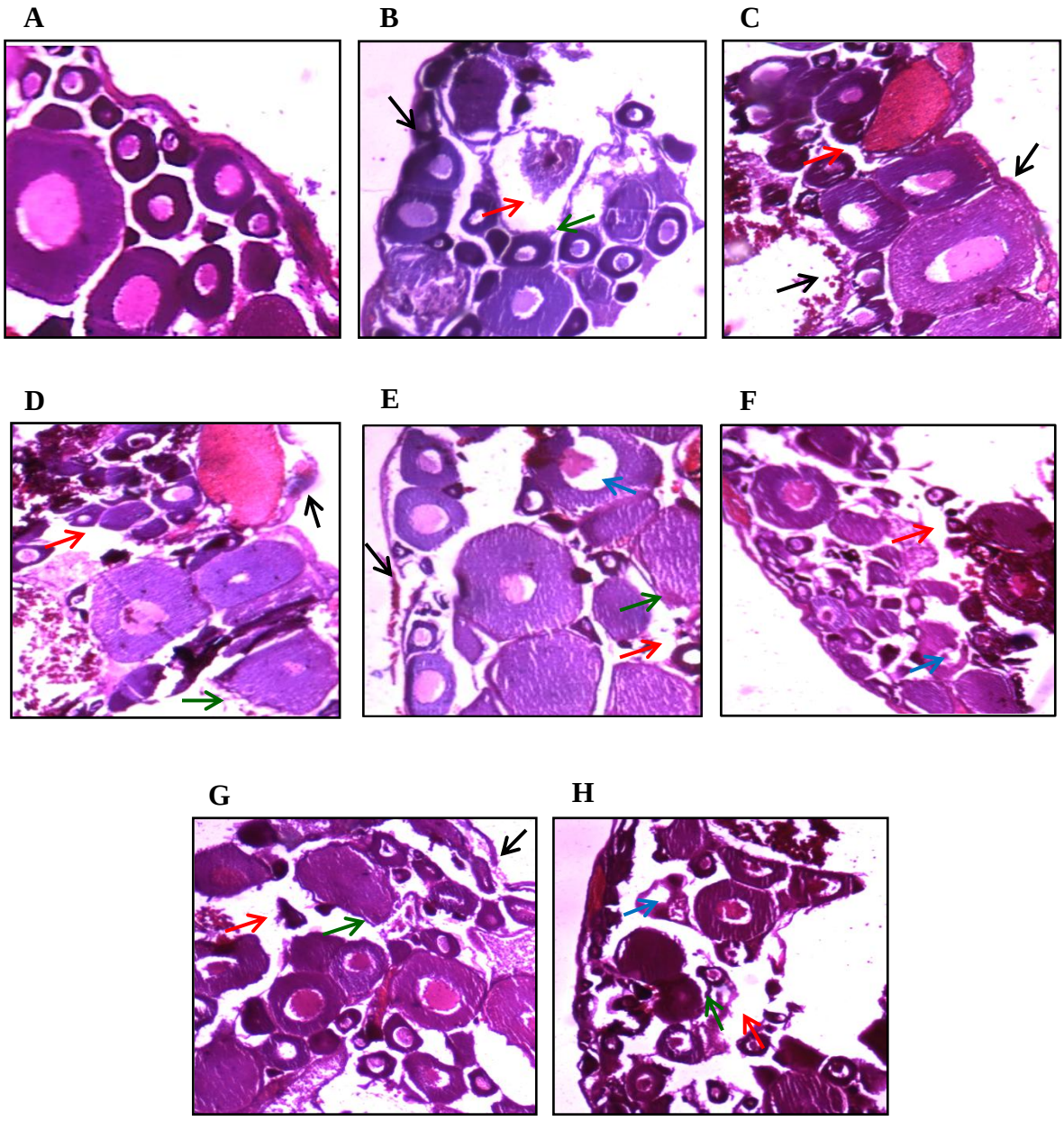
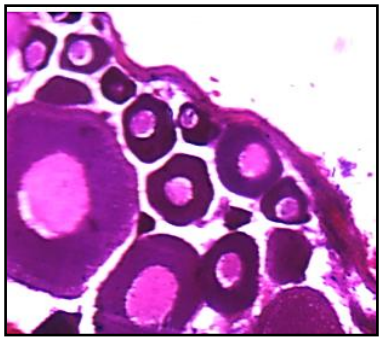
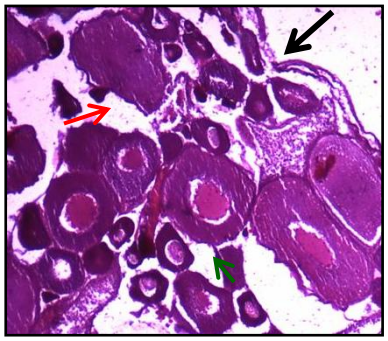


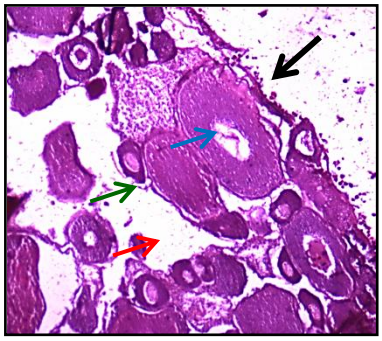
Figure 9: Transverse section of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)exposed to different concentrations of chlorpyrifos for 96 hr in *preparatory phase.* Histologicalphotographsshowed ruptured ovarian wall (black arrow), inter-follicular space (red arrow), cytoplasmic liquefaction (blue arrow) and degenerated granulosa layer (green arrow). A- Control, B- 0.028 mM/l, C- 0.057 mM/l, D- 0.085 mM/l, E- 0.114 mM/l, F- 0.142 mM/l, G- 0.171 mM/l, H- 0.199 mM/l. Images were captured at 10X magnification.



Control fish



Short term (1/3rd of LC_{50})



Long term (1/10th of LC_{50})

Figure 10: Transverse section of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)exposed to 1/3rd and 1/10th of LC_{50} of 96 hr of CPF (0.041 mM/l and 0.12 mM/l) in *preparatory phase* for 15 and 30 days of exposure duration. Histologicalphotographsshowed ruptured ovarian wall (black arrow), inter-follicular space (red arrow), cytoplasmic liquefaction (blue arrow) and degenerated granulosa layer (green arrow). Images were captured at 10X magnification**.**

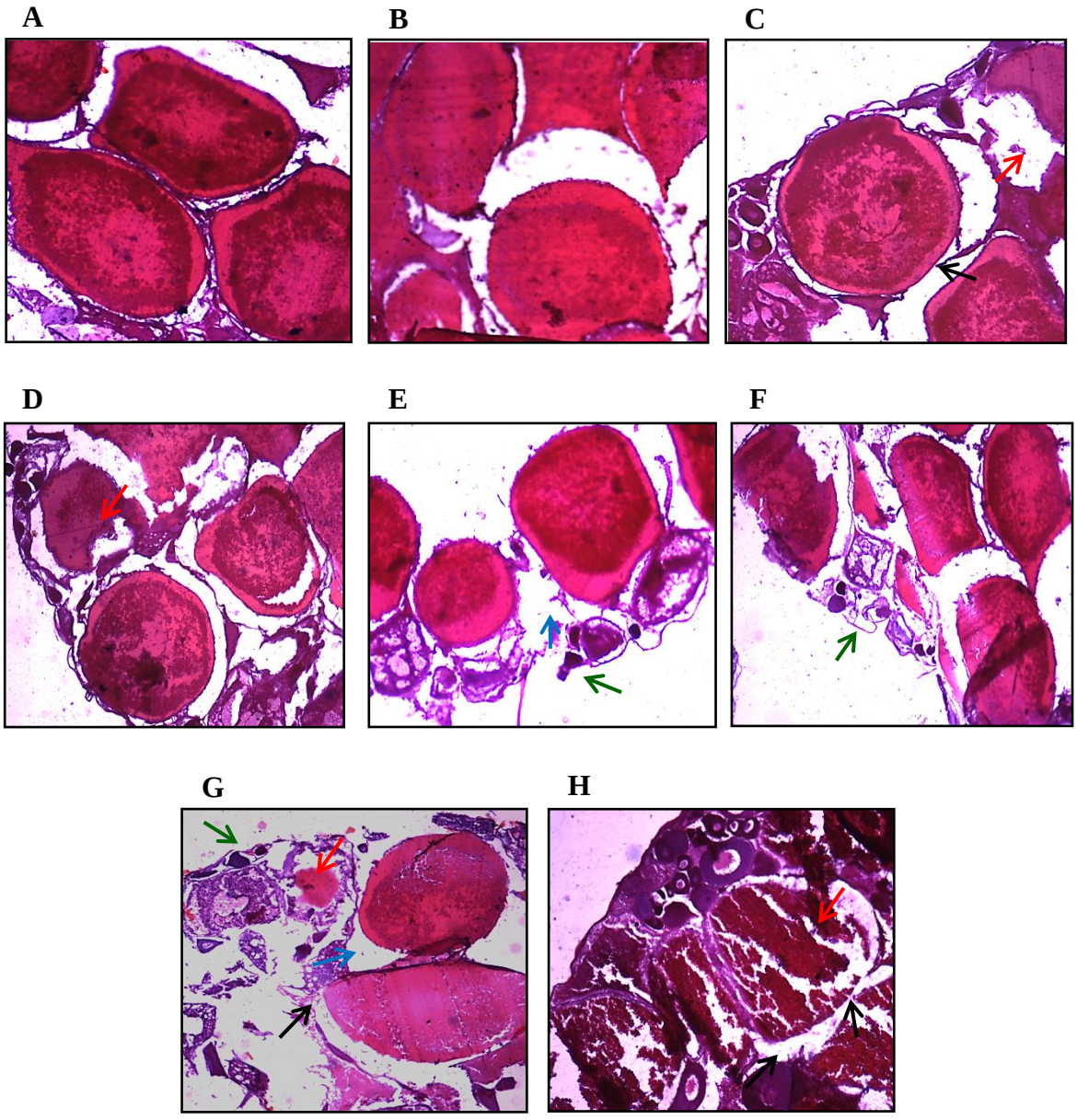
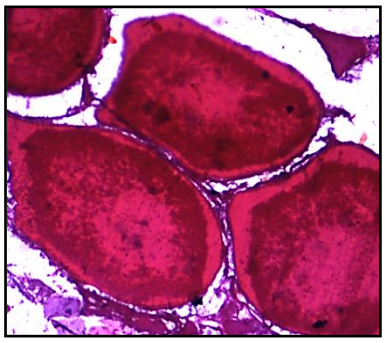
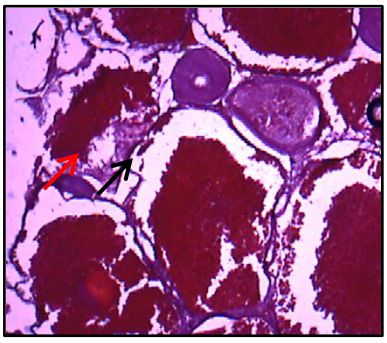


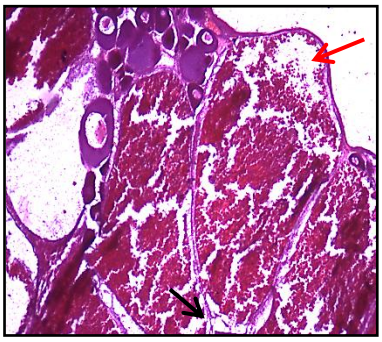
Figure 11: Transverse section of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)exposed to different concentrations of chlorpyrifos for 96 hr in *pre-spawning phase.* Histologicalphotographsshowed degenerated granulosa layer (black arrow), ruptured oocyte (red arrow), inter-follicular space (blue arrow) and degenerated of ovarian wall (green arrow). A- Control, B- 0.002 mM/l, C- 0.005 mM/l, D- 0.028 mM/l, E- 0.057 mM/l, F- 0.085 mM/l, G- 0.114 mM/l, H- 0.142 mM/l. Images were captured at 10X magnification.



Control fish



Short term (1/3rd of LC_{50})



Long term (1/10th of LC_{50})

Figure 12: Transverse section of ovary of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)exposed to 1/3rd and 1/10th of LC_{50} of 96 hr of CPF (0.008 mM/l and 0.002 mM/l) in *pre-spawning phase* for 15 and 30 days of exposure duration. Histologicalphotographsshowed degenerated granulosa layer (black arrow) and ruptured oocyte (red arrow). Images were captured at 10X magnification**.**

4. Discussion

The toxicity study is essential to find out toxicants limit and safe concentration, so that there will be minimum harm to aquatic fauna in the near future. The assessment of toxicity of chlorpyrifos (CPF) with reference to aquatic biota, especially fish is crucial in establishing the toxicity evaluation. In last few decades a lot of attention has been paid towards bioassay due to its proved potential to evaluate toxicity of chemicals. Several workers investigated the toxicity of organophosphorous pesticides in fish (Koundinya and Ramamurthi, 1979; Johnson and Finley, 1980; Kumar and Gupta, 1997; Santhakumar et al., 2000; Singh et al., 2010; Srivastava et al., 2010; Zhang et al., 2010; Barbieri and Ferreira, 2011; Maniyar et al., 2011).

In the present study, an attempt has been made to investigate the toxicity of CPF to freshwater catfish, *H. fossilis* by estimation of the median lethal concentration (LC_{50}). The investigation of its impacts on behavior, morphology and ovarian histology were also observed. The lethal concentration (LC_{50}) of CPF was varied with different reproductive phases in an annual cycle which clearly indicate that the acute toxicity of CPF varied with the fish species and even same species under the influence of number of factors including size and sensitivity to the toxicant, its concentration and duration of exposure and reproductive season as well. The safe concentration of chlorpyrifos for experimental fish, *H. fossilis* suggested that in resting phase, fish enjoy high tolerance limit (safe value) as compare to preparatory and pre-spawning phase. During pre-spawning phase even small concentration, i.e. safe in resting phase, may be lethal to fish. This suggested that as the fish moved toward their gonadal maturity, became more vulnerable to health hazards by aquatic toxicants.

The present investigation reported that as the maturity of fish was increased LC_{50} value become decreased. The sub-lethal concentration of CPF was different in different exposure duration and reproductive phases (Table 1). It is difficult to compare the toxicity of individual pesticides to different fish species (Schimmel et al., 1976).Variations in the LC_{50} depend upon many factors such as animal weight, developmental stages (Kamaldeep and Joor, 1975), period of exposure and temperature (Pathan et al., 2009) and number of biological and physiological factors which have been

reported by earlier workers (Srivastav et al., 2002; Jaroli and Sharma, 2005; Ali et al., 2008; Banaee et al., 2013; Padmanabha et al., 2015).A wide range of toxicant was found to increase the toxicity at higher temperature (Macek et al., 1969; Muirhead, 1971). It was related to the higher metabolic and respiration rate, which may largely be involved in toxicant response in an annual cycle (Macek et al., 1969; Wedemeyer et al., 1976; Gordon and McLeay, 1977). Fish in general have low metabolic rate in resting phase (Pickford and Atz, 1957). This low metabolic activity results in slowdown toxicant absorption and its bioaccumulation (Hussein et al., 2006). The result found that highest LC_{50} value was recorded in resting phase as compared to preparatory and pre-spawning phase. The toxicant absorption and its bioaccumulation were increased comparatively in preparatory and pre-spawning phase with increase in metabolic activity (Pickford and Atz, 1957).

The previously studies of acute toxicity of chlorpyrifos for *Heteropneustes fossilis* revealed that LC_{50} value for 96 hr was found to be 1.2 µl/L (Barbhuiya and Dey, 2014a). The value of LC_{50} 36 hr of chlorpyrifos was determined as 2.84 ppm for *H. fossilis* in the laboratory condition (Khatun and Mahanta, 2014). LC_{50} value of deltamethrin to *H. fossilis* was 1.86 µg/l (Srivastav et al., 2002). This is in agreement with Sprague (1969) who observed variation in LC_{50} for the same species and toxicant depending on size, age, sex and condition of test species along with experimental factors.

Reports on the acute toxicity tests by earlier workers revealed a clear idea of comparative lethal values of organophosphorous pesticide (CPF) for different fish species (Santhakumar and Balaji, 2000; Mathivanan, 2004; Ramasamy et al., 2007). The LC_{50} of chlorpyrifos and other organophosphates varied considerably with different fish species and environment viz., 297.63 µg/l in *Gambusia affinis* (Rao et al., 2005), 154 µg/l and 0.022 ppm in *Oreochromis mossambicus* (Rao et al., 2003; Padmanabha et al., 2015), 0.365 ppm and 811.98 µg/l for *C. punctatus* (Jaroli and Sharma, 2005; Ali et al., 2008), 0.002 mg/l in *Tilapia guineensis* (Chindah et al., 2004)and 0.16 mg/l, and 203 µg/l in *Cyprinus carpio* (Halappa and David, 2009; Banaee et al., 2013). The range of 96 hr LC_{50} of CPF was 0.57-3270 ppb for mosquito fish, Bluegill, Fathead minnow, Rainbow trout, Nila tilapia, Goldfish (Davey et al., 1976; Holcombe et al., 1982; Bowman, 1988a, b; Gul, 2005; Wang et al., 2009). Ramesh and Saravanan (2008) reported that 24 hr LC_{50}

of CPF in *Cyprinus carpio* was 5.28 ppm. The calculated LC_{50} for 96 hr of chlorpyrifos-methyl, for adult male Guppy *Poecilia reticulata* was 1.79 mg/l and for *O. niloticus* larvae was 0.92 mg/l (Ali, 2005). Different LC_{50} values were reported in previous studies for different organophosphate pesticides. LC_{50} value of malathion was 9 µl/l for *Labeo rohita* (Patil and David, 2008) and 16 µl/l for *Ophiocephalus punctatus* (Pugazhvendan et al., 2009). 96 hr LC_{50} of different organophosphate pesticides- monocrotophos, diazinon, nuvan and dimethoate in *Channa punctatus, Silurus glanis, Ctenopharyngodon idella* and *Heteropneustes fossilis* were 18.56 ppm (Agrahari et al., 2006), 4.142 mg/L (Koprucu et al., 2006), 6.5 mg/l (Tilak and Kumar, 2009) and 11.34 mg/l (Srivastava et al., 2010) respectively. The organophosphorous compound Coroban (20% EC- CPF) LC_{50} for 96 hr was 2.2 mg/l of *H. fossilis* (Srivastav et al., 1997). 24 hr, 48 hr, 72 hr and 96 hr LC_{50} value of Tricel (20% EC-CPF) for *Labeo bata* was as 257.03 µg/l, 208.92 µg/l, 177.82 µg/l and 109.64 µg/l respectively (Samjadar and Mandal, 2015). The LC_{50} value of other organophosphate pesticide, dimethoate for *Clarius batrachus* was 65 mg/l (Begum and Vijayaraghavan, 1995), 17.9 mg/l in *Channa punctatus* (Srivastav and Singh, 2001), 2.98 mg/l in *H. fossilis* (Pandey et al., 2009), 0.007 ppm of *Catla catla* (Kumar and Singh, 2000) and 1.61 mg/l for *Cyprinus carpio* (DeMel et al., 2005). Thus LC_{50} value of organophosphorous pesticides showed considearble variation reflecting different tolerance limit of different fish species.

Gafar et al. (2010) observed that toxicants in the aquatic environment may not necessarily result in the outright mortality of aquatic organisms but can result in several physiological dysfunctions in fish. The fish response towards toxic medium is important since it reflects the internal changes and also externally as behavioral and morphological toxicity. Behavioral changes as a result of stress are accepted as the most sensitive indication of potential toxic effects. The level of aquatic toxification can be assessed by behavioral changes at very first and by morphological changes in a while. These are considered as first signal of any kind of stress.

In the present study, the observation of behavioral changes was started immediately after CPF treatment as compared to control group. The pesticides were influenced the behavior pattern of fish by interfering with the nervous systems and sensory receptors (Keizer et al., 1995; Pan and Dutta, 1998; Cong et al., 2009). The

degree of distress was increased as the toxicant concentration increased. Noticeable changes in behavior were found at various concentrations of CPF.

Upon exposure to the toxicant, the immediate response of the fish was to drive to the bottom of the test chamber and stay there for few minutes of time. Diving to the bottom resembles the approach to the fish interpreted as avoidance behavior (Painter et al., 2009). The swimming pattern of exposed fish exhibited irregular, abrupt, erratic, jerky swimming movements, hyper excitability and loss of equilibrium. Fish also showed lateral swimming with their pectoral fin forward towards the head and the movement of fins was concentrations dependent however it was reduced with increasing exposure period. The exposed group of fish showed increased locomotor activity and entails increased expenditure of energy (Rathnamma et al., 2008; Halappa and Muniswamy, 2009) due to stress. Abnormal behaviors such as convulsion (fish was showed no swimming, continuous ataxia with intermittent body spasm and violent sacking to avoidance of toxicant), restlessness, surface to bottom movement and sudden quick movement observed in the current study were similar to the observations of Omitoyin et al. (2002) and Halappa and Muniswamy (2009). The observed behavioral changes showed by the exposed fish to the chlorpyrifos were similar to those observations in other fish exposed to organophosphate pesticides (Ahmed, 1975; Ghose, 1986; Shukla, 1995).

The physiological changes of any organism altered neurotransmitter levels or hormones that can affect external behavioral features ((Shah, 2002; Scott and Sloman, 2004). Altered behavior may result from nervous system damage, because of neurotransmitters are sensitive to contaminant exposure. Neurotoxicants that alter dopaminergic systems can affect swimming behavior and memory in fishes (Panula et al., 2006). Some pesticides, target the enzyme cholinesterase that breaks down the neurotransmitter cholinesterase for example mosquito fish *Gambusia affinis* exposed to sublethal concentrations of the pesticide CPF for 20 days reduced their locomotary behavior and swimming speed and accumulated acetylcholine at synaptic junctions (Rao et al., 2005). Abnormal swimming and loss of balance was caused by the deficiency in nervous and muscular coordination by toxicant (Venkata et al., 2008; Halappa and Muniswamy, 2009). It might be due to some neurological impairment in central nervous

system as evident by inhibition of AChE when they exposed fish to acute concentrations of different toxicants (Devi and Fingerman, 1995; Lata et al., 2001; Gracia et al., 2003; Patro, 2006; Ricceri et al., 2006; Ezike and Ufodike, 2008; Oshode et al., 2008; Angelis et al., 2009). The behavioral changes observed in the present study indicated the loss of physiological equilibrium may be due to inhibition of the acetylcholinesterase enzyme in the brain and leading to acetylcholine accumulation at synaptic junctions (Fulton and Key, 2001; Rao et al., 2005; Agrahari et al., 2006; Patil and David, 2008; Ramesh and Saravanan, 2008; Anita et al., 2010) which was likely to caused prolonged excitatory post synaptic potential. This may first lead to stimulation and later caused a block in the cholinergic system. The stimulation of peripheral nervous system resulted in to increased metabolic activities and more oxygen utilization (Rao, 1989).

The control groups of fish were calm, quiet and preferred to confine themselves to the bottom of the aquarium. They swam horizontally and slowly as compared to the treated fish that swam in a slanting manner from a lower level to an upper level. It is likely that the region in the brain (telencephelon) which is associated with the maintenance of equilibrium should have been affected.

In the present study, the freshwater catfish, *H. fossilis* showed an increment in the opercular movements and in surfacing behavior with the increasing concentration of CPF, to avoid breathing in the poisoned water (Lata et al., 2001) as observed by Shwetha and Hosetti (2009). The increase in opercular movement and corresponding increase in rate of surfacing of fish clearly indicated that fish adaptively shift towards aerial respiration (by obtaining atmospheric oxygen surfacing) and fish tried to avoid contact with CPF through gill chamber. Opercular movement is one of the early warning systems as an indicator of respiratory stress. It gives direct relation towards oxygen stress. Since fish breath in water in which they live, changes in chemical properties there will surely be reflected in their respiratory activity (Jeane et al., 2009). The increased opercular movements in the initial period of exposure might be to support enhanced physiological activities in stressful habitat and later decreased with exposure duration, may be due to accumulation of mucus over the gill filaments. Similar findings were observed by Pandey et al. (2005), Omitoyin et al. (2006), Yadav et al. (2007), Koprucu et al. (2006), Srivastava et al. (2010) in *Channa punctatus, Clarias gariepinus, Channa straita, Silurus glanis,* and

Heteropneustes fossilis exposed to mercuric chloride and malathione, lindane, fertilizer, diazinon and dimethoate respectively. Rapid opercular movement was also confirmed by Wasu et al. (2009) in *Clarias batrachus* treated with carbaryl and malathion. Shivakumar et al. (2006) also observed increased opercular movement in *Cyprinus carpio* exposed to endosulfan, cypermethrin and fenvalerate. Chindah et al. (2004) reported an initial increase in opercular beat frequency in chlorpyrifos exposed tilapia, followed by a marked decline with exposure time and explained the initial increase as sudden response to shock.

Treated fish slowly reached the water surface, probably due to gill damage that caused respiration malfunctions (Rathnamma et al., 2008) or as a result of difficulties in gas exchange due to mucous accumulation. Moreover, CPF cause severe damage to the gill membranes (Johal et al., 2007; Velmurugan et al., 2007), hampering the branchial exchange of gases and leading to asphyxia. Surfacing and gulping of air might be a compensatory device to cope with the oxygen deficiency (Israeli and Kimmel, 1996). The increased gulping activity by the exposed fish may be the reflection of an attempt by the fish to extract more oxygen to meet the increased energy demand to with stand the CPF toxicity. These behavioral patterns are the indication of respiratory impairment due to the effect of toxicant (Shwetha and Hosetti, 2009). Gulping of air may help to avoid contact of toxic medium (Katja et al., 2005). Similar observation has been reported by Patil and David (2008) in malathion treated fish *Labeo rohita* and by Parithabhanu (2013) in cypermethrin treated *Orechromis mossambicus*.The surfacing phenomenon of fish might be a demand for higher oxygen level during the exposure period due to hydro toxic condition (Katja et al., 2005) and these results are supported by Cook et al. (2005), Shivakumar et al. (2006), Balasubramani et al. (2008), Charjan et al. (2008), Appa et al. (2009), Halappa and David (2009). According to Gabriel et al. (2010), this behavior may have resulted from enzyme dysfunction and paralysis of respiratory muscle or depression of respiratory center and disturbances in energy pathways leading to depletion of energy.

Fish were lethargic and at the time of death they exhibited transient hyperactivity before collapsing. The fish were found to be lying down motionless at the bottom before death and died by opening their mouth. Same behaviors were found by Koprucu et al. (2006), Patil and David (2008), Susan et al. (2010) and Barbhuiya and Dey (2014b).

Among morphological deformities, fish showed fin necrosis, depigmentation in body colour, eye deformities and cupous mucous secretion. The occurence of these deformities was increased with increasing concentration and duration of CPF. The fade body colour can be considered as symptoms of stress and it has also been reported by Ree et al. (1997), Omitoyin et al. (2006), Koprucu et al. (2006) and Wasu et al. (2009) in fish like zebra fish, *Clarias gariepinus*, *Silurus glanis* and *Clarias batrachus* exposed to different pesticides. Pandey et al. (1990) also reported depigmentation in exposed fish and attributed it to reduction in number and size of chromatophores. The mucus accumulation also was observed all over the body surface of the exposed and dead fish in all concentrations which was found maximum at higher concentration of chlorpyrifos. This may due to the interference of toxicant with the gaseous exchange, secretion, waste products and osmoregulation (Oti, 2002; Adeyemo, 2005; Ayuba and Ofojekwu, 2005; Omitoyin, 2007; Okomoda et al., 2010). Fish secrete copious amount of mucus, as a defence mechanism to neutralize the insecticide effect, which gradually covers all over the body, gills and the buccal cavity. Moreover, Bisht and Agarwal (2007) observed hypertrophy and hyperplasia of mucous cells in $CuSO_4$ exposed fish and suggested it as adaptive and defense mechanism to prevent cutaneous entry of toxicant by coagulation through increased mucus production.

Researchers have reported the same alterations in *Oryzias latipes* exposed to CPF, permethrin, phenol, strychnine and 2,4-dinitrophenol (Rao et al., 1997), *Cyprinus carpio* exposed to CPF (Halappa and David, 2009), *Labeo rohita* malathion exposure (Patil and David, 2008), *Oncorhynchus tshawytscha* diazinon exposure (Scholz et al., 2000), *Oryzias latipes* endosulfan exposure (Gormley and Teather, 2003), *Cirrhinus mrigala* fenvalerate exposure (Mushigeri and David, 2005), *Oreochromis niloticus exposed to* fenitrothion (Benli and Ozkul, 2010), *Oreochromis niloticus* exposed to dimethoate (Auta et al., 2002) *Heteropneustes fossilis* exposed to dimethoate (Pandey et al., 2009).

The two type of eye malformations were also noticed in CPF treated group of fish such as pop eye and anapthalmia. Such abnormalities were found may be due to the apoptosis of cells in the eye or reduce in the diameter of eye socket (Tyor et al., 2012). The fin necrosis, skin lesions and discoloration of skin were also noticed in CPF exposed

fish. Similar reports were investigated by lger et al. (1994) in cadmium exposure treatment.

Further to know about the toxicological activity of chlorpyrifos on the ovarian tissue of freshwater catfish, *Heteropneustes fossilis*, the histological tool was used. CPF inhibited the growth of the ovary at different stages of maturation. Histological manifestations in ovaries may be caused by several factors and by a variety of effluents and aquatic pollutants (Sarojini and Victor, 1985; Davis and Cook, 1993; Farmer et al., 1995; Kumar et al., 2000). Fish exposed to different concentrations of CPF reported significant damage in ovarian tissue in all reproductive phases indicating that it was a useful methodology for monitoring the effects of pesticides on fish (Banaee, 2013). The result showed that the frequency of oocyte degenerations was more in higher concentration of CPF which was dose- dependent. The ovaries were predominated by oocytes which were at perinucleor stage with large nuclei and many nucleoli of various sizes. In *H fossilis*, after CPF exposure, oocytes exhibited degenerative changes, liquification of perinuclear cytoplasm, condensation of nucleus, disappearance of nuclear membrane, cytoplasmic clumping, degenerated granulose layer, and degenerated ovarian wall and wrinkled oocytes. Similar results were also found by Khillare (1992), Sukumar and Karpagaganpathy (1992), Ramachandra (2000) and Hossain et al. (2002). Partial disruption of the ovarian follicles, vacuolation in the cytoplasm of germinal cells, loss of connective tissue and secondary oocytes were reported in *H. fossilis* exposed to BHC (Hazarika and Das, 1998). Saxena and Agrawal (1986) have shown that cadmium chloride blocked all the oogonial activity at the vitellogenic phase in *C. btrachus* and suggested it that it might be due to retardation, oocytes proliferation and increase in number of atretic follicles. Similar finding were observed by Pawar and Katdare (1983), Khillare (1992), Sukumar and Karpagaganpathy (1992), Srivastava and Srivastava (1994), Dutta (1996), Adityakumar et al. (2002), Lee and Yang (2002), Chandra et al. (2004) and Ghosh and Nath (2005). Jyothi and Narayan (1999) reported the impact sub lethal concentration of carbaryl on the ovary of *Clarias batrachus*, resulted in vacuolation, necrosis, arrested ovarian recrudescence and inter-follicular oedema.

Benarji and Rajendranath (1991) studied cyto-architectural changes in the oocytes, including pronounced vacuolation, degeneration and deformation, clumping of the

cytoplasm and karyohypertrophy in *Clarias batrachus* exposed to dichlorvos. Degeneration of follicular walls, connective tissues and vacuolisation in the ooplasm of oocyte was observed in *A. testudineus* treated to carbofuran (Chatterjee et al., 1997). The formation of nucleolar extrusion has attracted the attention of many researchers because of its significance for the formation of proteins as reported by Khanna (1956). Muley and Mane (1987) observed ruptured follicles, completely dissolute nucleus and nucleoli in the gonads of Lamellibranch mollusks under cythion and malathion toxicity. Rastogi and Kulshrestha (1990) reported necrosis and fibrosis in connective tissue along with dilation of blood vessels and damage to yolk vesicles of maturing oocytes in carp minnow *Rasbora daniconius* under endosulfan, carbofuran and methyl parathion toxicity. The carbamates pesticides were also reported to induce similar degenerative changes in fish ovary (Kulshrestha and Arora, 1984; Sukumar and Karpagaganpathy, 1992).

During present study atretic oocytes and increase in the interfollicular spaces were observed. Same result was supported by findings of Nath and Kumar (1990) in *C. fasciatus* under nickel toxicity. Ramachandra (2000) reported that lower dose of Malathion caused reduction in ovarian weight and also retard the growth of the pre-vitellogenic oocytes, and on the other hand a higher dose resulted in the degeneration of the immature oocytes and rupture of follicular epithelium. Fish ovary exposed to other organophosphate compounds such as monocrotophos, methylparathion, phenthoate, malathion, and fenitrothion also resulted in follicular atresia and other degenerative changes in ovary (Pawar and Katdare, 1983; Shukla et al., 1984; Kumar and Pant, 1988; Dey and Bhattacharya, 1989; Rastogi and Kulshrestha 1990).

The present findings suggested that the histopathological changes in the ovary might be a reflection of disturbance in the endocrine/hormonal imbalance. Chatterjee et al. (1997) found that carbofuran altered both the area and the percentage occurrence of the various types of primary oocytes in the ovary of *H. fossilis*. He observed the degeneration of follicular walls, connective tissues and vacuolization in the ooplasm of the stage II and III oocytes. He stated that carbofuran at sub-lethal concentrations inhibits oocyte maturational processes in catfish. Giri et al. (2000) studied the effect of basathrin on the ovarian tissue of *H. fossilis*and he reported marked damage in germinal epithelium, atresia of oocyte, stromal hemorrhage, vacuolization of oocytes and general

inflammation. Kling (1981) reported that cells assimilation of yolk granules during the exposure and simultaneous arrest of vitellogenesis caused the reduction in size of oocytes, resulting in a total atresia of ovaries of Tilapia.

Dutta et al. (1994) reported notable microscopic changes in ovigerous lamellae, oocytes at different stages of development and the nucleus of the immature oocyte of the catfish *Heteropneustes fossilis*. He observed clumping of cytoplasm, degeneration in the follicular cells, shrinkage of nuclear material, increased atretic oocytes, along with ruptured follicular epithelium. Saksena et al. (1999) observed the toxicity of two organophosphorous pesticides Nuvan and Dimecron in freshwater murrel, *Channa orientalis*. He found that both pesticide decreased gonadosomatic index, reduced diameter of different stages of oocytes and number of later stages of oocyte development and significant increase in the percentage of atretic follicles in ovaries of exposed animals. Dutta and Meijer (2003) studied the effect of the pesticide, on the ovaries of bluegill (*Lepomis macrohirus*). He noticed adhesion of primary follicles, cytoplasmic retraction in oocyte II, cytoplasmic degeneration, increased atretic oocytes, damages to the oocyte IV, Partial destruction of the ovigerous lamellae and vitellogenic membrane, destruction of follicles, severe damage of the ovigerous lamellae, increased intrafollicular spaces, vacuolated cytoplasm, extrusion of karyoplasm and necrosis in the cytoplasm.

Khillare (1992), Sukumar and Karpagaganpathy (1992) observed that the exposed ovary to pesticide resulted in degenerative changes, liquification of perinuclear cytoplasm and condensation of nucleus, disappearance of nuclear membrane, cytoplasmic clumping, degenerated granulose layer, and degenerated ovarian wall and wrinkled oocytes. Prominent inter-follicular spaces were observed in the ovary which was probably formed due to shrinkage of the oocytes. Based on observations of the ovarian anatomical changes with following given exposure times, it becomes evident that the sublethal doses of CPF can and will alter the microscopic anatomy of the fish ovary. Therefore this investigation demonstrates a relation among pesticidal stress, behavioral and morphological disorders, survival and mortality rates and histopathological changes in the ovary which further are a reflection of the disturbance in the endocrine/hormonal imbalance.

CHAPTER – II

Effect of organophosphorous pesticide, chlorpyrifos on oocyte maturation and follicular steroids in freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)

ABSTRACT

The objective of the present study was to investigate *in vitro* effect of organophosphorous pesticide, chlorpyrifos (CPF) on hCG-induced oocyte maturation (OM) in the freshwater catfish, *Heteropneustes fossilis*. In this observation, interplay of different steroids involved in oocyte maturation was also measured. For this, the post-vitellogenic follicles were incubated with human chorionic gonadotropin (hCG) alone to induce oocyte maturation in a concentration and duration-dependent manner and with CPF on hCG induced OM post-vitellogenic follicles. The result showed that CPF caused an inhibitory effect on hCG induced GVBD in co-incubation experiments at the dose of 0.26 pM/ml. Pre- and post-incubation of follicles with CPF resulted a significant inhibition in hCG induced oocyte maturation in all durations and maximum inhibition 87.67% and 76% was recorded at 12 hr incubation duration ($p < 0.05$, Newman-Keuls test). Effective dose of hCG (1.7 mg/ml, 8 hr) induced OM coincided with a significant increase in follicular pregnenolone, progesterone, deoxycorticosterone (DOC) and dihydroxyprogesterone (DHP) as well as significant decrease in estradiol-17β (E_2) as compared to control group of post-vitellogenic follicles. Under CPF exposure follicles elicited a sharp significant decrease in pregnenolone, progesterone and DHP. However, DOC and E_2 were increased significantly. Co-incubation study revealed that CPF suppressed hCG induced concentrations of DHP, pregnenolone and progesterone significantly but side by side support DOC increase and maintain high E_2. Thus, the

present results suggested that the CPF inhibited hCG induced oocyte meiotic maturation due to suppression of maturation inducing steroids within follicle.

Keywords: CPF, Pregnenolone, Progesterone, 17,20βP, Estradiol-17β, DOC, Oocyte maturation.

1. Introduction

In vertebrates, meiotic maturation of prophase-I arrested oocytes is pre-requisite for ovulation and subsequent fertilization to occur in vertebrates (Nagahama and Yamashita, 2008). There are several studies revealed that oocyte maturation in teleosts are three step induction process which involving gonadotropins (GtHs), maturation-inducing hormone (MIH) and maturation-promoting factor (MPF) (Nagahama and Yamashita, 2008). Developmental events in the ovaries are regulated by steroids synthesized under the direct influence of two gonadotropins; GtH1, GtH2 (Patino et al., 2001; Rehman et al., 2001; Mishra and Joy, 2006; Nagahama and Yamashita, 2008; Skoblina, 2009). Gonadotropin plays an important role in the induction of final oocyte maturation by MIS synthesis in follicle cells (Kobayashi et al., 1988; Zhu et al., 1989; Nagahama, 1997; Patino et al., 2001; Senthilkumaran, 2004). Human chorionic gonadotropin (hCG) found in mammals is a structurally related variant of fish gonadotropin, which acts as an effective inducer of oocyte maturation in several teleost (Skoblina, 2009). It stimulates the *in vitro* steroidogenesis of granulosa cell and releases the MIS in the incubation medium (King et al., 1995; Sorbera et al., 1999). The follicular meiotic maturation in fish include germinal vesicle migration (GVM), a switch in follicular secretion from C_{18} to C_{21} steroids and the resumption of the meiotic process which are under the direct control of gonadotropin (Patino and Thomas, 1990; Nagahama et al., 1995; Patino et al., 2001; Mishra and Joy, 2006a; Nagahama and Yamashita, 2008; Skoblina, 2009). Meiosis is commenced with the surge in gonadotropin-releasing hormone (GnRH), followed by a rise in circulating luteinizing hormone (LH). Upon binding of LH with the granulosa cell receptors, the production of maturation-inducing steroid (MIS) begins. MIH synthesis occurs in the ovarian follicle layers under the stimulation of GTHs, pre-ovulatory luteinizing hormone (LH) surge being the stimulus for final oocyte maturation (Nagahama and Yamashita, 2008). Different classes of steroid

hormones (C_{18} to C_{21}) have shown to induce oocyte maturation in vertebrates including teleosts (Goswami and Sundararaj, 1974; Nagahama and Adachi, 1985; Trant and Thomas, 1989; Lutz et al., 2001; Senthilkumaran and Joy, 2001) apart from classical MIH, 17α-20β-dihydroxy-4-pregnen-3-one (1720βP) (Nagahama, 1997). The C_{21} steroids include pregnenolone (P_5: 5-pregnan,3β-ol-20-one), deoxycorticosterone (DOC: 4-pregnen-21-ol-3,20-dione), progesterone (P_4: pregn-4-ene-3,20-dione) and 17,20βP (DHP: 17α-20β-dihydroxypreg-4-ene-3-dione). Among these P_4, 17,20βP and DOC have been shown to be potent MIS or inducers of GVBD *in vitro* in teleosts (Goswami and Sundararaj, 1974; Sundararaj and Goswami, 1977; Young et al., 1982; Goetz, 1983; Nagahama et al., 1983; Jalabert, 1976, 1991). MIS directly acts through the plasma membrane receptor on the oocyte surface and induces follicular oocyte maturation (Nagahama, 1997). 17,20βP is the most effective maturation-inducing steroid that induces resumption of meiosis in most of the fish species (Goetz, 1983; Sundararaj et al., 1985; Scott and Canario, 1987; Jalabert et al., 1991; Thomas, 1994; Nagahama, 1997; Mishra and Joy, 2006a). The estradiol-17β (E_2: C_{18} steroid), is the major regulator of vitellogenesis, a complex and elaborate process responsible for the growth of follicles and yolk, so deposited are used during early embryonic development (Nagahama, 1997).

A complex series of enzymes are responsible for the biosynthesis of gonadal steroid in fish. The pathway initiates with the synthesis of the C_{21} steroid precursor pregnenolone via side-chain cleavage of cholesterol by cholesterol side-chain cleavage cytochrome P450 (P450scc) enzyme. Pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme. Progesterone is then converted to 17α-hydroxyprogesterone by 17α-hydroxylase activity of cytochrome P450c17 enzyme which is followed by the production of 17,20βP by 20β-hydroxysteroid dehydrogenase (20β-HSD) enzyme, the key enzyme is induced by GtH (LH) in granulosa cells of post-vitellogenic follicles immediately prior to oocyte maturation (Nagahama et al., 1985) and estradiol-17β by 17β-HSD enzyme and P450aromatase enzyme (P450arom). Progesterone is also converted in to deoxycorticosterone by 21-hydroxylase enzyme which further produced corticosterone by 11β-hydroxylase (Kagawa et al., 1982; Young et al., 1986; Nagahama, 1987; Nagahama and Yamashita, 2008).

Oocyte maturation (OM) has been widely studied in teleosts (Goetz, 1983; Scott and Canario, 1987; Jalabert et al., 1991; Nagahama et al., 1995; Patino et al., 2001; Mishra and Joy, 2006b). In the catfish *Mystus vittatus* and *C. batrachus*, 17,20βP has also been reported to be the most potent steroid for inducing OM (Upadhyaya and Haider, 1986; Haider and Rao, 1992). There have been several recent reports suggesting that MIS also plays an important role in ovulation. 17,20βP could induce ovulation *in vitro* in two catfish species, *Heteropneustes fossilis* (Tripathi and Singh, 1995) and *Pseudobagrus fulvidraco* (Lim et. al., 1997). In addition, a progestin was found to be an effective method for inducing OM and ovulation in *C. gariepinus (*Richter et al., 1985). Several studies have determined the effectiveness of various gonadotropin and pituitary preparations to induce oocyte maturation *in vivo* (Haniffa et al., 2000; Rehman et al., 2001; Chuda et al., 2002; Mishra and Joy, 2006a) and *in vitro* (Kagawa et al., 1994; Sorbera et al., 1999; Rehman et al., 2001; Skoblina, 2009) in many species.

Chlorpyrifos [*O, O*-diethyl-*O*-(3, 5, 6-trichloro-2-pyridinyl)-phosphorothioate] (CPF), one of the broad-spectrum organophosphorous pesticide used to control the pest in agriculture areas. The recent increase in the agricultural use of organophosphorous pesticides may threaten the reproductive health of humans, wildlife and aquatic life. CPF is bio-activated by cytochrome P450 (CYP)-mediated mono-oxygenases enzyme through desulfuration reaction or dearylation reaction to a more potent cholinesterase inhibitor, chlorpyrifos-oxon (CPO) (Chambers, 1992). Organophosphates may act as neuroendocrine disruptors via inhibition of AChE activity and increase of acetylcholine level in the brain (Herken and Neubert, 1953; Whitney et al., 1995). The inhibition of acetylcholine esterase (AChE) enzyme affects pituitary and hypothalamus functions and the release of gonadotropins (Sarkar et al., 2001). Cholinesterase inhibitors were known to modify the pituitary–thyroid or pituitary–adrenal axes and to alter progesterone levels (Smallridge et al., 1991; Prakash et al., 1992). It notably targets the cell signaling cascades governing neuronal and hormonal signals that are essential for cell differentiation and homeostatic regulation (Pope, 1999; Schuh et al., 2002; Slotkin, 2004). CPF was described as a potent inhibitor (Hodgson and Rose, 2008) of human liver cytochrome P450-dependent (CYP450) metabolism of testosterone (Usmani et al., 2003) and oestradiol (Usmani et al., 2006). Viswanath et al., (2010) reported chlorpyrifos as a most potent anti-androgenic compound. CPF significantly decreased the expression of

cytochrome P450scc, 2β-HSD and 17β-HSD, and decreased luteinizing hormone receptor stimulated cAMP production (Viswanath et al., 2010). Chlorpyrifos is an endocrine disruptor (Oruc, 2010).

There are various studies reporting that organophosphates act like suppressor of gene expression related to gonadotropin synthesis (LH and FSH level) or steroidogenesis (Walsh et al., 2000; Gore, 2001; Kitamura et al., 2003). Importantly, this compound blocks both adrenocorticotropin and cAMP-mediated steroidogenesis but not pregnenolone-driven steroid production, indicating that they target the steroidogenic pathway between the formation of cAMP and the production of pregnenolone (Civen and Brown, 1974; Civen et al., 1977).

Heteropneustes fossilis has been used, since 1960s, as a model for oocyte maturation studies due to best suitability for laboratory studies (Sundararaj and Goswami, 1977). The objective of the present study was to explore the impact of CPF in gonadotropin induced oocyte maturation of freshwater catfish, *Heteropneustes fossilis*. To see direct involvement of gonadotropin and CPF in oocyte maturation, *in vitro* study and analysis of maturation inducing steroid profile of post-vitellogenic follicles were performed.

2. Materials and Methods

2.1. Chemicals

The standard 5-pregnan,3β-ol-20one (Pregnenolone: P_5), 17β-dihydroxy-1,3,5(10)-estratriene (Estradiol-17β: E_2), 4-pregnen-21-ol-3,20-dione (deoxycorticosterone: DOC), pregn-4-ene-3,20-dione (Progesterone: P_4) and 17α-20β-dihydroxypreg-4-ene-3-dione (17,20βP: DHP) were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). The Ovidac® (human chorionic gonadotropin: hCG, Zydus Pharma Pvt. Ltd.) and Hilban® (20% EC CPF: chlorpyrifos, Hindustan insecticide Ltd.) were purchased from local medical stores. All other chemicals and reagents used were of analytical grade and were bought from HiMedia Laboratory Pvt. Ltd. India.

2.2. Animal collection and acclimatization

The experiments were performed in accordance with the local / national guidelines for animal experimentation and all care was taken to prevent any kind of cruelty to them.

The gravid female catfish, *Heteropneustes fossilis* (180±10 g) were collected from an aquatic dealer of local fish market during pre-spawning season (June) of annual reproductive cycle. They were maintained in laboratory condition with external aquarium filters of constant photoperiod (12:12 hr; L:D) and water temperature (24±1^{o}C) for two weeks. Fish were fed a diet of dead shrimp daily up to experiments. During the acclimatization, few fish were sacrificed to locate germinal vesicle (GV) migration under a dissecting microscope.

2.3. Preparation of oocyte incubation medium and clearing medium

The incubation medium was prepared (Mishra and Joy, 2006c) as follows: NaCl: 3.74, KCl: 0.32, $CaCl_2$: 0.16, $NaH_2PO_4.2H_2O$: 0.10, $MgSO_4.7H_2O$: 0.16, glucose: 0.40 and phenol red: 0.008 (in g) were dissolved in 1 L triple distilled water. The pH was adjusted to 7.5 with 1 N sodium bicarbonate and autoclaved. Penicillin (2,00,000 U) and streptomycin sulfate (200 mg) were added and filtered. The medium was stored at 4^{o}C and prepared fresh every week. The clearing solution to see the germinal vesicle within gravid oocyte was prepared as follows: ethanol/acetic acid/formalin; 6:1:3.

2.4. Collection and preparation of post-vitellogenic follicles

The acclimatized fish were sacrificed by decapitation and their ovaries were transferred to a petri-dish containing fresh cooled incubating medium. The rounded dark green colored post-vitellogenic follicles were separated from each other with the help of the fine brush and forceps without causing any mechanical injury to them. Each experimental and control group consisted of a triplicate set of incubation of about 30-40 post-vitellogenic follicles from three fish. During incubation experiment, if incubation medium showed change in colour, immediately replaced with fresh medium (colour change in incubation medium reflects a pH change of medium).

2.5. Experiments

2.5.1. Effect of hCG on oocyte maturation: Concentration and Time response study

About 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were incubated with different concentrations (0.5, 1.0, 1.2, 1.5, 1.7, 2.0, 2.2, 2.5, 3.0, 3.5 mg/ml) of hCG for 24 hr. Same number of post-vitellogenic follicles were incubated in plain incubation medium as a control. After 24 hr of incubation, the follicles were removed from the medium, cleared in clearing solution and examined under a stereo-binocular microscope for determining germinal vesicle breakdown (GVBD) percentage as an index for oocyte maturation.

For time response study, about 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were incubated in effective concentration (1.7 mg/ml, as per the result of above experiment) of hCG for 2, 4, 6, 8, 12, 16, 24 hr interval. Control was used as described above. At the end of each interval, the incubation medium was removed with the fresh plain incubation medium and incubation maintained further to complete 24 hr. After completion of experiment duration, follicles were processed for calculating the GVBD percentage as discussed earlier.

2.5.2. Effect of CPF on hCG induced oocyte maturation: Concentration and Time response study

About 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were incubated in medium containing different concentrations of CPF (0.0026, 0.026, 0.26, 2.6, 13, 26 pM/ml) with effective dose and duration of hCG (1.7 mg/ml, 8 hr; based on the result of above experiment). After 8 hr of duration of hCG, medium was replaced with incubation medium having only CPF concentration to complete 24 hr of the total incubation period. Control groups were maintained side by side. After 24 hr, the follicles were cleared in clearing solution and examined for calculating the GVBD percentage.

For time response study, about 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were co-incubated in medium containing an effective concentration of CPF (0.26 pM/ml; as examined earlier) for 4, 8, 12, 16, 24 hr interval with hCG (1.7 mg/ml, 8 hr). Control groups were maintained concurrently. After completion of each

interval, the incubation medium was replaced by medium having desired drug to complete 24 hr. In brief, at the end of 4 hr CPF, the incubation medium was removed with the effective concentration of hCG for remaining of effective duration. After the completion of total 8 hr of incubation of hCG (1.7 mg/ml), the incubation medium was replaced with the fresh plain medium for remaining period to complete 24 hr. At the end of 8 hr CPF, the incubation medium was replaced by plain medium to complete 24 hr. In remaining 12 and 16 hr CPF group, medium was first replaced at 8 hr with medium having CPF only for desired duration then in plain medium to complete total 24 hr. In 24 hr CPF effect, one change was done after 8 hr with plain incubation medium having CPF. After 24 hr of total incubation, follicles were processed for calculating the GVBD percentage.

2.5.3. Pre-incubation study

About 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were first incubated with the medium containing CPF (0.26 pM/ml) for 4, 8, 12 hr time periods. After the respective time intervals, the follicles were rinsed in fresh incubation medium and then transferred into the incubation medium containing hCG (1.7 mg/ml). After 8 hr of hCG, they were rinsed in fresh incubation medium and maintained in the plain incubation medium to complete of total incubation duration 24 hr. Control group was maintained side by side. At the end of 24 hr, follicles were processed for scoring the GVBD percentage.

2.5.4. Post-incubation study

About 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were first incubated with the medium containing hCG (1.7 mg/ml) for 8 hr. Then the follicles were rinsed in fresh incubation medium and transferred into the incubation medium containing CPF (0.26 pM/ml) for 4, 8, 12 hr. After each interval, the follicles were rinsed in fresh incubation medium and maintained in the plain incubation medium for up to 24 hr. Control incubation set was maintained side by side. At the end of incubation follicles were processed for scoring the GVBD percentage.

2.5.5. In vitro effects of hCG and CPF on steroidal hormone levels in post-vitellogenic follicles

About 30-40 post-vitellogenic follicles in triplicate from each fish (n=3) were co-incubated in medium containing effective concentration and duration of hCG (1.7 mg/ml, 8 hr) CPF (0.26 pM/ml, 12 hr). After each interval, the follicles were replaced with fresh incubation medium with desired drug and further maintained in plain incubation medium upto 24 hr. Positive and negative control incubation sets were maintained side by side as described above. At the end of incubation, follicles were pooled for each animal and kept in -20^{o}C for further steroid (P_5, P_4, DOC, E_2 and DHP) extraction and estimation by HPLC.

2.6. Study parameters

2.6.1. Germinal vesicle breakdown

At the end of each experiment, the follicles were cleared in a clearing solution (ethanol: formalin: acetic acid; 6: 3: 1; Trant and Thomas, 1988) for 3-4 minutes to visualize germinal vesicle and examined at 4X magnification under Bright field microscope (Olympus CX41) using micropublisher 3.3 RTV camera for determining the GVBD. The translucent follicles without germinal vesicle (GV) and opaque follicles containing GV were captured separately. The percentage of GVBD was determined as number of translucent follicles divided by the total number of the follicles incubated multiplication of hundred.

2.6.2. Extraction of steroids from the sample

The extraction was performed as per Mishra and Joy (2006 b). In brief, the pooled post-vitellogenic follicles form different experiments and control was homogenized separately in 4 volumes of cold PBS (0.02 M, phosphate buffered-saline pH 7.4) with a homogenizer at 0^{o}C. The homogenate was centrifuged at 20,000 g for 20 min. at 4^{o}C. The supernatant was extracted with two volumes of hexane, thrice and three volumes of diethyl ether, three times. The ether phase was collected, pooled, evaporated and dried

under N_2 gas and stored at -20^oC until chromatography. The incubation medium was directly extracted with diethyl ether and stored at -20^oC.

2.6.3. Chromatography

The chromatographic analysis of the steroids was conducted with a HPLC system (Waters, United States) consisting with two pumps (515 HPLC Pump) and a digital ultraviolet detector (2489 UV/ visible detector) with a variable wavelength (190-700 nm) based on Fastie-Ebert monochromator. The system was operated with Empower Pro-software (version: 6.00.00.00 copyright 2005 Waters corporation). The analysis was performed with a reversed phase C_{18} column (4.6 x 25. Mm; Waters Spherisorb$^{(R)}$ 5 µm ODS2). The isocratic mobile phase was 60% methanol in water. The flow rate was 1.5 ml/min. and the total run time was 30 min. (Nagahama and Adachi, 1985). The mobile phase and samples were degassed just before use. Before injecting the standard/sample, the column was activated with 100% methanol, followed by conditioning with the mobile phase. The analysis was monitored at 240 nm wavelength.

2.6.4. Preparation of standards and retention time determination

The steroids (P_5, P_4, DOC, E_2 and DHP) were dissolved in methanol separately to prepare stock solutions. From the stock solutions serial dilutions were made with methanol. The diluted solutions were filtered (0.2 µm) and injected into 20 µl loop of HPLC system with the help of microlitre syringe (Hamilton). The standards were tested individually at different concentration to record retention time and peak area under isocratic condition. This analysis was repeated three times with each standard.

2.6.5. Validation of the assay

2.6.5.1. Response-linearity

Different concentrations of standards in triplicate were injected into the column to set up concentration vs peak area curve. The response was linear with the concentration ranges used (E_2: 16 ng/ml-10 µg/ml; P_4: 0.67-80 µg/ml; P_5: 0.04-40 µg/ml; DOC: 0.4-80

µg/ml; and DHP: 12 ng/ml-20 µg/ml). The minimum detection limit for the assay was 0.24 ng per injection.

2.6.5.2. Recovery and sensitivity

Known concentrations of the standards in different dilutions were processed in the same manner as tissue samples (described above) and were injected into the column, after filtration. This study was repeated three times for each dilution. Percentage recovery was calculated from the concentrations of the standards injected directly and measured after extraction. The percentage recovery was 90-92% for $E_{2,}$ 96-98% for P_4, 93-95% for P_5, 97-99% for DOC and 94-98% for DHP. The values were not corrected for loss.

2.6.5.3. Inter- and intra-assay variations

Inter- and intra-assay variations were determined from five chromatograms each, using the same set or different sets of diluted standards. The inter- and intra-assay variations were, respectively, 14 and 5% for $E_{2,}$ 18 and 5% for P_4, 12 and 8% for P_5, 20 and 12% for DOC and 12 and 6% for DHP.

2.6.6 Sample analysis

The -20^{o}C stored dried samples of follicle were pooled (30-40 follicles in triplicate from three fish) and reconstituted in 1ml methanol. The reconstituted samples were filtered (0.2 µm) and 20 µl each of sample was injected into the column and eluted for 30 minutes. The samples were also co-eluted with known concentrations of the standards and compared with the elution of the respective standards in the mixture for identification and quantification of the steroids. Each sample was analyzed in triplicate in this manner. Chromatograms for blank were run with the vehicle (methanol and the mobile phase) to check any interference in the elution of the steroids. The blank had eluted before the steroids peaks appeared. The differences in the peak area between the standard and the standard with the sample in the chromatograms were recorded with the help of Empower Pro-software and the concentrations of steroids in samples were calculated.

2.7. Statistical analysis

The data were expressed as mean±SEM. The data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test ($p < 0.05$) for multiple group comparisons. The data of HPLC steroid quantification in different experimental condition were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$) for comparisons with control group.

3. Results

3.1. Morphological changes in post-vitellogenic follicles

In the control group, the oocytes were generally opaque in size at the beginning of migration of germinal vesicle. The germinal vesicle was located at the center or near the center of the oocyte. The hCG (human chorionic gonadotropin) incubated follicles showed migrated germinal vesicle (GV) from the center to the animal pole and finally the nuclear membrane underwent dissolution or GVBD (Figure 13). The follicles showing GVBD response were translucent as against the opaque ones in the control group.

3.2. Effect of hCG on percentage of GVBD: Concentration and time response study

The hCG induced a significant elicited effect on percentage of GVBD during the 24 hr incubation period ($F = 8769.42$; $p < 0.001$, one-way ANOVA; Figure 14A). The GVBD response was increased significantly over the concentration and gave maximum response (96.33%±0.87) at 1.7 mg/ml as compared to control group (2.33%±0.32) ($p < 0.05$, Newman-Keuls test; Figure 14A). This was used as effective concentration in forthcoming experiments.

A pulse study was also performed to know the effective minimum duration of hCG (1.7 mg/ml) to elicit GVBD response. The GVBD response was significantly high from 2 hr onwards but maximum at 8-24 hr as compared to control ($p < 0.05$, Newman-Keuls test). At 8 hr interval, hCG produced a significant increase in % GVBD (97.19%±0.87) ($F = 17631.07$; $p < 0.001$, one-way ANOVA) (Figure 14B). Therefore,

the minimum effective duration of hCG that produce maximum maturation was 8 hr. All the incubation sets recorded nil mortality of follicles.

3.3. Effect of CPF on hCG induced GVBD response: Concentration and time response study

CPF showed an inhibitory effect on hCG induced oocyte maturation in a concentration dependent manner as compared to alone gonadotropin (hCG) incubated follicles (F = 6255.47; p <0.001, one-way ANOVA; Figure 15A). The inhibition was significantly higher with 0.26-26 pM/ml concentrations where it had shown 90% inhibition as compared with a hCG group (p <0.05, Newman-Keuls test). Based on this result, the minimum concentration of CPF that inhibited maximum hCG induced GVBD was taken 0.26 pM/ml in next experiments.

A time response study of CPF (0.26 pM/ml) was conducted to know the effective minimum duration to inhibit hCG induced GVBD. The results showed that GVBD % was significantly inhibited 90%±1.2 from 12 hr onwards as compared to hCG group (p <0.05, Newman-Keuls test). Although the CPF inhibition was significant at all intervals compared with the hCG groups (F = 1782.81; p <0.001, one-way ANOVA; Figure 15B). Therefore, in successive experiments 4, 8, 12 hr treatment duration was selected to record CPF response. All the set showed nil mortality of follicles.

3.4. Pre-incubation and Post-incubation study

Both pre- and post-incubation study showed significant inhibition by CPF in hCG induced GVBD in all tested durations (4, 8, 12 hr). The percentage of CPF inhibition in hCG induced oocytes was increased with duration of CPF treatment in both pre- and post-incubation treatment in a duration dependent manner (pre-incubation inhibition (%): 52±0.06, 75.67±0.13, 87.67±0.14; post-incubation inhibition (%): 18±0.87, 44±1.15, 76±0.57; for 4, 8, 12 hr). However, the level of CPF inhibition in hCG induced GVBD was more in pre-incubation experiments as compared to post-incubation (Figure 16).

3.5.Steroid separation and retention time

With the chromatographic assistance and its adoptive condition, P_5 was eluted first, followed by E_2, DOC, P_4 and DHP. The retention times were 4.7, 5.9, 6.5, 11.6 and 21.3 min. respectively. However, in case of samples elution, the retention time of identified steroids showed minor shifts (Figure 17, 18). The authentication of steroids elution in sample was performed by co-running with known concentration of the standard in the sample mixture and compared with the peak of standard alone.

3.6. In vitro effects of hCG and CPF on steroid hormone levels in postvitellogenic follicles

Human chorionic gonadotropin (hCG) enhanced the steroid synthesizing capability of oocyte and stimulated the meiotic resumption promoting steroids (P_5, P_4, DOC and DHP) significantly as compared to control group (p <0.001, two-way ANOVA; Figure 18). However, the E_2 level was decreased significantly (p <0.05, Tukey's test) (Figure 19). The co-exposure of CPF and hCG incubated follicles showed significant decreased in concentration of P_5, P_4 and DHP as compared to control group and hCG group (p <0.05). However, the DOC and E_2 levels were increased in the CPF alone and in co-incubation with hCG exposed follicles as compared to control group (p <0.05) (Figure 18). In control group, all hormones were detected, maximum level was of E_2. Other maturation inducing steroids were in their lower concentration in control group.

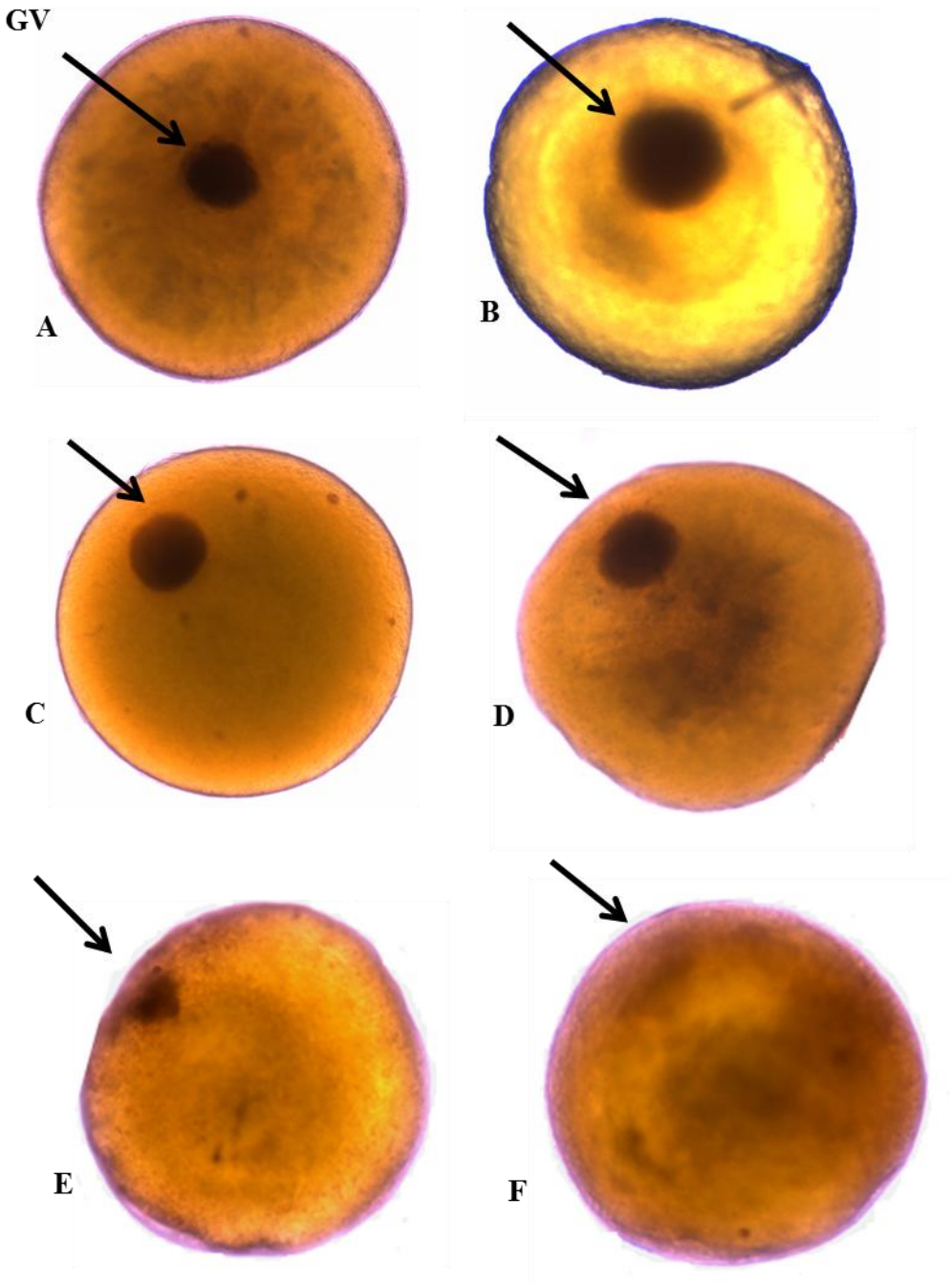


Figure 13: Process of oocyte maturation activity of *Heteropneustes fossilis* after the hCG treatment. A-E: showed visible movement of germinal vesicle (GV) and F: showed the germinal vesicle breakdown (GVBD).

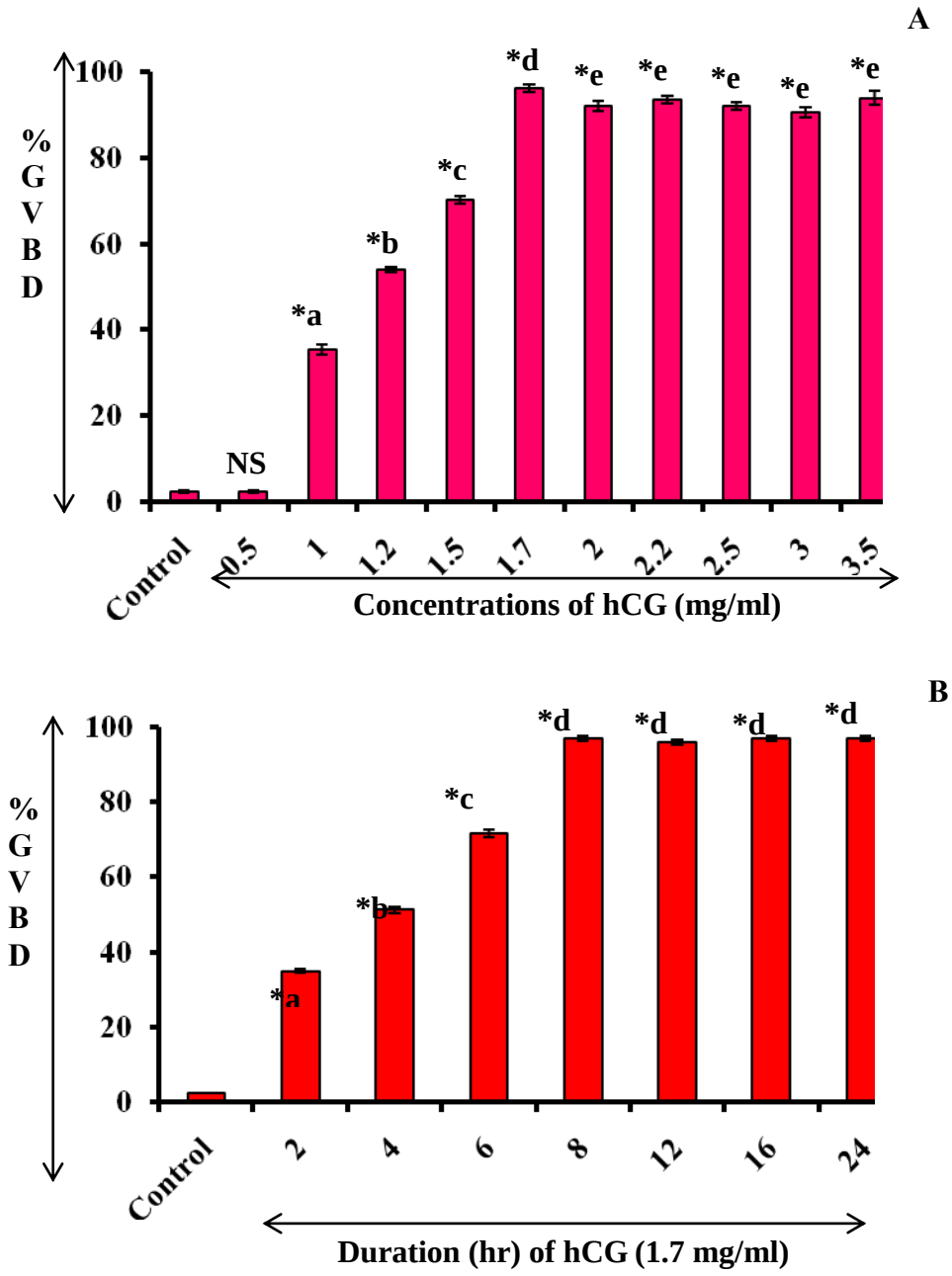


Figure 14: (A) Effect of different concentrations of hCG on *in vitro* oocyte maturation of gravid *H. fossilis* post-vitellogenic follicles. (B) Pulse study of effective dose (1.7 mg/ml) of hCG on *in vitro* oocyte maturation. Values were mean±SEM of percentage GVBD of 30-40 follicles incubated in triplicate (n=3) for 24 hr. Data were analyzed by one way ANOVA ($p < 0.001$), followed by Newman-Keuls test ($p < 0.05$) for comparison within the groups. Asterisk (*) showed significant different from the control group at $p < 0.05$. NS showed non-significant from the control group. Group superscripted with different letter shows significant difference ($p < 0.05$) whereas same letter showed non-significance.

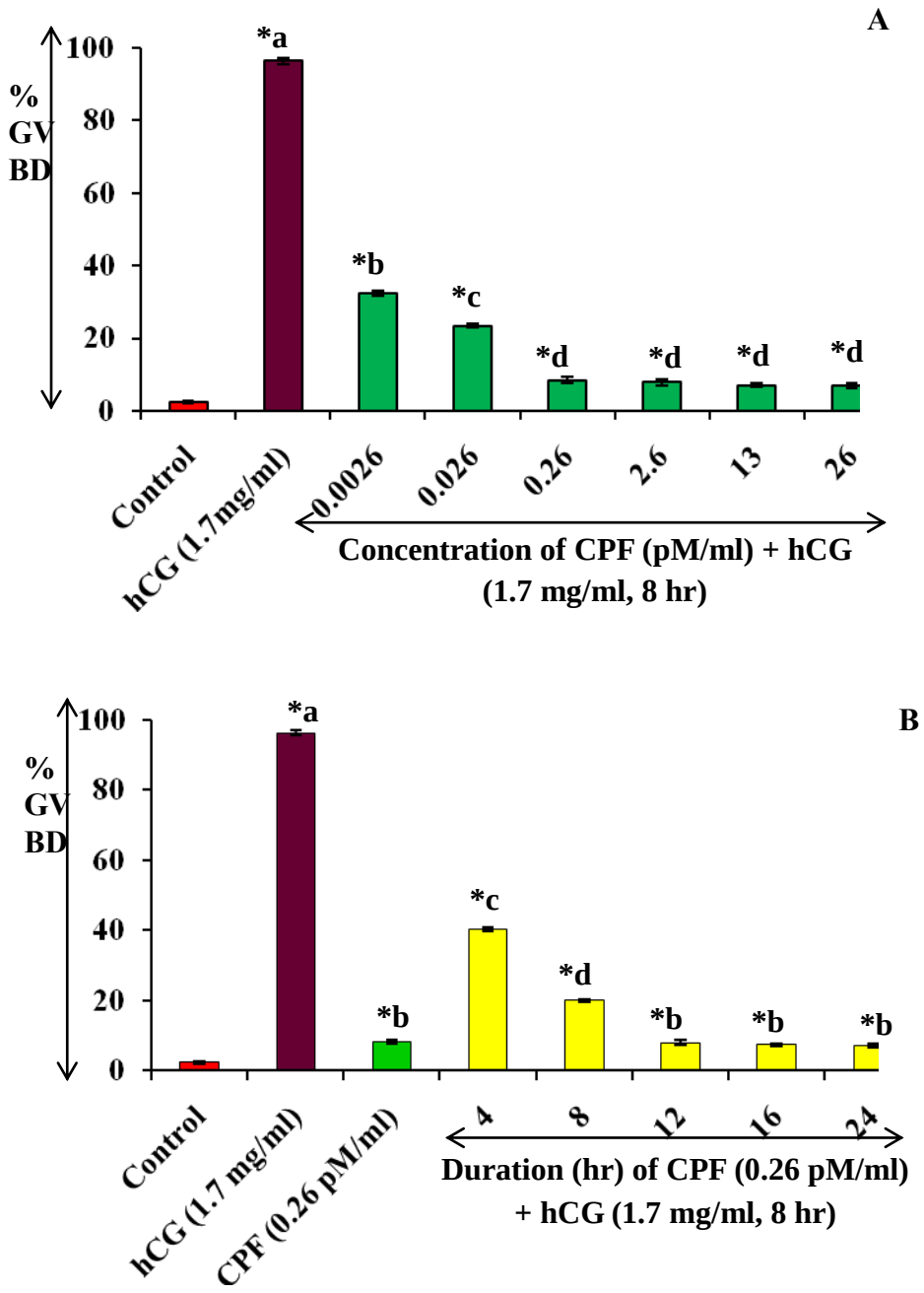


Figure 15:(A)Effect of different concentrations of CPF on hCG induced *in vitro* oocyte maturation. (B) Duration effect of CPF (0.26 pM/ml) on hCG (effective dose and duration: 1.7 mg/ml, 8 hr) induced *in vitro* oocyte maturation of gravid *H. fossilis*post-vitellogenic follicles for 24 hr. Values were mean±SEM of percentage GVBD of 30-40 follicles incubated in triplicate (n=3) for 24 hr. Data were analyzed by one way ANOVA ($p < 0.001$), followed by Newman-Keuls test ($p < 0.05$) for comparison within the groups. Asterisk (*) showed significant different from the control group at $p < 0.05$. Group superscripted with different letters showed significant difference ($p < 0.05$) whereas same letter showed non-significance.

+ hCG (1.7 mg/ml, 8 hr)

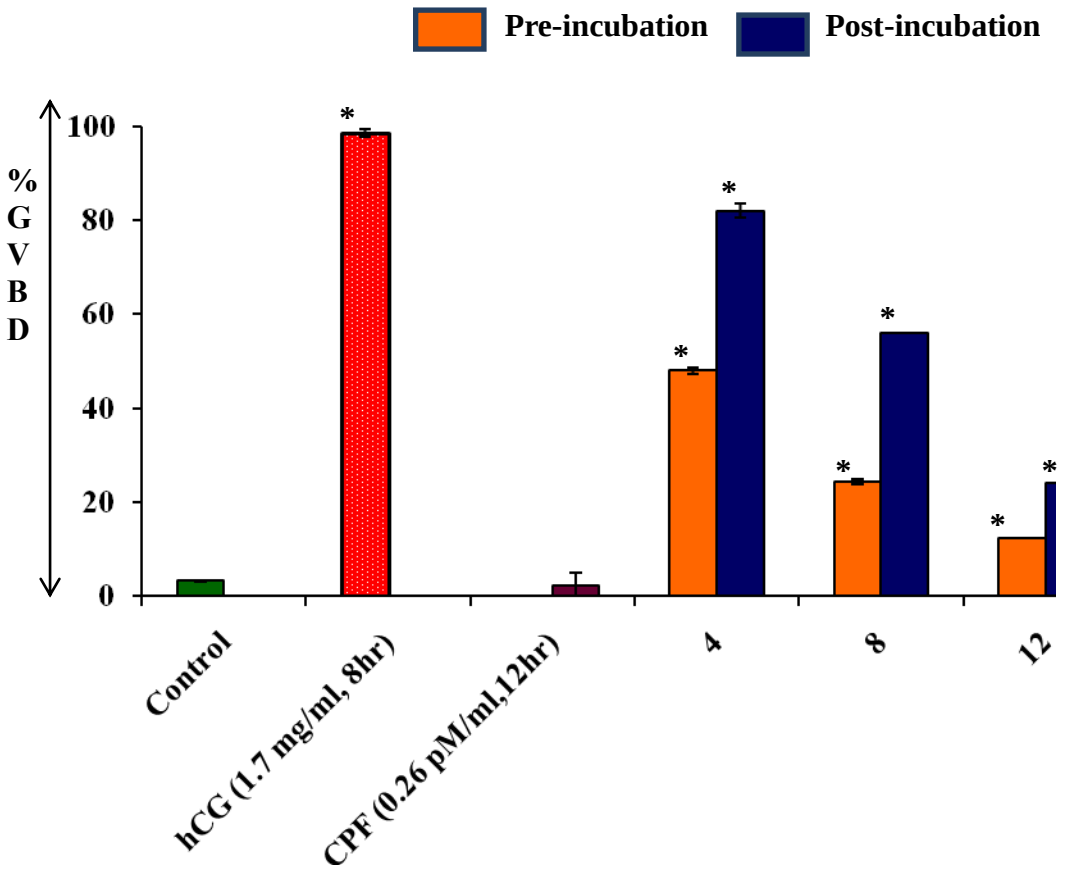


Figure 16: *In vitro* effects of pre- and post-incubation of post-vitellogenic follicles with CPF (0.26 pM/ml) for 4, 8 or 12 hr on hCG induced GVBD. Values were mean±SEM of percentage of germinal vesicle breakdown (GVBD) of 30-40 follicles each incubated in triplicate (n=3). Data were analyzed by one way ANOVA ($p < 0.001$), followed by Newman keul's test ($p < 0.05$) for comparison within the groups. Asterisk (*) showed significant different from the control group at $p < 0.05$.

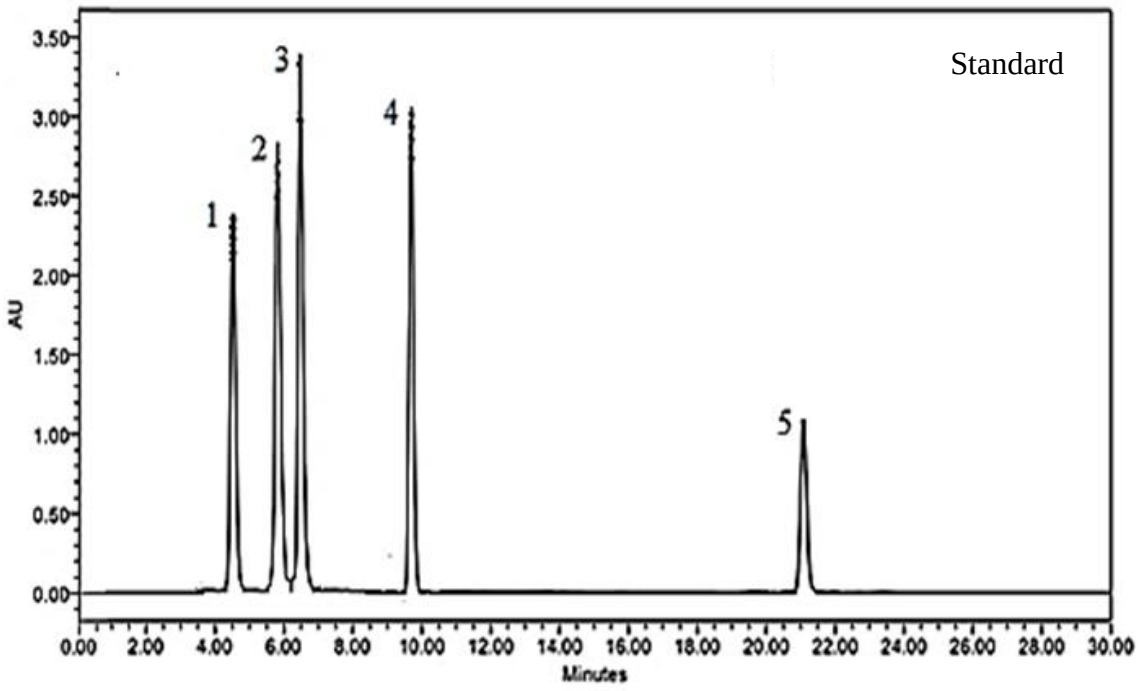


Figure 17: HPLC chromatogram showing separation profile of different steroids examined in the current study. Peak 1- P_5: 5-pregnan,3β-ol-20one (retention time = 4.7 min.; 0.15 µg/µl concentration); peak 2- E_2: 17β-dihydroxy-1,3,5(10)-estratriene (retention time = 5.9 min; 0.2 µg/µl concentration); peak 3- deoxycorticosretone: 4-pregnen-21-ol-3,20-dione (retention time = 6.5 min; 0.15 µg/µl concentration); peak 4- P_4: Pregn-4-ene-3,20-dione (retention time = 11.6 min; 0.15 µg/µl concentration) and peak 5- 17,20βP: 17α-20β-dihydroxypregn-4-ene-3-dione (retention time = 21.3 min; 0.2 µg/µl concentration).

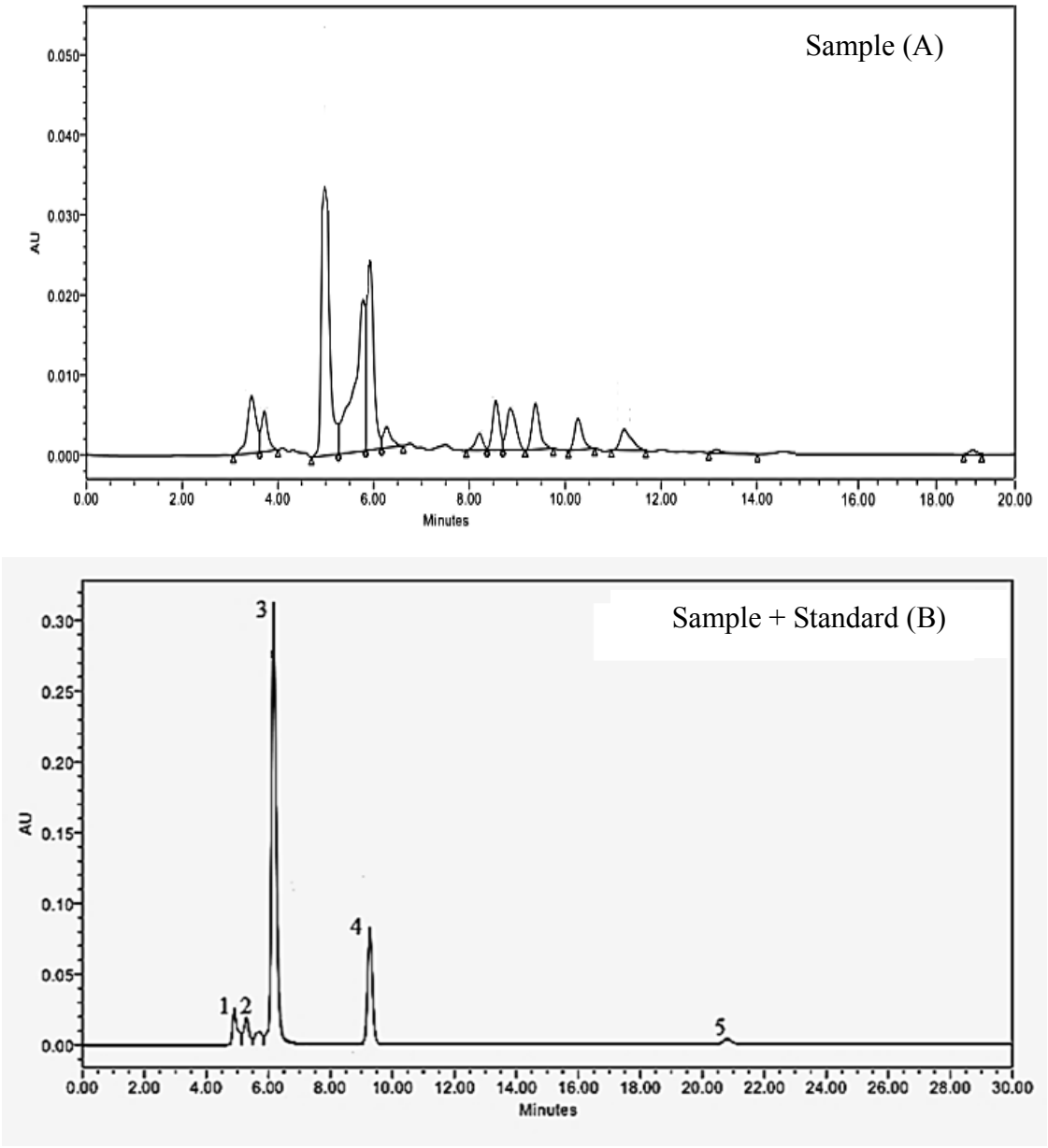


Figure 18: HPLC chromatograms showing elution profile of induced hCG (1.7 mg/ml) post-vitellogenic follicles exposed to CPF (0.26 pM/ml) for 24 hr and (B) sample in the presence of known concentration of standards (Peak 1- P_5: 5-pregnan,3β-ol-20one, peak 2- E_2: 17β-dihydroxy-1,3,5(10)-estratriene, peak 3-deoxycorticosretone: 4-pregnen-21-ol-3,20-dione, peak 4- P_4: Pregn-4-ene-3,20-dione and peak 5- 17,20βP: 17α-20β-dihydroxypregn-4-ene-3-dione).

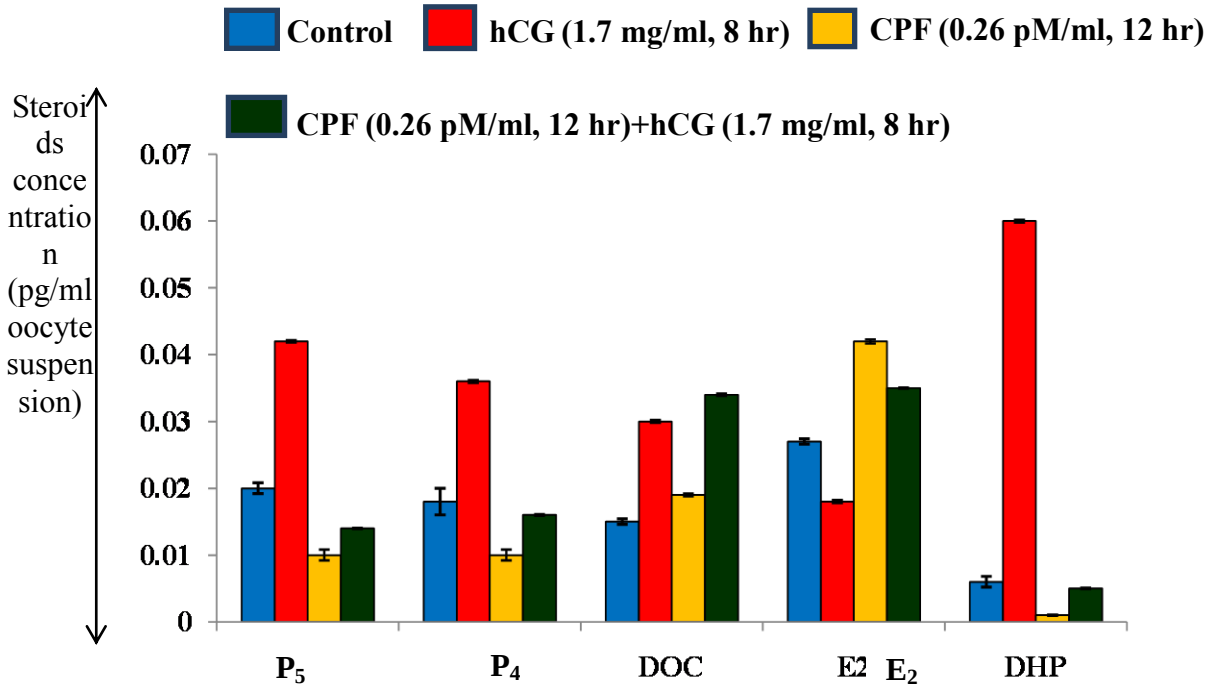


Figure 19: A comparative steroid profile of co-incubated post-vitellogenic follicles in hCG (1.7 mg/ml, 8 hr) and CPF (0.26 pM/ml, 12 hr) in comparison with different positive and negative control groups. After the respective drugs incubation duration, the experiment was continued up to 24 hr with plain incubation medium.. Values were mean±SEM (n=3). Data were analyzed by two way ANOVA ($p < 0.001$), followed by Tukey's test ($p < 0.05$). Asterisk (*) showed significant difference from the control group ($p < 0.05$). Note: P_5: Pregnenolone; P_4: Progesterone; DOC: deoxycorticosterone; E_2: Estradiol-17β; DHP: 17,20βP.

4. Discussion

The migration and breakdown of germinal vesicle are the first two morphological signs of oocyte maturation. GVBD which involves undulation, folding, fragmentation and disintegration of nuclear membrane at or before metaphase I (Masui and Clarke, 1979) has been the commonly used bioassay system of oocyte maturation. The oocytes were ready for resumption of meiosis after completion of the growth phase. Post-vitellogenic oocytes possess a large nucleus (germinal vesicle: GV) in the center in meiotic prophase I (Nagahama, 1983). Following the migration of germinal vesicle, the GV membrane disappears known as Germinal vesicle breakdown (GVBD), which indicates the end of prophase I. In teleost fishes, final oocyte maturation consists of the migration and breakdown of GVBD, chromosome condensation and formation of the first polar body (Goetz, 1983; Thomas, 1994; Mishra and Joy, 2006c).

The present result support that hCG (human chorionic gonadotropin) was capable of stimulating *in vitro* oocyte maturation in post-vitellogenic follicles of catfish, *H. fossilis* in a concentration and duration dependent manner. The hCG incubated follicles showed GV migration from the center to animal pole and finally the nuclear membrane underwent dissolution or germinal vesicle breakdown (GVBD). The follicles showing GVBD was translucent as against the opaque ones in the control follicles (Sundararaj et al., 1985). The response is similar to the earlier reported studies in different fish species *in vitro* and *in vivo* (Scott and Canario, 1990; York et al., 1993; Kagawa et al., 1994; Joy et al., 1998; Sorbera et al., 1999; Matsuyama et al., 2001; Lubzens et al., 2010). This species variation reflects with difference in the effective dose and duration of hCG (Sorbera et al., 1999; Zuberi et al., 2002).

The organophosphorous pesticide, chlorpyrifos (CPF) showed an inhibitory effect on hCG induced oocyte maturation in a concentration and duration manner. The earlier investigations also supported CPF exposure significantly knocked down the gonadotropin induced GVBD (Haider and Upadhyaya, 1986; Haider and Inbaraj, 1988). Haider and Upadhyaya, (1986) also reported that organophosphates significantly reduced the rate of GVBD in oocytes of the catfish, *M. vittatus*, *in vitro* even in the presence of

gonadotropin. The inhibition of hCG induced oocyte maturation was more in pre-incubation set as compare to post-incubation set. This suggest that CPF inhibit gonadotropin induced GVBD in a receptor dependent mechanism (Gore, 2001).

Gonadal steroids are the terminal or subterminal regulators of gametogenesis, oocyte maturation and ovulation. In teleosts, E_2 is the major regulator of vitellogenesis, a complex and elaborate process responsible for the growth of the follicle and the yolk so deposited are used during early embryonic development. 17,20βP is the maturation-inducing steroid that induces resumption of meiosis (Nagahama, 1997). In post-vitellogenic ovary, the C_{18}-C_{19} steroidogenic pathway is down regulated and the progestin pathway is up regulated by the LH surge to induce oocyte final maturation and ovulation. *In vitro* gonadotropins do not act directly on the oocyte to induce resumption of meiosis, but instead initiates maturation of the oocyte by synthesis of a MIS by the ovarian follicles (Senthilkumaran et al., 2004).

Control group follicles registered the presence of all measured hormones but high E_2 level was sufficient to keep follicles in their meiotic arrest form (Sundararaj and Goswami, 1977; Goetz, 1983; Jalabert et al., 1991; Mishra and Joy, 2006c). The result registered in a sharp decrease in E_2 level which may be due to conversion of E_2 in its metabolites (hydroxyl-metabolites) that enhanced the maturation process (Fostier et al., 1983; Mishra and Joy, 2006c). This increase and decreased level of the steroids may interpret their conversion in another or may adopt another pathway. Among the measured steroids DHP, P_4 and DOC associated with the maturational activity and ovulation. *In vitro* oocyte maturation by hCG resulted in the stimulation of progestin pathway (C_{21}) and inhibition of estrogen pathway (C_{18}) (Kobayashi et al., 1988; Zhu et al., 1989; Pinter and Thomas, 1999; Senthilkumaran et al., 2004). The *in vitro* treatment of hCG triggered the level of these steroids in post-ovulatory follicles as compared to control. The pregnenolone (P_5) level was also increased about two fold with hCG stimulus. The rise in P_4 and DHP could be correlated with its MIS activity within follicles (Goetz 1983; Jalabert et al., 1991; Nagahama, 1997; Matsuyama et al., 2001; Rahman et al., 2002; Garcia-Alonso et al., 2004; Semenkova et al., 2006). Many researchers found that the increased production of DHP was associated with oocyte germinal vesicle migration and GVBD (Sundararaj et al., 1985; Zhoa and Wright, 1985; King et al., 1995; Amiri et al.,

2001; Garcia-Alonso et al., 2004). DOC is a known maturation inducing steroid in *H. fossilis* (Sundararaj and Goswami, 1977; Sundraraj et al., 1979). The result stated that hCG act as a possible maturation inducing agent *in vitro* which was supported by the findings of York et al. (1993), Joy et al. (1998), Sorbera et al. (1999), Matsuyama et al. (2002). Apparently increased production of these steroids in response to hCG is correlated with stimulation of P450c17 and 20β-hydroxysteroid dehydrogenase (20β-HSD) (Senthilkumaran et al., 2004; Nagahama and Yamashita, 2008).

However, CPF exposure caused suppression of maturation inducing steroids (P_4, P_5 and DHP) in post-vitellogenic follicles of *H. fossilis*. CPF inhibited oocyte maturation via manipulating hormone level that was triggered by hCG within follicle. In CPF alone, E_2 level was too high that maintains arrest. The inhibition of DHP by CPF seems to be the plausible mechanism to explain the adverse effect of this known endocrine disruptor (Sonnenschein and Soto, 1998). The E_2 and DOC concentrations were significantly increased in co-incubation with CPF groups as compared to hCG alone group. However, the increased concentration of E_2 was more pronounced than DOC. Presence of corticosteroids in *in vitro* follicles study was also reported earlier (Sundararaj et al., 1979; Mishra and Joy, 20006c; Radha et al., 2010). Control group shown the presence of DOC that was increased in all treated groups, hCG, CPF and co-incubation group. Though level of increase, when compared among the groups, was more in combination group of hCG and CPF followed by alone hCG. It may be due to gonadotropin influence synthesis of P_4. In the presence of CPF, hCG induced progestin pathway was decreasing. The increased level of DOC due to CPF alone and in co-incubation with hCG group suggested an additional mineral operated channel, that may linked with hydration of oocyte (Bry, 1985; Milla et al., 2006; Radha et al., 2010). On the basis of current result it may concluded that CPF inhibits DHP by keeping estradiol in higher level and induced DOC. The catfish ovary has reported DOC importance in oocyte maturation, oocyte hydration and ovulation (Sunderaraj and Goswami 1977; Bry 1985; Radha et al., 2010). Increase in DOC concentration occurs in the peripheral blood during the initiation of spawning indicates its possible importance in fish ovulation (Colombo et al., 1978; Milla et al., 2006). This supports an idea that CPF may involve in pre-mature hydration or ovulation process.

The steroid profile of P_5, P_4 and DHP hormone after the exposure of CPF on hCG induced post-vitellogenic follicles resulted into three to tenfold decrease. Since P_4 and P_5 are precursors, of DHP, therefore their reduction reflected may be due to their conversions via another pathway (21 hydroxylase, P450 with 17-20 Lyase activity) in to estradiol and deoxycorticosterone. In alone group, CPF increased both E_2 and DOC. This increase in E_2 maintains oocyte meiotic arrest (Jalabert et al., 1991; Mishra and Joy, 2006c). This might be due to the fact that CPF might have differentially altered the key enzymes involved in P_4, DHP and E_2 production. CPF are known to inhibit cytochrome P450-dependent enzymatic activities (Walsh et al., 2000), and thus might disrupt steroidogenesis and, consequently, DHP biosynthesis (Pope, 1999; Schuh et al., 2002; Gupta, 2004; Slotkin, 2004).

Thus, organophosphorous could have directly targeted the enzyme's active site or affected the availability and/or activity of cofactors and reducing equivalents (Walsh et al., 2000). The P450 side chain cleavage (P450scc) enzyme is part of the cholesterol side chain cleavage enzyme system (CSCC) (Simpson, 1979). CPF decreased the activity of P450scc enzyme. These pesticides disturb steroid secretion, either by impairing their synthesis or release, resulting in reproductive deficiencies in fish (Singh and Singh, 1980a, b). The pesticides interfere with the production of free cholesterol, the sex hormone precursor, and hence reduce steroid production (Lal and Singh, 1987).

CHAPTER – III

Effect of organophosphorous pesticide, chlorpyrifos on embryogenesis of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)

ABSTRACT

The present investigation was aimed to study the effect of chlorpyrifos on hatchlings of freshwater catfish, *Heteropneustes fossilis*. For this, *in vitro* and *in vivo* experiments were performed. In *in vitro* experiment, the fertilized eggs stripped from normal control catfish were exposed to different concentrations of chlorpyrifos viz., 0.52, 0.26, 0.052, 0.026, 0.013, 0.006, 0.004 µM/l. For *in vivo* study, the induced female catfish with synthetic hormone (hCG) was exposed to same concentrations of chlorpyrifos. The experiment was done in triplicates along with the control group. The result showed that both, fertilization and hatching rate was decreased significantly in exposed group in comparison to control. *In vivo* exposure showed significantly low embryo mortality (27.2±0.76%) ($F=893.67$; $p < 0.05$) and high hatching rate (90.5±0.5%) ($F=271.87$; $p < 0.05$) as compared to *in vitro* study(35.2±0.81% and 85.4±1.25%) ($F=1070.28$, $F=267.23$; $p < 0.05$) in lower concentration of CPF (0.004 µM/l). The percentage of malformed hatchlings was higher in *in vitro* experiment as compared to *in-vivo*. The occurrence of all studied parameters was observed in a concentration dependent manner, as the concentrations of CPF were increased, the percentage of embryo mortality and malformed hatchlings was decreased. However, the hatching rate was increased. The result showed that exposed group of hatchlings were affected by many morphological and notochordal deformities viz., ventral or lateral tail flexure, abnormal spinal bending, irregular head shape and size, loss of eye, reduced barbel, pericardial edema, yolk sac edema, notochordal defect, tail fin flexure, reduction of brain development and reduction of pigmentation. The result suggested that OPs play an important role in inducing the

muscular dystrophy and morphological deformities during embryonic development and organogenesis.

*Keywords:*Chlorpyrifos, fertilization rate, embryo mortality, hatching rate, morphological deformities

1. Introduction

The study of fish embryogenesis is an important aspect to know the developmental processes of any fish spp. and also to understand species-specific adaptations and their ecological value in the course of speciation (Meijide and Guerrero, 2000). Fish are largely used for the assessment of the quality of aquatic environment and as such can serve as bio-indicator species of environmental pollution (Lopes et al., 2001; Whitefield and Elliott, 2002; Dautremepuits et al., 2004). However, to understand the effects of environmental contaminants on fish, their action should be studied during the main life cycle, especially during the first stages of development. Measures of toxicity derived from these early life stage tests can provide a strong indication of the potential range of biological effects of toxicant action (U.S. EPA 2002). The slight alterations or modifications in the external environment, may lead to developmental malformations. These changes produced as a result of metabolism of teratogens and interacting factors. Embryonic responses to teratogens occur at various developmental stages and at different levels of organogenesis that may lead to modification of normal development.

The hazardous effects of rapidly degraded organophosphorous pesticides (OPs) are less evident. Organophosphorous pesticides may disrupt normal patterns of neuronal connectivity in the developing nervous system (Howard et al., 2005). The role of cholinesterase during organogenesis (Moody and Stein, 1988) and anti-ChE activity of organophosphorous compounds should not be ignored. The history of OP compounds and anti-ChE activity is more than a century old but the study on this topic seems to be highly neglected. The scanty literature on the subject and consistent reports of harmful effects on vertebrates and invertebrates (Rull et al., 2006; Peiris-Johna and Wickremasinghe, 2008) is contradictory. The importance of AChE in the function of the nervous system has been recognized for a long time, yet its role in development remains

mysterious (Brimijoin and Koenigsberger, 1999). AChE is transiently expressed during discrete periods of neural development of the thalamocortical pathways, and transient AChE activity correlates with the specific growth of thalamic axons into the cortex and synaptogenesis with cortical neurons (Robertson and Yu, 1993). In addition, significant sequence similarity exists between AChE and cell adhesion proteins that function in morphogenic phenomena. These observations have led to the hypothesis that AChE may play key roles in neural development.

Chlorpyrifos (CPF) an organophosphorous pesticide is a most potent neuro-toxicant. AChE inhibition is the primary manifestation of toxicity during OP exposure (Thomson et al., 1991; Sultatos, 1994; Calumpang et al., 1997; Fulton and Key, 2001). CPF neurotoxicity mediated by the phosphorylation and subsequent inhibition of acetylcholinesterase (AChE) (Oehmichen and Besserer, 1982; Finkelstein et al., 1988), whose primary function is to terminate nerve impulse transmission at cholinergic synapses by hydrolyzing the neurotransmitter acetylcholine (ACh) (Kennedy, 1991; Murty and Ramani, 1992). This inactivation of AChE results to nerve exhaustion, nervous system failure and ultimately to death. Most chemicals in this group require oxidative desulfuration to achieve their greatest cholinesterase-inhibiting potencies.

Developmental exposure to CPF elicits long-lasting alterations in cell-signaling cascades. Further, they are shared by various neurotransmitter and hormonal inputs. Thus resulting in a abnormalities of synaptic communication and affect the corresponding behaviors (Meyer et al., 2004b). There are several studies which were controversial regarding fetal and embryotoxicity of OPCs. For example some organophosphorous pesticide like: parathion, diazinon, malathion and dichlorvos induced maternal toxicity but there is no evidence of teratogenicity (Vogin et al., 1971; Talens and Wooley, 1973; Spyker and Avery, 1977; Baksi, 1978). However another OP, dipterex has shown to cause teratogenic effect at high concentration (Baksi, 1978) and acephate was found to cause developmental toxicity at maternal toxic dose to mice (Staples et al., 1976). Chung et al. (2002) also reported that flupyrazofos, a new OP, causes fetal growth retardation at maternal toxic doses in rats. Similarly in Swiss albino mice, Ambali et al. (2010) reported that chlorpyrifos affected conception and pre-implantation losses in dose dependent manner. Eskenazi et al. (1999) reviewed that there

are numerous animal studies have shown that in utero or early exposure to OP pesticides affect neurodevelopment. There are growing data showing that they are teratogenic on the grounds of experimental concentrations in non-mammal developing embryos, such as amphibians (Richards and Kendall, 2002) and birds (Meiniel, 1981). Exposure to CPF can affect both the fertility and reproductive outcomes because of its ability to interact with steroid hormones receptors (Peiris-John and Wickremasinghe, 2008). CPF target the early stage of development of the cerebral and cerebellum cortice (D'Arcangelo et al., 1995; Eksloglu et al., 1996; Gleeson et al., 1998; Keller and Persico, 2003). It's*in vivo* administration has been associated with deficits in neurons and cholinergic functions (Jameson et al., 2006).

Organophosphorous compounds are able to induce muscular damage (Gupta et al., 1987; Karalliedde and Henry, 1993; De Bleecker et al., 1994; John et al., 2003). The teratogenic effects may be observed as morphological abnormalities or functional defects that may only occur later in life. Developmental toxicity also involves non-permanent manifestations such as growth retardation and edema. Thus, the present work was dealt with the teratogenic potential of chlorpyrifos (CPF) in freshwater catfish, *Heteropneustes foosilis* hatchlings in relation to the fertilization rate, hatching rate, embryo mortality and percentage of malformations during development.

2. Materials and Methods

2.1. Chemicals

The organophosphorous pesticide, chlorpyrifos (CPF) was used which was procured from the local market of Lucknow, Uttar Pradesh, India, under the trade name Hilban® (20% EC CPF), supplied by Hindustan insecticide limited. The Ovidac® (human chorionic gonadotropin: hCG, Zydus Pharma Pvt. Ltd.) was purchased from local medical stores. All other chemicals and reagents used were of analytical grade and were bought from HiMedia Laboratory Pvt. Ltd. India.

2.2. Animal collection and their acclimatization

Experiment was performed in accordance with local/ national guidelines of ethical committee for experimentation in animals to avoid any type of cruelty.

The healthy brooders of freshwater catfish, *Heteropneustes fossilis* of relatively same size (17±2 cm) and weight (185±20 gm) were collected from commercial fisherman of Lucknow, Uttar Pradesh, India. Brooders were brought to the laboratory in wide mouthed large plastic containers in natural water avoiding stresses and injuries as possible. Later, they were acclimatized in 120 l glass aquaria containing water having a pH of ≈ 7.5, dissolved oxygen 5-6 mg/l and a temperature of 24±1^{o}C for one week. The photoperiod was 12h: 12h (light: dark). Water was renewed daily to remove faecal matter and waste metabolite of fish during acclimatization. During this period, Fish were fed a diet of dead shrimp.

2.3. Teratogenic assay

2.3.1.In vitro experimental setup

To study the *in vitro* effect of chlorpyrifos, male and female fish were selected based on the external morphological features. The female fish were artificially induced by synthetic hormone (hCGhormone: 6.95 IU/g dose of body mass) intramuscularly. Experiment was conducted in five replicates.Approximately 12 to 14 h after the injection, eggs were obtained by stripping method into fertilization tray and fertilized by sperm suspension that was previously obtained by mincing of adult male testes in 0.4% fish saline. Successful fertilization was achieved when the eggs were oriented with the red cap animal pole side up. Screening of unfertilized eggs was performed. After obtaining fertilized eggs, one fifty eggs were kept in different concentrations of chlorpyrifos viz., 0.52, 0.26, 0.052, 0.026, 0.013, 0.006 and 0.004 µM/l along with control set of fertilized eggs in five replicates (n=3). These concentrations were selected on the basis of sub-lethal toxicity experiment of CPF in pre-spawning phase as discussed earlier in Chapter 1. Dead eggs become white due to coagulation and precipitation of protein. Dead eggs were counted and removed daily. At the end of incubation period, the hatched eggs were counted. The teratogenic changes were recorded after 24 hr of hatching.Water was changed daily with same concentration of pesticide.

2.3.2. In vivo experimental setup

For *in vivo* study, a set of injected female brooders with hCG (6.95 IU/g dose of body mass) were transferred in different concentrations of chlorpyrifos viz., 0.52, 0.26, 0.052, 0.026, 0.013, 0.006 and 0.004 µM/l along with control set upto their desired ovulation time. Approximately after 10 to 12 hr of latency period, the eggs were obtained by applying gentle pressure on the female's abdomens were put into rectangular plastic fertilization tray and then artificially inseminated with a milt of sperm suspension previously obtained by mincing adult male testes in 0.4 % fish saline. The fertilized eggs were identified by the appearance of red cap at animal pole side up. Thereafter, within a one minute the screening of unfertilized eggs was performed. The obtained fertilized eggs were transferred into normal tap water for analysis of further observations.

2.4. Data collection

The fertilization and hatching rate were calculated by formula as given below:

Fertilization rate (%) = (No. of fertilized eggs/ Total no. of eggs) × 100

Hatching rate (%) = (No. of eggs hatched/ Total no. of fertilized eggs) × 100

The data were expressed as mean±SEM. The data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test ($p < 0.05$) for multiple group comparisons. For the morphological examination of surviving hatchlings, the images were captured under Bright field microscope (Olympus CX41) using micropublisher 3.3 RTV camera per 24 h interval.

3. Results

The purpose of the present investigation was to study the effect of chlorpyrifos (CPF) on the fertilization rate, hatching rate and malformations of freshwater catfish, *Heteropneustes fossilis*. All the observed parameters were concentration dependent of chlorpyrifos.

3.1. Fertilization rate and embryo mortality

The result showed that chlorpyrifos (CPF) exposure caused lower fertilization rate as compared to control (98.7±0.5%). The fertilization rate was depending on the concentrations of CPF. At higher concentration of chlorpyrifos (0.52 µM/l), the fertilization rate was significantly lower i.e., 42.7±0.94% and at lower concentration of CPF (0.004 µM/l), rate was significantly higher 80.37±0.76% (F=289.23; $p < 0.05$) (Figure 20A).

The organophosphorous pesticide, chlorpyrifos caused severe embryo mortality. The present findings represented that in *in vitro* exposed group, the percentage of embryo mortality was higher as compared to *in vivo* exposure. The severity of toxicity was occurred in a concentration dependent manner. The control group had negligible mortality (0.2±0.12%) as compared to exposed group of CPF. *In vitro* exposure of CPF caused significant high embryo mortality (90.0±0.42%) at high concentration of CPF (0.52 µM/l) whereas at low concentration of CPF (0.004 µM/l), 35.2±0.81% embryo mortality was found (F=1070.28; $p< 0.05$). However *in vivo* exposure of CPF showed significantly low embryo mortality (27.2±0.76%) at low concentration of CPF (0.004 µM/l) and high mortality (79.7±0.94) was found at high concentration of CPF (0.52 µM/l) (F=893.67; $p< 0.05$) (Figure 20B).

3.2. Hatching rate and malformed hatchlings percentage

The hatching rate was also depending on different concentrations of CPF. *In vitro* exposure caused decreased hatching rate as compared to *in vivo* exposure of CPF. In *in vitro* exposure, the significant low hatching rate (33.83±2.12%) was noticed in higher

concentration of CPF (0.52 µM/l) as compared to lower concentration of CPF (0.004 µM/l), it was 85.42±1.25% (F=267.23, $p< 0.05$). The control group had highest hatching rate 98.3±2.5%. In case of *in vivo* exposure, at 0.004 µM/lconcentration of CPF, the rate of hatching was 90.5±0.5%, which was significantly higher than higher dose of CPF i.e., 0.52 µM/l, rate was 40.8±1.91% (F=271.87, $p< 0.05$) (Figure 21A).

The percentages of malformed hatchlings were also following a concentration dependent manner of CPF. These were increased in *in vitro* exposure as compared to *in vivo* exposure. At higher concentration of CPF (0.52 µM/l), the percentage of malformed hatchlings was significantly increased in both the exposures (*in vitro* and *in vivo*) i.e., 86.6±0.87% *in vitro* and 68.9±0.94% *in vivo* (F=640.81, F=225.93; $p < 0.05$). As the concentration of CPF was decreased, the percentage of malformed hatchlings was also decreased in both the exposure of CPF. At lower concentration of CPF (0.04µM/l), the low malformed hatchlings were found significantly (37.5±1.07 and 20.4±1.11; *in vitro* and *in vivo*) (F=640.80, F=225.93; $p< 0.05$) (Figure 21B).

3.3. Embryonic development and malformations due to chlorpyrifos

The effect of organophosphorous pesticide, chlorpyrifos (CPF) on embryonic development of freshwater catfish, *Heteropneustes fossilis* was observed in the present study. The result showed that *in vivo* exposure caused delayed embryonic development whereas *in vitro* exposure of CPF faster the embryonic development as shown in Table 10.The present study resulted that CPF produced a concentration dependent inhibitory response in normal development of the hatchlings. There are many malformations noticed due to chlorpyrifos toxicity. These malformations were formation of eyes and barbel, brain development, pericardial edema, yolk sac edema, fin fold defect (tail fin flexure),reduction of pigmentation and different notochordal defects. In higher concentrations of chlorpyrifos (0.52 µM/l), the hatchlings were recorded with combinations of all these abnormalities (Figure 22B-23B).

Four major categories of gross morphological abnormalities (irregular head development, cardiac edema, yolk sac edema and notochordal defect) and three minor deformities (body shortening, tail fin flexure and loss of pigmentation) were observed in

the present study (Figure 22-23). Among major categories, the chlorpyrifos treated hatchlings showed an irregular head shape. This abnormality was observed in CPF exposures i.e., *in vitro* and *in vivo* exposure (Figure 22-23: B, C). The malformed head beard swelling like protrusion on lateral side or the ventral (Figure 22-23: B, C). The malformation was often associated with a reduced barbel and reduced eye development (Figure 22-23). Another major malformation was cardiac and yolk sac edema. The cardiac edema was found as an enlargement in the pericardial sac or pericardial edema (Figure 22-23: B, C). Also yolk sac edema was often associated with irregular development of head and notochordal (spinal cord) curvature. The yolk sac and cardiac edema were observed in exposed group of hatchlings to different concentrations of chlorpyrifos both in *in vitro* and *in vivo* exposure. Malformed hatchlings were characterized by poorly developed mouth.

Further the most frequently observed gross morphological deformations in both of the exposures of CPF *in vitro* and *in vivo*, were bent body or a notochordal curvature. Different types of notochordal abnormalities were recorded (Figure 24): (1) C-shaped curvature (Figure 24A), (2) lordosis (dorso-ventral curvature) (Figure 24B), (3) Kyphosis (ventro-dorsal curvature) (Figure 24C), and (4) Scoliosis (lateral curvature) (Figure 24D). The presence of these defects was following the concentration manner of CPF.

Table 10: Effect of organophosphorous pesticide, chlorpyrifos on embryonic development of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794)

Parameters (h:min)	**Control**	**Treatment**	
		In vitro	***In vivo***
Latency period	12±2	16±2	10±2
Fertilization	0	0	0
Blastodisc formation	0:10	0:05	0:15
2 celled stage	0:15	0:10	0:22
4 celled stage	0:30	0:25	0:40
8 celled stage	1:15	1:10	1:25
16 celled stage	1:25	1:20	1:30
32 celled stage	2:00	1:40	2:15
Morula	2:30	2:10	2:45
Blastula stage	3:30	3:10	3:55
Gastrula stage	6:30	6:10	6:45
Yolk plugged stage	7:00	6:45	7:15
Kidney shaped embryo	09:00-10:00	08:40	09:30-10:30
Twisting movement	20:00-21:00	19:00-19:30	20:30-21:30
Fully active embryo	22:00	21:30	23:00
Hatching	23:00-24:00	23:00-23:30	24:00

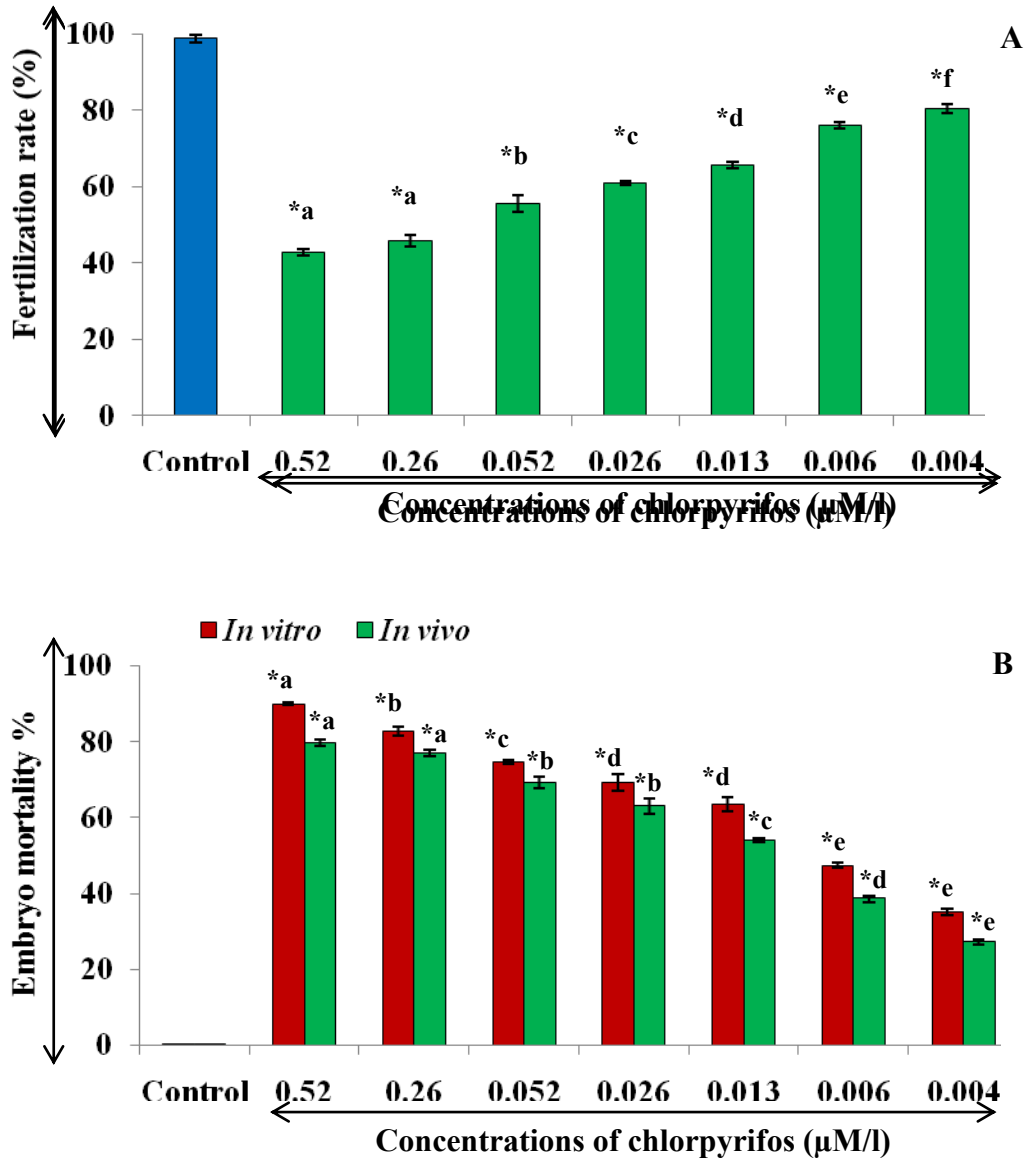


Figure 20: (A) Effect of *in vivo* exposure of different concentrations of chlorpyrifos on the fertilization rate (%). (B) *In vitro* and *In vivo* exposure of different concentrations of chlorpyrifos on embryo mortality (%) of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). Data were presented as mean±SEM. Values were analyzed by one way ANOVA ($p < 0.05$), followed by Newman-Keuls test ($p < 0.05$) for comparison within the groups. Asterisk (*) shows significant difference from the control group at $p < 0.05$. Group superscripted with different letter shows significant difference ($p < 0.05$) whereas same letter shows non-significance.

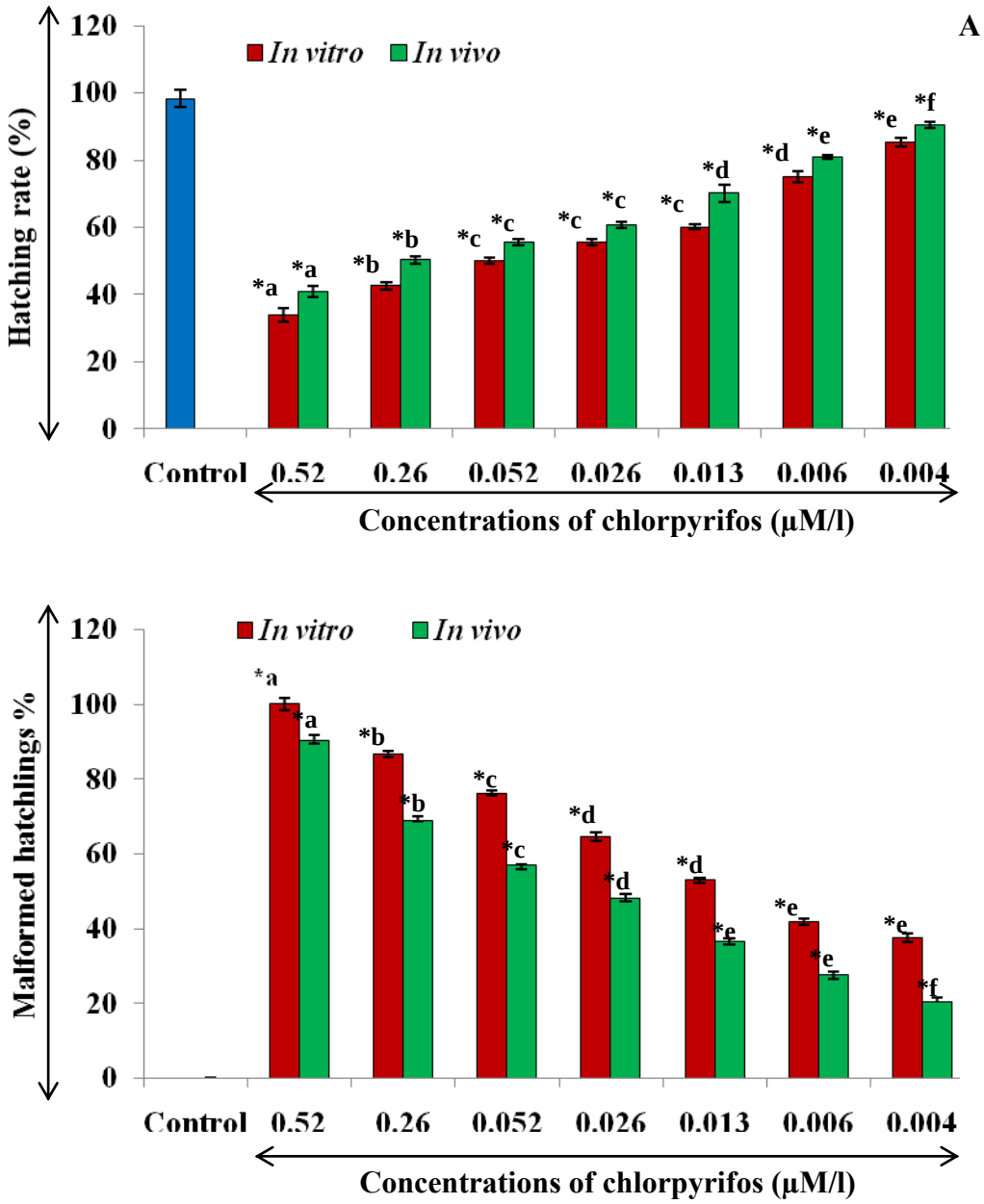


Figure 21: Effect of *in vitro* and *in vivo* exposure of different concentrations of chlorpyrifos on hatching rate (%) (A), and malformed hatchlings (%)(B)of freshwater catfish, *Heteropneustes fossilis* (Bloch, 1794). Data were presented as mean±SEM. Values were analyzed by one way ANOVA ($p < 0.05$), followed by Newman-Keuls test ($p < 0.05$) for comparison within the groups. Asterisk (*) shows significant difference from the control group at $p < 0.05$. Group superscripted with different letter shows significant difference ($p < 0.05$) whereas same letter shows non-significance.

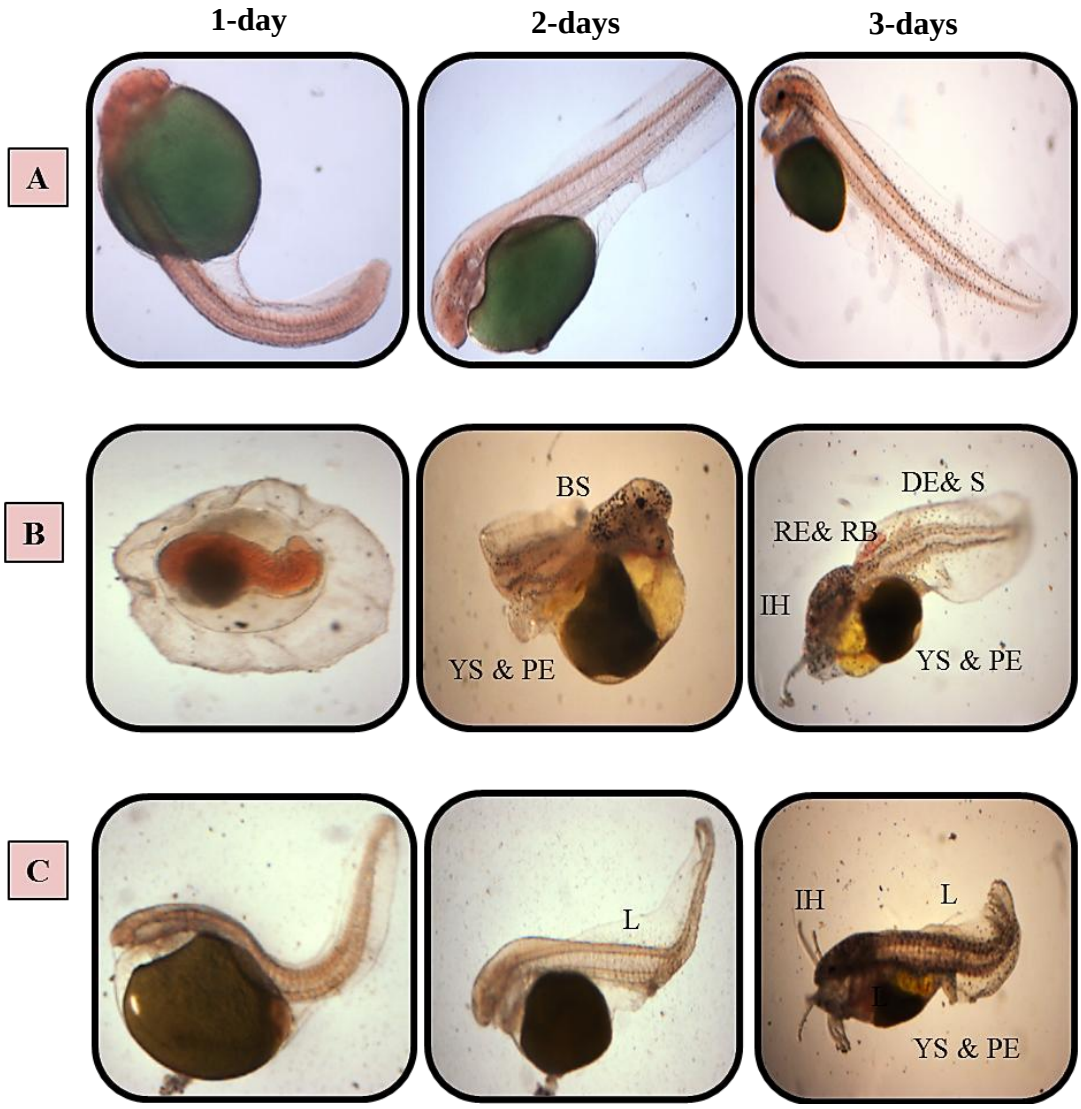


Figure 22: Photographs of 1-day, 2-days and 3-days hatchlings of freshwater catfish, *Heteropneustes fossilis*. A: Control group, B: *in vitro* exposure of maximum dose of chlorpyrifos i.e., 0.52 µM/l and C: *in vitro* exposure of minimum dose of chlorpyrifos i.e., 0.004 µM/l. Note showing: IH: Irregular head shape, BS: body shortening, YS & PE: yolk sac & pericardial edema, L: lordosis, RE & RB: reduced eye & reduced barbel, LP: loss of pigmentation, DE&S: deformed eye & skull.

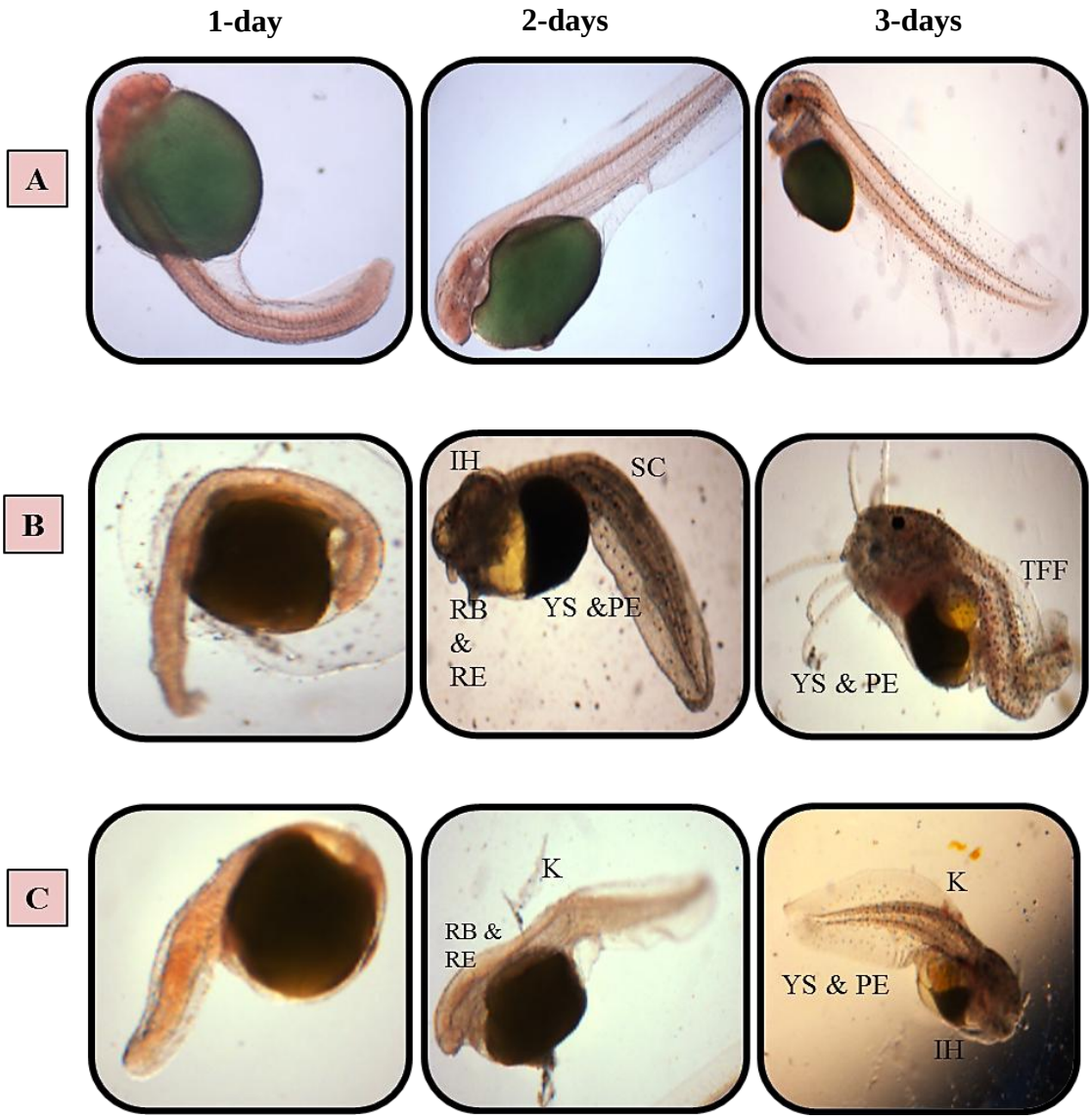


Figure 23: Photographs of 1-day, 2-days and 3-days hatchlings of freshwater catfish, *Heteropneustes fossilis*. A: Control group, B: *in vivo* exposure of maximum dose of chlorpyrifos i.e., 0.52 µM/l and C: *in vitro* exposure of minimum dose of chlorpyrifos i.e., 0.004 µM/l. Note showing: IH: Irregular head shape, BS: body shortening, YS & PE: yolk sac & pericardial edema, RE & RB: reduced eye & reduced barbel,TFF: tail fin flexure, SC: scoliosis, K: kyphosis

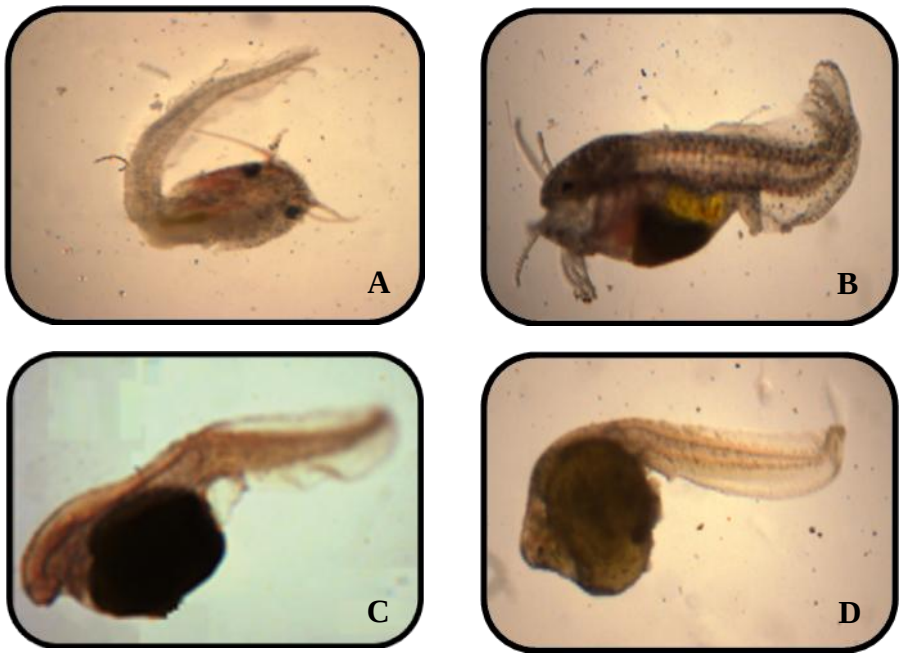


Figure 24: Photographs showing notochordal defects found in the chlorpyrifos exposed group of hatchlings of freshwater catfish, *Heteropneustes fossilis*. Note showing; A: C-shaped, B: Lordosis, C: Kyphosis, D: Scoliosis.

4. Discussion

The result of the present study showed that organophosphate pesticide, chlorpyrifos (CPF) not only affect embryo survival and hatching rate but it also influence period of embryonic development. The embryonic development was faster in *in vitro* exposure of CPF, as compared to control and *in vivo* exposure of CPF. CPF also caused teratogenic changes in term of morphological and notochordal defects. According to Dawson and Bantle (1987), CPF must be considered as a powerful teratogenic compound.

The fertilization rate was decreased in chlorpyrifos treated group of experimental fish as compared to control. The decreased embryo mortality was also observed in CPF exposed group of fish in a concentration manner as compared to control group. As comparison to *in vitro*, *in vivo* exposed fish had higher embryo mortality. Same observation was noticed by Rand and Petrocelli (1985) who reported that dimethoate, an organophosphorous pesticide caused a large increase in mortality associated with a small dose of pesticide. The hatching rate was low in *in vitro* exposed group as compared to *in vivo* exposed group of fish and control group. It has been observed that chlorpyrifos had significant effects on hatchability. Toor and Kaur (1974) also reported various adverse effects of pesticides on survival of developing eggs, hatchability and hatchlings of carp. Kaur and Dhawan (1993) found that at higher concentrations of pesticides (carbaryl, carbofuran, malathion and phosphamidon), egg development was arrested and heavy mortality (>50%) was occurred at this stage, indicating the greater sensitivity of early embryonic stages to pesticides. Many environmental pollutants (phosphor-organic pesticides, some herbi- and fungicides, heavy metals, PCBs, petroleum hydrocarbons) caused increased embryo mortality, decreased hatching rates and teratogenic effects (Hoffman and Sileo, 1984; Deli and Varnagy, 1985; Garrison and Wyttenbach, 1985; Indyk, 89/90a, b, c; Hoffman, 1990; Kumar and Devi, 1992; Lenselink et al., 1993). Kaur and Toor (1977) reported that chlorpyrifos was inhibited some hatching enzymes which resulted in lower hatching rate. According to Boone and Chambers, (1997), the main factor of high CPF toxicity was its property of AChE-target enzyme sensitivity.

There were not so much studies of pesticidal exposure towards the embryo toxicity, and the past investigations in this regards have indicated alarming situation in infertility, abnormal gamete, defective embryos, poor fertilization and birth defects (Fuortes et al., 1997; Smith et al., 1997; Amer et al., 2001; Greenlee et al., 2003; Yamauchi et al., 2003). In present study, a high tolerance of CPF was expressed in terms of survivability of hatchlings. The result found that at lower concentration of chlorpyrifos (0.004 µM/l), 37.5% of malformed hatchlings in *in vitro* and 20.4% in *in vivo* exposure was noticed. In the present observation, various types of malformations were observed in hatchlings of freshwater catfish, *H. fossilis* due to chlorpyrifos toxicity such as axial (lordosis or kyphosis) or lateral (scoliosis) notochordal curvature, deformed skull and eye, yolk sac and cardiac edema, shortened body, reduced barbels and tail fin flexure.

The result showed that the hatchlings of freshwater catfish, *Heteropneustes fossilis* showed gross alterations in tail flexure coupled with a decrease of neuromuscular activity. These results were well correlate with findings of other authors (Snawder and Chambers, 1989; Vismara et al., 1996; Richards and Kendall, 2002).The abnormal tail flexure observed in CPF could be the consequence of the cholinergic phase, where the AChE inhibition causes repetitive firing of muscle fibres leading to the axis tail folding and consequence of uncontrolled and continuous contraction of the tail musculature (Lien et al., 1997). This hypothesis was confirmed by Behra et al. (2002) who noticed that the first movement of zebrafish embryos homozygous for AChE gene mutation was characterized by tail twitching. Anti-ChE has an important role in neural development in function of nervous system. It is transiently expressed during discrete periods of neural development of the thalamo-cortical pathways, and transient AChE activity correlates with the specific growth of thalamic axons into the cortex and synaptogenesis with cortical neurons (Robertson and Yu J, 1993). There is a significant sequence similarity exists between AChE and cell adhesion proteins that function in morphogenic phenomena. Mack and Robitzki (2000) investigated a functional role of AChE in regulation of cell proliferation and the onset of differentiation during early neuronal development which was independent of its enzymatic activity. The most common and important malformation viz., Yolk sac edema, has been previously reported by a number of author's viz., Dushkina (1973), Eaton (1974), Linden (1978), Helder (1980),

Dominguez and Chapman (1984), Middaugh et al. (1988), Marty et al. (1990), Spitsbergen et al. (1991), Walker et al. (1991, 1992), Prince and Cooper (1995).

The observed deformities in the present study were also reported in many fish by many researchers due to pesticidal exposure viz**.,** Mehrle and Mayer (1975) in *Pimephales promelas*, Weis and Weis (1976) in *Cyprinodon variegatus*, Lien et al. (1997), in *Clarias gariepinus,* Solomon (1977) in *Oryzias latipes,* Kumar and Ansari (1984) in *Brachydanio rerio,* Kumar and Ansari (1984) in Zebra fish, Srivastava and Srivastava (1990) in *H. fossilis,* Jezierska et al. (2000) in carp, Sanchez-Bayo et al. (2005) in *Oryzias latipes* andMochida et al. (2008) in *Fundulus heteroclitus*. Many researchers also reported that pesticides such as toluene, toxaphene, malathion, kepone, trifularin and pyrithione can cause morphological and skeleton abnormalities in many freshwater fish (Weis and Weis, 1987; Lien et al., 1997; Mochida et al., 2008). The occurrence of these notochordal deformities could arise due to decreasing the collagen amount in spinal column, changing amino acid composition (Mehrle and Mayer, 1975), deficiencies of vitamin C (Kumar and Ansari, 1984), neuromuscular spasms (Meiniel, 1981; Couch et al., 1977) and absence of a functional swim bladder (Chatain, 1994). The observed morphological and muscular damages may be related to inhibition of acetylcholinesterase activity that showed a possible role played by organophosphorous on induction of muscular dystrophy and morphological deformities during development. Abu-Qare et al. (2001a, b) found that diazinon and methyl parathion exposure caused inhibition of cholinesterase (ChE) enzymes during maternal and fetal tissues assessment. The ChE enzyme plays a significant role during organogenesis (Moody & Stein, 1988). Behra et al. (2002) stated that AChE is required for embryo muscular development. In mammals, OP compounds produce typical signs of anti-cholinesterase toxicity such as complex posturing movements, notochordal morphogenesis and skeletal muscle fasciculation associated with muscle fibre damages (Meiniel, 1981; Gupta et al., 1987, Karalliedde and Henry, 1993, De Bleecker et al., 1994, John et al., 2003; Snawder and Chambers, 1993).

Consolidated summary and conclusion

Organophosphorous pesticide (OP) has been widely used in agricultural practices. It is second largest selling organophosphorous pesticide and found to be highly toxic to aquatic animals. Chlorpyrifos is an organophosphate insecticide, acaricide and nematicide. Its direct application to soil, vegetation and animals can result in exposure to non-target organisms or aquatic organisms.

The present research included the acute toxicity of chlorpyrifos (CPF) to freshwater catfish, *Heteropneustes fossilis.* Fish were exposed to varied range of concentration of CPF (0.002 - 2.28 mM/l) for 96 h in three respective reproductive phases viz, resting, preparatory and pre-spawning phase. The finney's probit analysis (1971) was used to determine median lethal concentration (LC_{50}). The safe concentration of the studied pesticide was calculated as per Hart et al. (1945) and Henderson et al. (1959) in all respective seasons. The obtained LC_{50} were found to 1.547, 0.678, 0.299 and 0.174 mM/l in resting phase, 0.332, 0.193, 0.152 and 0.123 mM/l in preparatory phase and 0.296, 0.107, 0.044 and 0.026 mM/l in pre-spawning phase for 24, 48, 72 and 96 hr respectively. The safe concentrations were 0.039 mM/l in resting phase, 0.19 mM/l in preparatory phase and 0.004 mM/l in pre-spawning phase. Susceptibility of catfish, *H. fossilis* to CPF was found to be duration and concentration dependent. Their tolerance to CPF was increasing with inactiveness of gonadal activity (resting season). At higher concentration of CPF, fish showed alterations in morphological and behavioral responses, especially erratic and jerky swimming, frequent surfacing and ingulping, mucus secretion, an increase in opercular movement and copious secretion of mucus all over the body. The histological assessment resulted that CPF caused reduction in size of oocytes, vacuolation in cytoplasm, damaged ovarian follicles, increase in inter-follicular space, breaking of ovarian wall, occurrence of necrosis, damaged structure of ovarian follicles, formation of fragmented ova, atretic oocytes and nucleolar extrusion.It is concluded that CPF is highly toxic to catfish and severely affect their physiology and behavior.

To investigate *in vitro* effect of chlorpyrifos (CPF) on hCG-induced oocyte maturation and different steroids involved in oocyte maturation of post-vitellogenic follicles of freshwater catfish, *Heteropneustes fossilis*, the follicles were incubated with human chorionic gonadotropin (hCG), CPF and in combination of hCG and CPF in a concentration and duration dependent manner. The result showed that CPF caused an inhibitory effect on hCG induced GVBD in co-incubation experiments at the dose of 0.26 pM/ml. Pre- and post-incubation of follicles with CPF resulted a significant inhibition in hCG induced oocyte maturation in all durations and maximum inhibition 87.67% and 76% was recorded at 12 hr incubation duration ($p < 0.05$, Newman-Keuls test). Effective dose of hCG (1.7 mg/ml, 8 hr) induced OM coincided with a significant increase in follicular pregnenolone, progesterone, deoxycorticosterone (DOC) and dihydroxyprogesterone (DHP) as well as significant decrease in estradiol-17β (E_2) as compared to control group of post-vitellogenic follicles. Under CPF exposure follicles elicited a sharp significant decrease in pregnenolone, progesterone and DHP. However, DOC and E_2 were increased significantly. Co-incubation study revealed that CPF suppressed hCG induced concentrations of DHP, pregnenolone and progesterone significantly but side by side support DOC increase and maintain high E_2.

To observe the effect of CPF on developmental embryo, *in vitro* and *in vivo* experiments were performed. For this, *in vitro* and *in vivo* experiments were performed. In *in vitro* experiment, the fertilized eggs stripped from normal control catfish were exposed to different concentrations of chlorpyrifos viz., 0.52, 0.26, 0.052, 0.026, 0.013, 0.006, 0.004 µM/l. For *in vivo* study, the induced female catfish with synthetic hormone (hCG) was exposed to same concentrations of chlorpyrifos. The experiment was done in triplicates along with the control group. The result showed that both, fertilization and hatching rate was decreased significantly in exposed group in comparison to control. *In vivo* exposure showed significantly low embryo mortality (27.2±0.76%) ($F=893.67$; $p < 0.05$) and high hatching rate (90.5±0.5%) ($F=271.87$; $p < 0.05$) as compared to *in vitro* study(35.2±0.81% and 85.4±1.25%) ($F=1070.28$, $F= 267.23$; $p < 0.05$) in lower concentration of CPF (0.004 µM/l). The percentage of malformed hatchlings was higher in *in vitro* experiment as compared to *in-vivo*. The occurrence of all studied parameters was observed in a concentration dependent manner, as the concentrations of CPF were increased, the percentage of embryo mortality and malformed hatchlings was decreased.

However, the hatching rate was increased. The result showed that exposed group of hatchlings were affected by many morphological and notochordal deformities viz., ventral or lateral tail flexure, abnormal spinal bending, irregular head shape and size, loss of eye, reduced barbel, pericardial edema, yolk sac edema, notochordal defect, tail fin flexure, reduction of brain development and reduction of pigmentation.

From the present study it can be concluded that CPF caused a significant impact on behavioral, morphological, histopathological, endocrinological and developmental parameters of freshwater catfish, *Heteropneustes fossilis.* CPF acts as a potent endocrine disruptor affecting ovarian steroidogenesis and ovulation. It inhibits the meiotic oocyte maturation involved reducing enzymatic activity as well as MIS inhibition which may lead to adverse effect on fish reproduction. It also plays an important role in inducing the muscular dystrophy and morphological deformities during embryonic development and organogenesis. The findings of this research proposed that CPF may be teratogenic for catfish. The present study of chlorpyrifos toxicity of air-breathing freshwater catfish, *Heteropneustes fossilis* concluded that this fish is very sensitive to low level of organophosphorous pesticide toxicity. Therefore, these pesticides should be used with great caution and in a sustainable way.