Research Article

Chlorpyrifos Effect on Vitellogenin, Ovarian Steroid in Adult and *NR5A1* Expression in Fry of the Freshwater Catfish, *Heteropneustes fossilis* (Bloch, 1794)

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ABSTRACT

Aim: To study the interference of organophosphate pesticide chlorpyrifos (CPF) in gonad function at the steroidal and gene levels during prespawning adult and fry stage respectively of the freshwater stinging catfish, *Heteropneustes fossilis*. **Methods:** The matured adult fish of early prespawning stage were exposed to different combinations of CPF (0.174 µM, 10^{-1th} of 96 h LC_{50}), gonadotropin (hCG; 100 IU.fish-1) and natural antioxidants (curcumin; 10 mg.L-1 and ascorbic acid; 5 mg.L^{-1}) for 24 h to measure vitellogenin and ovarian steroid. Twenty days post-hatch, fish fry were exposed to CPF for 96 h to understand the influence on *NR5A1* gene expression pattern. **Results:** The CPF increased vitellogenin level at liver, ovary and serum. Among steroidal cascade CPF induced highest estradiol followed by a significant increase in progesterone and 17α, 20β-dihydroxyprogesterone as compared to control fish. The gonadotropin further increased CPF induced effects on vitellogenin and ovarian steroidal level. Though, antioxidants co-incubation with CPF did not produce any significant change on either vitellogenin or in ovarian steroids content. CPF triggers *NR5A1* gene expression in a fry that indicates a role in early gonad differentiation and development. **Conclusion:** The present study clearly indicates the major influence of CPF in gonadal physiology of both adult and fry stages of freshwater catfish, *Heteropneustes fossilis*.

Key words: Chlorpyrifos, Estradiol-17β, Progesterone, 17α, 20β-dihydroxy-4-pregnen-3-one.

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INTRODUCTION

Chlorpyrifos (CPF) is the most extensively used broad-spectrum chlorinated organophosphate insecticide. The extensive use of pesticide has allowed it to reach the water bodies via surface runoff and bioaccumulation in aquatic organisms including fish. Despite being an acetyl cholinesterase inhibitor,[1] CPF is an endocrine disruptor,[2] genotoxic,[3] mutagenic,[4] teratogenic,[5] developmental neurotoxin.[6] However, study reporting the role of chlorpyrifos in catfish gonadal steroid and vitellogenin are nil. Antioxidant, curcumin (Cur), derived from the rhizome *Curcuma longa*, is a powerful antioxidant, anti-inflammatory and hepato-protective agent and has been reported to improve the growth and quality of fish in aquaculture.[7] Ascorbic acid (Asc) is another antioxidant to contribute an important role in fish health.[8,9] Both these antioxidants are promising suppressor of CPF induced stress on fish that influenced most of the tissue physiology.[9] The teleost are seasonal breeder whose reproductive events regulated by gonadotropins.[10] In response to various endogenous and environmental factors such as innate biorhythms, nutritional status, water temperature and photoperiod,[11] the brain (hypothalamus) produces gonadotropin-releasing hormone (GnRH) which stimulates the pituitary gonadotrophs to secrete two gonadotropins, GTH I and GTH II which are homologous to follicle stimulating hormone (FSH) and luteinizing hormone (LH).[12,13] The FSH stimulates

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the theca and granulosa cells of the ovarian follicle to produce estradiol-17β (E_2), which commands the liver to synthesize vitellogenin (Vtgs) and secrete them, into the bloodstream.[12,14,15] In aquaculture, circulations of Vtgs are the sign of puberty onset and progression of gonad maturation in female fishes.[16]

In fish, E_2 regulates important reproductive function by binding and activating the intracellular estrogen receptors (ERs). They also play an important role in the regulation of oogenesis and gonadotropin, testicular development, non-gonadal tissue gene expression, sex change or sex differentiation through regulation of hormonal synthesis.[17,18] The steroidogenic pathway initiates with the side-chain cleavage of cholesterol by cytochrome P450 (P450scc) forming pregnenolone which further gets converted to progesterone (P_4) via 3β-hydroxysteroid dehydrogenase (3β-HSD) or to 17α-hydroxypregnenolone via cytochrome 17α-hydroxylase (P450c17).[17] P_4 is an important progestin that mediates not only the oocyte growth and maturation but also spermatogenesis and sperm maturation in teleosts.[18] Progesterone and 17α-hydroxypregnenolone are converted to 17α-hydroxyprogesterone followed by the production of 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) by 20β-hydroxysteroid dehydrogenase (20β-HSD). 17α, 20β-DP is a maturation inducing steroid (MIS). It is an important steroid regulating oocyte maturation occurring before ovulation, an essential step for successful fertilization.[13] Chlorpyrifos reported as ova-toxin and embryo-toxin in most of the ruminants.[19] But how pesticide, CPF, will influence gonadotropin induced ovarian steroids to influence reproductive physiology of fish is an interesting part of the study.

In the catfish critical period for gonad differentiation started 30 - 40 days post-hatch. In this duration sex-specific gene expression started to multiply.[20] The *NR5A1* gene (nuclear receptor subfamily 5, group A, member 1) regulates the transcription of genes that controls the expression of the cytochrome P450 enzymes in the endocrine tissues, such as the adrenal cortex, testis, and ovary[21] and metabolism.[22] *NR5A1* plays an important role in the transcriptional regulation of cyp19a1a, converts androgens into estrogens, it considered as a rate-limited step in estrogen synthesis.[23,24] Besides this, it is also involved in the sex determination and differentiation in vertebrates including mammals and fish.[25,26] There is no report on analyzing the impact of pesticide on gonadal differentiation in the case of stinging catfish.

H. fossilis is a freshwater catfish. It is commonly used as laboratory fish due to its table size and sturdy nature. It is a favourite model to understand the reproductive limitation of the catfish and their alternate mechanism. The present study is an attempt to understand the interference of the CPF with the steroidogenesis and gene expression in *H. fossilis*. This is for the first time where the interplay of chlorpyrifos, hCG and antioxidants have been studied in *H. fossilis* with the reference of gonadal physiology.

MATERIALS AND METHODS

Materials

The steroid standards were purchased as 17β-Estradiol (CAS *Number*: 221093-45-4; Sigma Chemicals, USA), 4-Pregnene-3, 20-Dione (CAS *Number:* 57-83-0; Sigma Chemicals, USA) and 17α, 20β-Dihydroxy-4-pregnen-3-one (CAS Number: 1662-06-2; Cayman Chemicals, USA). The solvent, methanol and acetonitrile were of HPLC grade (Merck, India). The synthetic hormone, human chorionic gonadotropin (hCG: Ovidac®; Bayer Zydus Pharma Private Limited, India) was purchased from local medical stores. The CPF testing was done in a commercial-grade pesticide, Hilban® (20 % EC CPF, rest of constituent are emulsifier and additives to increase CPF availability for its pesticide effect) purchased from the retail agricultural shop (Hindustan Insecticide Limited, India). All other chemicals and reagents were of analytical grade (HiMedia Laboratory Private Limited, India).

Fish collection and acclimatization

The adult catfish *H. fossilis* of early prespawning (1 - 5 May) season of the relatively same size (16 ± 2 cm) and weight (140 ± 15 gm) was purchased from a local fish market in Lucknow, Uttar Pradesh, India. The fish were brought to the laboratory and acclimatized for a week under normal photoperiod and temperature (12 h: 12 h; light: dark and 25 ± 2°C) with chopped goat liver twice a day. The water was renewed daily to remove metabolic wastes accumulated during acclimatization.

National guidelines of the ethical committee were followed to exhibit experiments and to avoid any cruelty to the animals.

CPF effect on vitellogenin and ovarian steroid

Acclimatized adult females of *H. fossilis* were divided into eight groups having five fish in each and maintained for 24 h. The group I was a control having only freshwater and group II received fish treated with synthetic hormone (hCG hormone: 100 IU.fish^{-1}).[27] The group III and IV received hCG treated fish in curcumin (Cur: 10 mg.L^{-1})[28] water and ascorbic acid (Asc: 5 mg.L^{-1})[29] water respectively. The group V received hCG treated

fish in freshwater containing a combination of Cur and Asc. The group VI and VII received untreated fish and hCG treated fish respectively into CPF (0.174 µM, 10^{-1} of 96 h LC_{50})[30] mixed water. The last group VIII received hCG injected fish in freshwater containing CPF in addition to Cur and Asc.

Sampling

At the end of the experimental period, blood from fish of each group was collected by caudal severance and allowed to clot by leaving it undisturbed at room temperature for at least 30 min. The clotted blood was centrifuged at 2,000 g for 10 min in a refrigerated (- 4°C) centrifuge and the resulting supernatant called serum was decanted into clean tubes, sealed, and frozen at – 20°C until assayed. The fish from each group sacrificed and tissues such as liver and ovary were dissected out and stored at – 20°C until assayed.

Vitellogenin measurement

Vitellogenin in serum and tissues (liver and ovary) were determined by the alkali-labile protein phosphorus method.[15,31] The absorbance of the phosphomolybdate complex was determined at 815 nm (SpectraMax® ABS plus microplate reader).

Steroid measurement

The steroids were extracted from the ovary[27] and HPLC analysis was performed.[32] The ovary of each fish was homogenized in 4 volumes of cold 0.2 N perchloric acid with an ultrasonic homogenizer at 0°C for 10 - 15 sec. The homogenate was centrifuged at 21,000 *g* for 20 min at 0°C The supernatant collected was extracted with hexane twice followed by diethyl ether three times. The ether phase was then pooled, evaporated and dried under liquid N_2 and stored at – 20^0 C till chromatography. The sample was subjected to reversed-phase HPLC (Waters 2998) on an X-Bridge C_{-18} column (250 × 4.6 mm) packed with 5 um particles size. The mobile phase used was acetonitrile-water (40: 60, $v.v^{-1}$) at a constant flow rate of 1 $mL.min^{-1}$ for 30 min. The absorbance of the effluent was measured at 230 nm with Photodiode Array Detector. A chromatogram showing retention time, peak height and peak area for each standard/ sample were obtained using Empower 2.0 software. The quantitative estimation of steroid was obtained based on the peak area of the standards (Figure 1).

CPF EFFECT ON *NR5A1* GENE EXPRESSION IN FRY

For *NR5A1* expression study, live and healthy fry of *H. fossilis* (20 days post-hatch, dph; 6 ± 1 cm) was procured from the local area hatchery (Lucknow). The fry were allowed to acclimatize for three days in the laboratory under natural photoperiod and temperature (12 h: 12 h; light: dark and 25 ± 2°C). The water was renewed daily to remove metabolic wastes accumulated during acclimatization. They were fed with a minced boiled egg. After acclimatization, the fry were divided into three groups with five in each. The group I was a control. For groups II and III, the fry were exposed to CPF (0.174 µM, 10^{-1th} of 96 h LC_{50}) for 24 and 96 h respectively. At the end of the experimental duration, the fry were snap-freeze in liquid N_2 and given to Acube Life-Sciences Private, Lucknow for expression study of *NR5A1* gene. In brief, total RNA was isolated from each sample and 1000 ng was used for cDNA synthesis using reverse transcriptase following the manufacturer's protocol (Thermofisher). The RNA and oligo d_{1}. random hexamer $primers^{-1}$ were used for the first strand of cDNA synthesis (Table 1). The real time PCR reaction mixture of 20 µl containing 10 µl of SYBR green supermix (Bio Rad, USA) and 2 µl of cDNA was run for 35 cycles followed by denaturation 95°C for 30 sec, annealing 55°C (gradient) for 30 sec in Bio-Rad CFX96 system. The mRNA expression levels were normalized to that of housekeeping gene (GAPDH) and the results were analyzed against the control, untreated, group.

Statistical analysis

Data were represented as means ± SEM of minimum five animals in each group (n = 5). The significance of values obtained from different groups was tested by using one-way analysis of variance (ANOVA). The

Table 1: Primer sequences used in the *NR5A1* expression study.

Experiment	Gene	Direction	Sequence 5'-3'
Gene specific primer for qPCR study	*NR5A1*	Forward primer	ATGCTGGAAGCGCAGAGC
		Reverse primer	AATGGTGGTCGCGCGTTT
Housekeeping gene	*GAPDH*	Forward primer	ACCCACTCCTCCACCTTTGA
		Reverse primer	CTGTTGCTGTAGCCAAATTCGT

intergroup comparisons were done by Newman-Keuls' test ($P < 0.05$).

RESULTS

CPF effect on vitellogenin in liver, ovary and serum

The hCG increased the vitellogenin (Vtg) content significantly in liver and serum but not in the ovary of early prespawning phased *H. fossilis* (Figure 2, liver, $F = 34.32$, $P < 0.001$; Figure 3, serum, $F = 37.23$, $P < 0.001$; Figure 4, ovary, $F = 5.49$, $P < 0.05$) when compared to the control group in 24 h. The gonadotropin induced Vtg was not influenced by the presence of a singular or dual combination of antioxidants as compared to hCG injected group. The administration of CPF promoted a significant increase in the Vtg content both in the tissues (liver and ovary) and the serum about the control. However, gonadotropin injected fish when kept in CPF water surpassed CPF induced increase in Vtg in case of the liver and serum but not of the ovary. In this case, antioxidants could not produce any significant influence on Vtg level in tissue or serum.

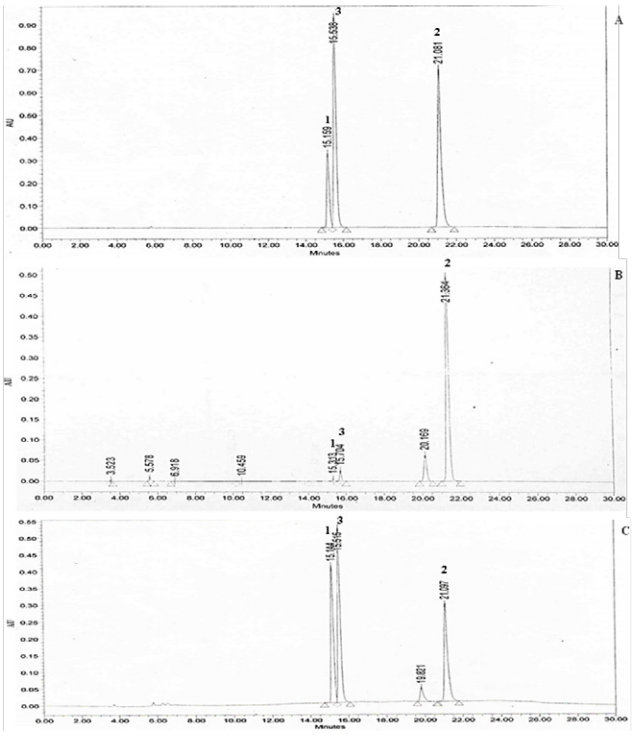

Figure 1: The typical HPLC chromatograms of standards mix (A) control (B) and experimental (C) showing respective peaks as: 1: 17β-estradiol (E2); 2: 4-pregnene-3, 20-dione (P4); 3: 17α, 20β-dihydroxy-4-pregnen-3-one (17α 20β-DP; MIS) when applied on reversed-phase HPLC (Waters 2998) on an X-Bridge C-18 column (250 X 4.6 mm) packed with 5 µm particles size. The mobile phase used was acetonitrile-water (40: 60, v/v).

CPF effect on ovarian steroid

The gonadotropin and CPF treatment alone and in combination with antioxidants produced significant changes in the concentrations of E_2, P_4 and 17α,20β-DP in early prespawning phased *H. fossilis* in 24 h (Figure 1,

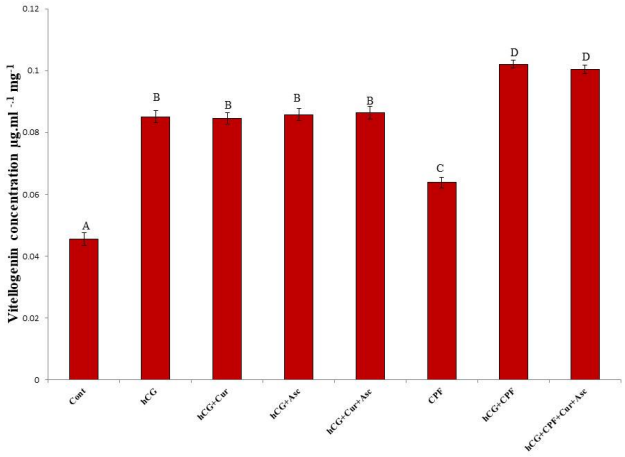

Figure 2: Effect of human chorionic gonadotropin (hCG; 100 IU.fish^{-1}) and chlorpyrifos (CPF; 0.174 µM) with a different combination of antioxidants (curcumin, Cur; 10 mg.L^{-1} and ascorbic acid, Asc; 5 mg.L^{-1}) for 24 h on vitellogenin content in liver of freshwater catfish, *Heteropneustes fossilis* during early prespawning period (May).Values are mean ± SEM of five fish in duplicates. Data were analyzed by one way ANOVA ($P < 0.001$) and Newman- Kuels' test ($P < 0.05$). Groups marked with the same symbols are not significant and those with different symbols are significantly different in intergroup comparison.

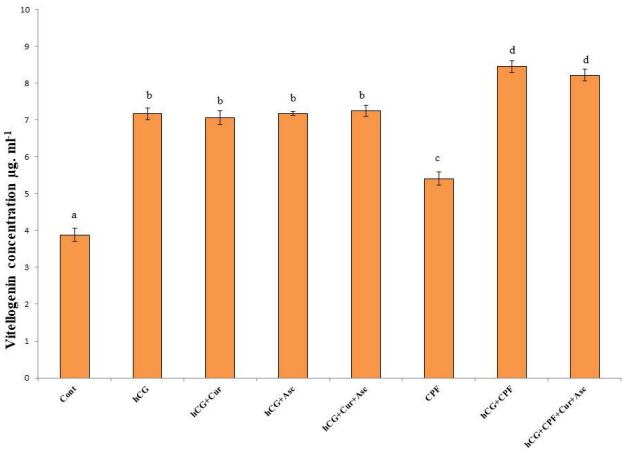

Figure 3: Effect of human chorionic gonadotropin (hCG; 100 IU.fish^{-1}) and chlorpyrifos (CPF; 0.174 µM) with a different combination of antioxidants (curcumin, Cur; 10 mg.L^{-1} and ascorbic acid, Asc; 5 mg.L^{-1}) for 24 h on vitellogenin content in the serum of freshwater catfish, *Heteropneustes fossilis* during prespawning period (May). Values are mean ± SEM of five fish in duplicates. Data were analyzed by one way ANOVA ($P < 0.001$) and Newman- Kuels' test ($P < 0.05$). Groups marked with the same symbols are not significant and those with different symbols are significantly different in intergroup comparison.

5; $F = 192.14, 586.71, 435.99$ respectively; $P < 0.001$). In the control group, a definite concentration of P_4 was noticed with very low concentrations of E_2 and 17α, 20β-DP. The administration of hCG stimulated the progestin pathway involving P_4 and 17α, 20β-DP with a simultaneous increase in the concentration of E_2. The administration of curcumin in hCG injected fish brought no significant change in the concentrations of E_2 and 17α, 20β-DP with an increase in the P_4 concentration as compared to the hCG injected group. However, the administration of Asc in gonadotropin injected fish induced a significant increase in P_4 and 17α, 20β-DP with a simultaneous decrease in E_2 concentration as compared hCG alone group. However, when gonadotropin injected fish kept in co-incubation with the antioxidants (Cur and Asc) a significant increase (2-fold) noticed in the concentration of P_4 and 17α, 20β-DP with a decreased (1-fold) E_2 concentration in comparison to the other groups of hCG. In CPF treated group, E_2 and P_4 were increased significantly as compared to control group. The administration of CPF to hCG injected fish showed similar E_2 levels with a slight but significant increase in P_4 and 17α, 20β-DP in comparison to CPF alone group. The combination of CPF, antioxidants and hCG marked a significant decrease in the E_2 but no significant change in 17α, 20β-DP level as compared to CPF and hCG group.

CPF effect on *NR5A1* gene expression

The 20 dph fry of *H. fossilis* exposed to a sublethal dose of CPF for 96 h showed a 5-fold increase in the *NR5A1* gene expression as compared to the control group but there was no change in gene expression level at 24 h duration study (Figure 6; $F = 5.39$, $P < 0.05$).

DISCUSSION

In this study, for the first time, interplay of organophosphorus pesticide (CPF), gonadotropin (hCG) and antioxidants (Cur and Asc) were studied on the vitellogenin, steroid and *NR5A1* gene expression in the freshwater stinging catfish, *H. fossilis*. The CPF acts as an endocrine disruptor with a potential to perturb sensitive steroidal and genetic pathway that regulate reproductive functions. Due to the estrogenic nature of CPF, it induced vitellogenesis and also promotes P_4 level in catfish which may lead to early ripening and ovulation of follicle in females and further increased with gonadotropin. By increasing *NR5A1* gene expression, CPF suggested its effects in early gonad differentiation in fry of catfish as compare to control fry.

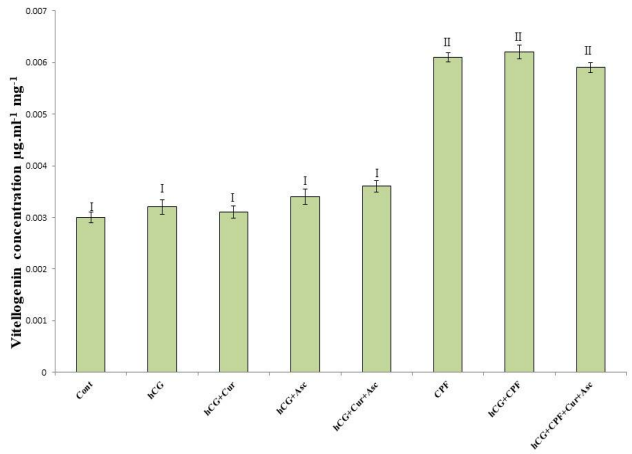

Figure 4: Effect of human chorionic gonadotropin (hCG; 100 IU.fish^{-1}) and chlorpyrifos (CPF; 0.174 µM) with a different combination of antioxidants (curcumin, Cur; 10 mg.L^{-1} and ascorbic acid, Asc; 5 mg.L^{-1}) for 24 h on vitellogenin content in the ovary of freshwater catfish, *Heteropneustes fossilis* during prespawning period (May).Values are mean ± SEM of five fish in duplicates. Data were analyzed by one way ANOVA ($P < 0.001$) and Newman- Kuels' test ($P < 0.05$). Groups marked with the same symbols are not significant and those with different symbols are significantly different in intergroup comparison.

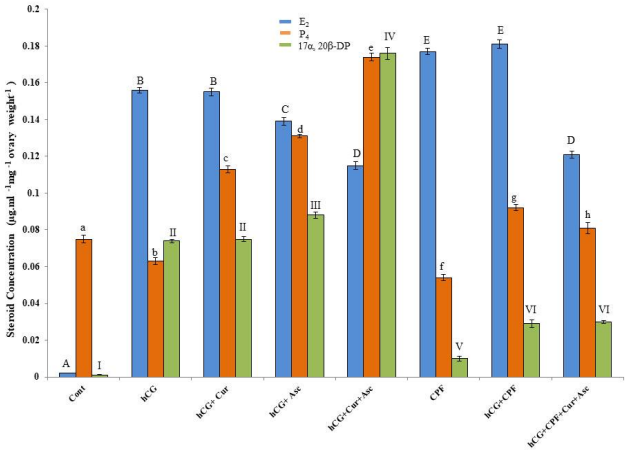

Figure 5: Effect of human chorionic gonadotropin (hCG; 100 IU.fish-1) and chlorpyrifos (CPF; 0.174 µM) with a different combination of antioxidants (curcumin, Cur; 10 mg.L-1 and ascorbic acid, Asc; 5 mg.L-1) for 24 h on steroid concentration (E2: 17β-Estradiol; P4: 4-Pregnene-3,20-dione; 17α,20β-DP: 17α,20β-dihydroxy-4-pregnen-3-one) in the ovary of freshwater catfish, *Heteropneustes fossilis* during prespawning period (May).Values are mean ± SEM of five fish in duplicates. Data were analyzed by one way ANOVA ($P < 0.001$) and Newman-Kuels' test ($P < 0.05$). Different symbols used to denote different steroid for intergroup comparison viz., capital letters denoted comparison for E2, small letter for P4 and roman number for 17α,20β-DP. Groups marked with the same symbols are not significant and those with different symbols are significantly different.

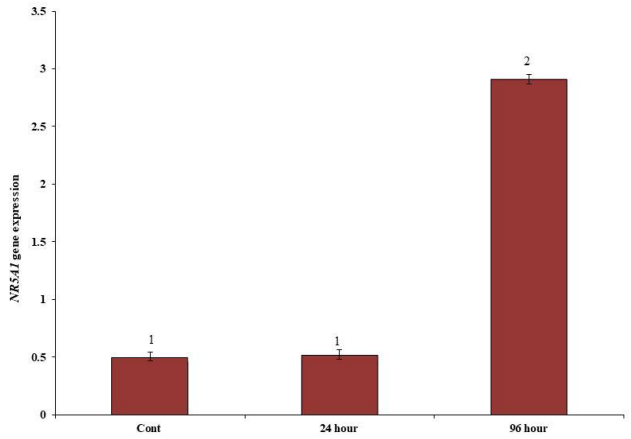

Figure 6: Effect of chlorpyrifos (0.174 µM) on the NR5A1 expression in 20 day fry of freshwater catfish, *Heteropneustes fossilis*. Values are mean ± SEM of five fry in duplicates. Data were analyzed by one way ANOVA ($P < 0.001$) and Newman-Kuels' test ($P < 0.05$). Groups marked with the same number are not significant and that with different number is significantly different in intergroup comparison.

Interplay on vitellogenin

In the present study, control group *H. fossilis* showed the presence of vitellogenin in the liver, serum and ovary owing to its prespawning period as reported earlier.[15] The level of E_2 starts increasing during the preparatory period, reaches a maximum during the prespawning period and drop steeply during the spawning period.[33] During this period, pituitary gonadotropin increased estrogen in gonad that further promotes the hepatic vitellogenesis which sequestered and incorporated into the ooplasm.[15] Results registered that in gonadotropin injected *H. fossilis* the level of Vtg was increased significantly in the liver, serum but not in the ovary as compared to the control. This is because the administration of hCG is effective enough to induce Vtg synthesis by promoting estradiol secretion but do not promote the uptake of Vtg and formation of yolked oocytes as that was already being done by the intrinsic gonadotropin. The pattern of change in Vtg was similar in serum as in liver that reflects a change in overall Vtg synthesis and will influence yolk accumulation in growing oocytes. The hCG injected fish, in presence of individual antioxidants, Cur, Asc or co-incubation, had a steady level of Vtg in 24 h treatment. The Cur has been reported to improve both the hepatic Vtg and nutrient deposition in the ovulating eggs of teleost fish improving the quality of the embryo and the larvae[34,35] whereas the Asc may act as a stabilizer, protector, enhancer or inhibitor to the steroid concentration in endocrine functioning.[36] CPF immersed as a follicle growth enhancer in this, 24 h *in vivo* study. It has been found to promote Vtg synthesis in tissues (liver and ovary) and serum. As per results, the ovary recorded an increase in Vtg as compared to the liver and serum due to CPF, whereas external gonadotropin increased Vtg in liver and serum, but not in the ovary. Both hCG and CPF increased Vtg in the liver as compared to the control group. Though an increase of Vtg was less in CPF incubation as compared to hCG injected fish. This increase was further enhancing when hCG injected fish kept in CPF alone or with Cur and Asc water. The results interpreted that both hCG and CPF have an additive role in Vtg synthesis in the liver that promote yolk accumulation *in vivo* condition within 24 h. Results suggest CPF did its estrogenic function in ovary without the influence of gonadotropin or antioxidants alone or in combination.[37-39]

Interplay on ovarian steroids

The results showed that the teleost ovary detected with low E_2 and 17α, 20β-DP but the good detectable amount of P_4 as per their early prespawning phase.[13] The gonadotropin injection increased the E_2 to enhance vitellogenesis process to bring follicle toward maturation by the liquidation of yolk.[40] The gonadotropin injected fish also registered an increase in P_4 and 17α, 20β-DP providing maturational competence that may culminate into ovulation. There are many reports to support this fact.[41,42] The hCG induced *in vitro* oocyte maturation of freshwater cyprinid, *Barilius vagra*[43] and catfish *H. fossilis*.[27] The administration of hCG induces the expression of 20β-HSD (hydroxysteroid dehydrogenase) required for the production of 17α, 20β-DP, a reported MIS (maturation inducing steroid).[13] The hCG injected fish in curcumin water maintains the 17α, 20β-DP production with a significant increase in the P_4. So far, there has been no thorough study eliciting the role of Cur in the gonadal maturation in fish except few to show it as an assistant.[35,44] The hCG injected fish in Asc water, has significantly increased the P_4 and 17α, 20β-DP levels followed by a decrease in the E_2 level. The findings of Dabrowski and Ciereszko (2001) also reported stimulation of P_4 synthesis by ascorbic acid by avian and bovine ovarian cells. The shift towards MIS indicates an impressive role of Asc in gonad maturation in fish.[45] The combination of antioxidants with hCG has significantly elaborated the MIS production as compared to the other groups which indicate the synergistic approach of the antioxidants (Loftsson, 2014).

Chlorpyrifos has been found to stimulate estradiol synthesis. It also triggered P_4 and 17α,20β-DP level which might be due to the inhibition of 17α-hydroxylase or C17,20 lyase (P450c17) which leads to an increased

amount of substrate available for 20β-HSD, the enzyme needed for the synthesis of 17α,20β-DP.[46,47] Similarly, as another estrogenic compound, Bisphenol A, induces ovarian steroidogenic genes.[24] CPF inhibited hCG induced 17α, 20β-DP production which might be due to the suppression of C_{21} steroids within the follicle.[48] Similarly, the administration of CPF to the combination of hCG and antioxidants decreased the E_2 level when compared to the CPF alone. The gonadotropin injected fish in CPF water alone or with antioxidants significant increase in P_4 and MIS facilitates oocyte maturation and ovulation *in vivo* as compared to the control group. CPF significantly reduced gonad major biochemicals (carbohydrate, protein and lipid) that are essential for healthy gametes.[49] The present study suggested CPF as an inducer of gonad steroid (17α, 20β-DP, P_4) that are necessary for maturation and ovulation. Because of this CPF will be resulted in the production of week ova, which can mature early *in vivo* condition in 24 hr study. In our unpublished data, we found that adult spawning phase fish spawn early with gonadotropin stimulation when pre-treated with CPF as compared to without pre-treatment. The antioxidants reported as a balancer to CPF induced toxicity,[9,50] emerged ineffective in the maintenance of the tonicity of hormones in *H. fossilis*.

CPF effect on *NR5A1* gene expression

A sublethal dose of CPF increased *NR5A1* gene expression multi-folds in a duration-dependent manner among young fry. The *NR5A1* is an activator of the aromatase enzyme.[23] CPF seems to be altered the mRNA expression level of *NR5A1* and epigenetic regulation.[24,26] CPF supports the early onset of gonad differentiation may lead to early sexual maturity in young fish. This may result in poor fecundity power and week gamete development as compare to healthy environment fish. Long term duration study is required further to set a trend for CPF impact on catfish reproductive physiology. Thus, the CPF has a detrimental effect both at the steroid and genetic level in catfish.

CONCLUSION

The present study has highlighted the interference of CPF on vitellogenin, hormonal levels and gene expression of *NR5A1* in freshwater catfish, *Heteropneustes fossilis*. Vitellogenins (Vtgs) are glycolipophosphoproteins essential for the successful development of oocytes and embryos of oviparous vertebrates, including fish. Steroids such as 17β-Estradiol (E_2), 4-Pregnene-3, 20-dione (P_4), 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) play an important role in the regulation of oocyte growth and maturation whereas *NR5A1* gene not only controls the expression of the steroidogenic cytochrome P450 enzymes in the endocrine tissues but is also involved in the sex determination and differentiation in vertebrates including mammals and fish. These three (vitellogenin, steroid levels and *NR5A1* gene) are important key points in deciding proper development and reproductive fitness in fish. The involvement of antioxidants (Cur, Asc) and their impact on the Vtg and hormone levels is explained in the present research work. This study is for the first time where an interplay of CPF, human chorionic gonadotropin (hCG) and antioxidants (Cur and Asc) were studied on the steroidogenesis. The *NR5A1* gene expression was studied with and without CPF in fry of freshwater catfish, *Heteropneustes fossilis*. The study concludes that CPF is an endocrine disruptor with a potential to perturb sensitive steroidal and genetic pathway that regulate reproductive functions. It induces increased vitellogenesis in fish which may lead to decreased fertility and egg production in females, or lead to reduced gonad size or feminization of genetic male fish. The antioxidants act as a balancer to chlorpyrifos induced toxicity, maintains the tonicity of hormones. Thus, the excess use of the pesticide has deterimental effect both at the steroidal and genetic level in aquaculture.

Thus, the present work is an effort to draw the attention towards the hazardous effect of pesticide, chlorpyrifos on catfish, *Heteropneustes fossilis*. There is a need of awareness among the people who perform agriculture practices to optimize the usage of CPF in order to protect environment and the biota who could come in contact with toxicant.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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