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RESEARCH ARTICLE

Haemato-biochemical Responses in *Cyprinus carpio* (Linnaeus, 1758) Fry Exposed to Sub-lethal Concentration of a Phenylpyrazole Insecticide, Fipronil

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Abstract The present study evaluates the haemato-biochemical responses associated with fipronil exposure [(±)-5-amino-1-(2,6-dichloro-α,α,α-trifluoro-*p*-tolyl)-4-trifluoromethylsulfinyl-pyrazole-3-carbonitrile] in *Cyprinus carpio* fry. Fish were exposed to sublethal concentration (1/3rd of LC_{50}) (0.142 mg L^{-1}) for 15 days and corresponding changes in different haemato-biochemical parameters were recorded at the end of experimental period. Significant ($P < 0.05$) increase in white blood cell counts, blood glucose, serum complement reactive protein and serum cortisol level were noticed, whereas haemoglobin and serum total protein contents were significantly ($P < 0.05$) decreased. Aspartate amino transferase, catalase and super oxide dismutase activities were significantly ($P < 0.05$) increased while alkaline phosphatase and malate dehydrogenase activities were significantly ($P < 0.05$) decreased. Similarly, 47 % inhibition in acetylcholine esterase activity was noticed due to fipronil stress. Results indicated that sublethal exposure of fipronil can induce haemato-biochemical alterations causing stress to *C. carpio* fry. Thus, haemato-biochemical parameters can be used as biomarkers for the sublethal toxicity of fipronil in the water bodies.

Keywords Fipronil · Sublethal · Biochemical · Cortisol · CRP · Stress

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Introduction

Applications of pesticides have been increasing ever since the onset of first green revolution to feed the overgrowing population of the world. This phenomenon is evident over the last few decades especially in tropical countries like India, where majority of the population is reliant on agriculture for their livelihood. Pesticides are substances widely used in current agriculture practices and have become a necessity to ensure increased productivity through the pest control. However, indiscriminate use of pesticides ultimately leads to pollution of aquatic environment and becomes hazardous to the aquatic life. Kilgore and Li [1] emphasized that concentration of pesticide residues were found to be more in aquatic ecosystem rather than in terrestrial ecosystem because pesticides are transported to greater distances in the hydrosphere affecting many non-target organisms. In India as high as 70 % of the chemicals employed in the agricultural practices, find their way into freshwater bodies [2] and affect various non target organisms including fishes even at sublethal concentrations [3]. Fishes are one of the most important groups of aquatic organisms, which are vulnerable to such conditions and can be considered as an important indicator of environmental stressors [4].

Among the different species of fish cultured in India, common carp (*Cyprinus carpio*) is the most extensively

cultured species in paddy cum fish farming system due to its omnivorous feeding, tolerance to diseases, superior growth, prolific breeding and capability to sustain wide fluctuations in environmental conditions. In paddy cum fish farming various kinds of pesticides are applied as a means to protect rice crop from the attack of various pests. The use of the eco-friendly insecticides to suppress the pest population is gaining considerable attention in integrated pest management programmes.

A new class of insecticide, the phenylpyrazole, is replacing organochlorines and organophosphates as agricultural insecticide. Phenylpyrazole insecticide has been shown to enter the aquatic environment from agricultural runoff or drift from aerial or ground based spraying applications where they may pose threat to young fishes [5–9]. Fipronil is a new broad-spectrum phenylpyrazole insecticide which is commercially sold in the Indian market under various trade names. Because of impending bans on application of dieldrin, lindane and DDT, use of fipronil is gaining considerable attention in recent years [10]. Even minute concentration of fipronil is highly effective against various insects and pests of crops, notably rice insects, thrips, and termites [11] owing to its lipophilicity and persistency. Fipronil is used to protect paddy crop from the attack of various pests in paddy-cum fish integrated farming system and also to control bund-destroying crabs in rice field. Thus, there is potential threat on non-target species such as fish. Moreover, fipronil is reported to be toxic to rainbow trout (*Oncorhycus mykiss*) and bluegill sunfish (*Lepomis macrochirus*) with 96 h LC_{50} of 0.246 and 0.083 mg L^{-1}, respectively. It is also toxic to Japanese carp with 96 h LC_{50} value of 0.34 mg L^{-1} [12] and sheephead minnows (*Cyprinodon variegatus*) (LC_{50} = 0.13 mg L^{-1}) on acute basis [13]. However, there is not much information available pertaining to the toxicity of fipronil on the heamato-biochemical responses of *C. carpio*, one of the important aquaculture species with high commercial demand in the domestic market. Hence, the present study was aimed to delineate toxicity of fipronil to *C. carpio* fry in terms of median lethal concentration (LC_{50}) and subsequently to assess the risks of haemato-biochemical perturbations in *C. carpio* fry as a result of short term (15 days) exposure of sublethal concentration of fipronil which may prove to be useful indicator of the health status of a specific aquatic ecosystem.

Material and Methods

Experimental Animal and Site of the Experiment

Cyprinus carpio fry, with an average weight of 3.26 ± 0.43 g, were procured from Palghar fish farm (19°41′0″N; 72°45′0″E), Maharashtra during the month of February 2011. Fishes were transported in a big circular container (500 L) with sufficient aeration and were carefully transferred to cement tank. The next day, fishes were given a salt (NaCl) treatment (1 %) for 5 min to ameliorate the handling stress if any. The stock was acclimatized under aerated conditions for 15 days by using tap water supply, free from any pesticide or toxicant contamination. During acclimation, fish were fed to satiety (4 % of body weight) with control diet having 30 % crude protein (CP). Feeding was stopped 24 h before the treatment of the fish with fipronil and no feeding was done during 96 h of exposure to the fipronil. The laboratory analysis was carried out at the Fish Nutrition and Biochemistry Laboratory of Central Institute of Fisheries Education (CIFE), Mumbai.

Chemicals

Technical grade fipronil ($C_{12}H_4Cl_2F_6N_4OS$) (99.1 % pure) manufactured by Bio Quest International Private Limited Mumbai, India, consisting of alpha and beta isomers at the ratio of 50:50, was procured from Malti enterprises, Mumbai. It was kept in an airtight container in the refrigerated condition. All other chemicals used in the experiment were of analytical grade. Stock solution of fipronil was prepared using analytical grade acetone (solubility of fipronil in acetone is 545.9 g L^{-1}). Required quantity of fipronil was drawn from this stock solution for the further experiment. Range finding test was conducted using methodology of APHA [14]. Experiment was carried out to determine the median lethal concentration (LC_{50}) of fipronil in *C. carpio* fry for 96 h by probit analysis [15].

Bioassay

Cyprinus carpio fry of average weight, 3.26 ± 0.43 g were used for bioassay experiment in uniform sized rectangular glass aquaria (50 L capacity). In each glass aquarium total volume of water was maintained at 40 L and was provided with round the clock aeration. Test organisms were exposed to a logarithmic increasing range of concentrations such as 0.01, 0.1, 1.0 and 10.0 mg L^{-1}. Static non-renewable bioassay was conducted with 15 test animals for each concentration. Percentage mortality was observed every 24 h interval at 24, 48, 72 and 96 h. In range finding test of fipronil, it was found that mortality of 0 and 100 % lies in between 0.1 and 1.0 mg L^{-1}.

For definitive test, six fipronil concentrations 0.37, 0.39, 0.41, 0.43, 0.45 and 0.47 mg L^{-1} were selected and the tests were conducted in triplicates for each concentration containing 15 fishes in each replicates. Side by side one control was also kept slightly away from bioassay tank to avoid contamination. No feeding was done during this period. Percentage mortality was recorded at 24, 48, 72 and

96 h interval and dead fishes were removed immediately. The data obtained from the experiment was processed by probit analysis using statistical software package SPSS 16.0. During the experimental period different water quality parameters were measured using standard procedures and were within optimum range.

Sublethal Toxicity Studies

To study the haemato-biochemical alterations due to sublethal exposure of fipronil, an experiment was conducted using uniform sized rectangular tubs (50 L capacity) as experimental units. Ninety fishes were distributed into two experimental groups in triplicates following a completely randomized design. The treatment groups were control and exposed. The total volume of the water in each tub was maintained at 40 L throughout the experimental period. Round the clock aeration was provided. In the exposed group, sublethal concentration of (1/3rd of LC_{50}) (0.142 mg L^{-1}) was maintained to simulate the fipronil contaminated water bodies of India. Fishes were fed with control diet having 30 % CP and given to satiation level at 09:00 h and at 18:00 h daily for 15 days. The uneaten feed and faecal matter were removed by siphoning out about 50 % of the tank water on alternate days with care to avoid stress to the test organisms. About 50 % of the tank water which was siphoned out on alternate days was renewed with 50 % fipronil treated water prepared from stock solution in order to provide constant sublethal effect of the fipronil. Similar methodology was followed for sublethal toxicity studies by Das and Mukherjee [16]. The physico-chemical parameters of the rearing water were within the optimum range (dissolved oxygen: 6.56–7.1 mg L^{-1}; pH: 7.25–7.8; temperature: 26.6–28.2 °C; alkalinity: 46–58 mg L^{-1}; hardness: 48–64 mg L^{-1}; ammonia: 0.09–0.15 mg L^{-1}; and nitrite; 0.04–0.06 mg L^{-1}) throughout the experimental period which is in agreement with Prusty et al. [3].

Analysis of Fipronil

For analysis of fipronil, experimental water sample (200 mL) was ground in a high speed blender with excess anhydrous sodium sulfate (100 g). Acetonitrile and saturated petroleum ether were used for pesticide fractionation. Petroleum ether was then collected and evaporated to 10 mL. The concentrate (2 µL) was injected into GC (Shimazdu 14 B) with capillary column of 1.85 m length, 4 mm internal diameter, made of glass, packed with 10 % D.C. 200 (w/w) on solid support 80–100 mesh chromosorb WHP, and with electron capture detector (63 Ni). Nitrogen was the carrier gas with flow rate of 30 mL min^{-1}. The column temperature was increased from 150–250 °C at the rate of 10 °C min^{-1} held at 250 °C for 16 min. The temperature of the detector and injector was 270 °C. The α and β isomers of fipronil were identified using specific standards. Fipronil was analyzed in Reliable Analytical Laboratories, Thane, Mumbai, India.

Tissue Homogenate Preparation

At the end of 15 days of sublethal exposure of fipronil, six fishes per treatment groups were sampled and anaesthetized with CIFECALM (50 µL L^{-1}) for blood collection and were sacrificed for tissue collection for enzyme studies. CIFECALM is an herbal anesthetic formulation containing natural alcoholic extracts of *Eugenia caryophyllata* and *Mentha arvensis*. The muscle, liver, gill and brain tissues were dissected and weighed carefully. A 5 % homogenate was prepared with chilled sucrose solution (0.25 M) in a glass tube using Teflon coated mechanical tissue homogenizer. The tube was continuously kept in ice to avoid heating. The homogenate was centrifuged at 5,000×*g* for 10 min at 4 °C in a cooling centrifuge. The supernatant was collected and stored at −20 °C for enzyme studies [3, 10].

Haematological Studies

Blood was collected by puncturing the caudal vein using a medical syringe (No. 23), which was previously rinsed with 2.7 % EDTA solution (as an anticoagulant) and shaken gently in order to prevent haemolysis. The blood samples were used for determination of haemoglobin (Hb) content, total RBC (erythrocyte) counts, total WBC (leukocyte) counts and for nitroblue tetrazolium assay. For serum, two fresh fishes from each replicate with a total of six fish from each treatment groups were anesthetized with CIFECALM. The serum was collected without anti-coagulant and allowed to clot for 2 h, centrifuged (3,000×*g* for 5 min) and then kept at −80 °C until use. The Hb percentage was determined by estimating cyanmethemoglobin using Drabkin's fluid (Qualigens, India). Five mL of Drabkin's working solution was taken in a clean and dry test tube and 20 µL of blood was added to it. The absorbance was measured using a spectrophotometer (MERCK, Nicolet, Evolution 100) at a wavelength of 540 nm. The final concentration was calculated by comparing with standard cyanmethemoglobin (Qualigens, India). Total RBC and WBC were counted in a haemocytometer using erythrocyte and leukocyte diluting fluids (Qualigens, India), respectively. Twenty microliter of blood was mixed with 3,980 µL of diluting fluid in a clean glass test tube. The mixture was shaken well to suspend the cells uniformly in the solution. The cells were counted using a haemocytometer.

The following formula was used to calculate the number of RBCs and WBCs counts per ml of the blood sample

$$\text{Number of cells mm}^{-3} = (\text{Number of cells counted} \times \text{dilution})/(\text{Area counted} \times \text{depth of fluid})$$

Packed cell volume or Heamatocrit (Hct %) was determined by the Wintrobe and Westergreen method as described by Blaxhall and Daisley [17].

The mean corpuscular volume (MCV-fL), mean corpuscular Hb (MCH-pg) and mean corpuscular Hb concentration (MCHC-g dL^{-1}) were determined according to following formulae as proposed by Dacie and Lewis [18].

$$\text{MCV} = (\text{Hct})/\text{RBC} \times 10$$

$$\text{MCH} = (\text{Haemoglobin})/\text{RBC}) \times 10,$$

$$\text{MCHC} = (\text{Haemoglobin}/\text{Hct}) \times 100.$$

where Hct represents Haematocrit percentage.

Blood Glucose and Serum Cortisol

Blood glucose was estimated by the method of Nelson-Somogii [19]. Serum cortisol levels were determined using a commercially available cortisol specific competitive binding enzyme linked immunosorbent assay (ELISA) kit (Neogen Corporation, Lexington, KY, USA) from triplicate samples. These cortisol ELISA kits and methodologies have been successfully validated and utilized in many fish species [20–22].

Serum Protein, Albumin and Complement Reactive Protein (CRP)

Serum protein was estimated by biuret and BCG dye binding method of Reinhold [23] using the kit (total protein and albumin kit, Qualigens Diagnostics, division of Glaxo SmithKline Pharmaceutical Limited). Albumin was estimated by bromocresol green binding method of Doumas et al. [24]. The absorbance of standard and test were measured against the blank in a spectrophotometer at 630 nm. CRP determinations were carried out by Olympus AU commercial kits.

Enzyme Assays

Lactate Dehydrogenase (LDH) and Malate dehydrogenase (MDH)

Lactate dehydrogenase (L-lactate NAD + oxidoreductase; E.C.1.1.1.27) was assayed in the liver and muscle tissue homogenates using 0.1 M phosphate buffer (pH 7.5), 0.2 mM NADH solution in 0.1 M phosphate buffer. The reaction was initiated by adding 0.2 mM sodium pyruvate as the substrate and optical density (OD) was recorded at 340 nm as described by Wroblewski and Ladue [25]. A similar reaction mixture was used for the estimation of malate dehydrogenase (L-malate: NAD + oxidoreductase; E.C.1.1.1.37) except for the substrate (1 mg oxaloacetate/mL of chilled triple distilled water) by the method of Ochao [26].

Acetylcholine Esterase (AChE)

Acetylcholine esterase enzyme (E.C.3.1.1.7) was assayed in the brain tissue homogenate by the method of Hestrin [27]. Acetylcholine esterase assay system comprised of 1.0 mL of M/15 phosphate buffer (pH 7.2), 1 mL acetylcholine (0.004 M, pH 4.0) substrate buffer mixture (1:9 dilution), and 0.2 mL of homogenate and incubated for 30 min at 37° C. Alkaline hydroxylamine (2.0 mL) was added to terminate the reaction. The solution was mixed thoroughly and 1 mL of HCl (2:1) was added followed by thorough mixing. Enzyme solution was then added to the control tubes. The color was developed by addition of 1 mL of $FeCl_3$ (10 %) and OD was recorded at 540 nm after thorough mixing.

Alkaline Phosphatase (ALP)

ALP (E.C. 3.1.3.1) enzyme activity was determined in the liver tissue homogenate by the method of Garen and Levinthal [28]. The assay mixture comprised of 0.2 mL bicarbonate buffer (0.2 M), 0.1 mL of 0.1 M $MgCl_2$, 0.1 mL tissue homogenate, 0.5 mL of distilled water and 0.1 mL of freshly prepared 0.1 M para-nitrophenyl phosphate. The reaction mixture was incubated in water bath at 37 °C for 15 min and the reaction was stopped by 1.0 mL of 0.1 N NaOH. OD was taken at 410 nm. Phosphate liberated was estimated at OD of 660 nm [29].

Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST)

Alanine amino transferase (L-alanine 2 oxaloglutarate aminotransferase; E.C.2.6.1.2) activity was assayed in the liver and muscle tissue homogenates as described by Wotton [30]. The substrate comprised of 0.2 M D, L-alanine acid and 2 mM α-ketoglutarate in 0.05 M phosphate buffer (pH 7.4). In the experimental and control tubes, 0.5 mL of substrate was added. The reaction was started by adding 0.1 mL of tissue homogenate. The assay mixture was incubated at 37 °C for 60 min. The reaction was terminated by adding 0.5 mL of 1 mM 2,4-dinitrophenylhydrazine (DNPH). In the control tubes the enzyme source was added after DNPH solution. The tubes were held at room temperature for 20 min with occasional shaking.

Then 5 mL of 0.4 mL NaOH solution was added, the contents were thoroughly mixed. After 10 min, the OD was recorded at 540 nm against a blank. The procedure adopted for aspartate amino transferase (L-aspartate: 2 oxaloglutarate aminotransferase, E.C.2.6.1.1) activity was same as for ALT activity except the substrate comprised of 0.2 M D, L-aspartic acid instead of alanine.

Catalase and Super Oxide Dismutase (SOD)

Catalase (E.C.1.11.1.6) was assayed in the liver and gill tissue homogenates using 50 mM phosphate buffer (pH 7.0). The reaction was initiated by adding 30 % H_2O_2 as the substrate and O.D. was recorded at 240 nm [31]. Superoxide dismutase (E.C. 1.15.1.1) activity was estimated by the method of Misra and Fridovich [32]. This is based on the oxidation of epinephrine–adrenochrome transition by the enzyme. The reaction mixture consisted of 50 µL of sample, 1.5 mL phosphate buffer and 0.5 mL epinephrine. The solution was mixed well and OD was immediately read at 480 nm.

Total Protein Estimation

Quantification of protein in the different tissues was carried out using Lowry et al. [33]. All the colorimetric assays were carried out using UV–Vis spectrophotometer (E-Merck, Germany)

Statistical Analysis

The statistical significance for various parameters between the control and fipronil exposed groups were determined using student's '*t*' test according to the method of Baily [34] using SPSS 16.0. The data was presented as mean ± SE of six replicates per group and the significance were tested at $P < 0.05$.

Results

Data pertaining to acute toxicity test of technical grade fipronil on fry at 24, 48, 72 and 96 h and corresponding LC_{50} values with 95 % confidence interval are presented in Table 1. The 96 h LC_{50} value of fipronil to *C. carpio* fry was found to be 0.428 mg L^{-1} with 95 % confidence limit ranging between 0.418 and 0.439 mg L^{-1} at temperature range of 26–28 °C and pH 7.25–7.5. The values showed a gradual decrease with increase in exposure time. Concentration level of fipronil in control and exposed groups were confirmed by the results of fipronil analysis before the start of sublethal toxicity experiment. In the control group no fipronil was detected however sublethal concentration of 0.142 mg L^{-1} was found in the exposed group (Fig. 1).

Table 1 Median lethal concentration (LC_{50}) of *C. carpio* fry exposed to fipronil for a period of 96 h

Period of exposure (h)	LC_{50} (mg L^{-1})	95 % confidence interval	
		Lower limit	Upper limit
24	0.539	0.492	0.743
48	0.467	0.450	0.500
72	0.440	0.429	0.455
96	0.428	0.418	0.439

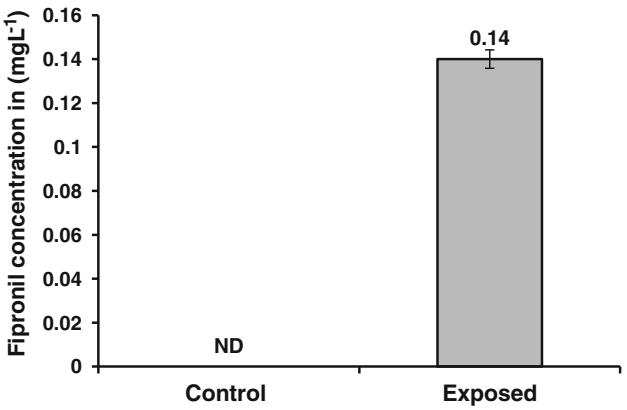

Fig. 1 Concentration of fipronil in control and exposed groups analysed before start of the sublethal experiment. Data represent the mean ± SE of three replicates. *ND* not detected

Effects of Sublethal Fipronil Exposure on Haematological Parameters

WBC count, MCH and blood glucose in fipronil exposed group was significantly ($P < 0.05$) increased over the control group whereas Hb percentage was significantly ($P < 0.05$) decreased (Table 2). No significant ($P > 0.05$) differences were observed in erythrocyte count, haematocrit percentage, MCV and MCHC between the control and exposed group (Table 2). Total serum protein was significantly ($P < 0.05$) decreased while albumin values were not affected significantly ($P > 0.05$) due to fipronil exposure (Fig. 2). Significant increase ($P < 0.05$) in serum CRP (Fig. 3) and cortisol (Fig. 4) were observed in fipronil exposed group.

Effects of Sublethal Fipronil Exposure on Enzymatic Activities

Changes in the activity of certain enzymes in fish exposed to fipronil were recorded at the end of experimental period (Table 3). AST activities in both liver and muscle were significantly ($P < 0.05$) higher in fipronil exposed group than the control (Table 3), whereas exposure of fipronil led to significant ($P < 0.05$) decline in acetylcholine esterase

Table 2 Changes in the indices of blood plasma in a *C. carpio* fry treated with sublethal concentration of fipronil

Parameters	Control	Exposed
WBC(10^3 cells mm^{-3})	238.25 ± 0.80	288.24 ± 1.49*
RBC (10^6 cells mm^{-3})	1.03 ± 0.02	1.01 ± 0.01
Hb (gm %)	7.16 ± 0.10	6.30 ± 0.11*
Hct (%)	16.20 ± 0.62	14.55 ± 0.59
MCV (fL)	144.67 ± 3.39	159.80 ± 7.08
MCH (pg)	42.48 ± 1.16	65.78 ± 2.60*
MCHC (g dL^{-1})	41.20 ± 2.44	46.66 ± 1.41
Blood glucose (g dL^{-1})	92.71 ± 4.22	121.16 ± 6.16*

Values are represented as the mean ± SE of six replicates. Asterisks indicate significant difference from control and fipronil exposure where * $P < 0.05$

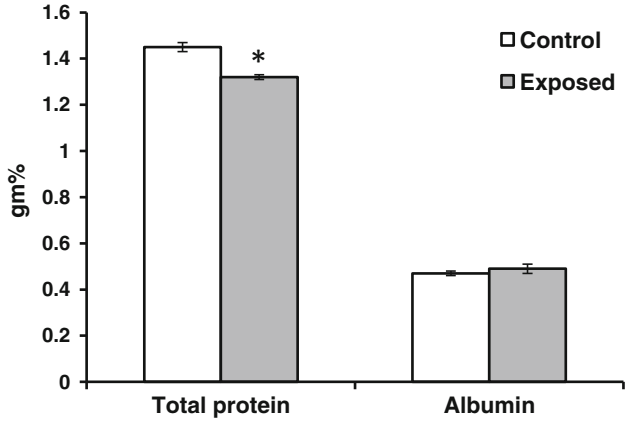

Fig. 2 Total serum protein and albumin content of control and fipronil exposed *C. carpio* fry at the end of 15 days exposure. Data represent the mean ± SE of six replicates. *Asterisks* indicate significant difference from control and fipronil exposure where *$P < 0.05$

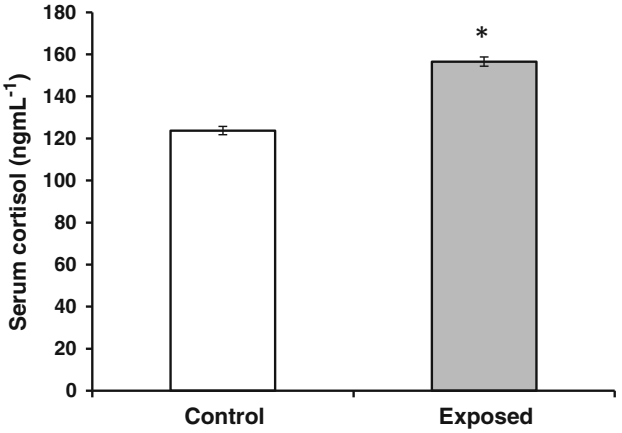

Fig. 4 Serum cortisol level of control and fipronil exposed *C. carpio* fry at the end of 15 days exposure. Data represent the mean ± SE of six replicates. *Asterisks* indicate significant difference from control and fipronil exposure where *$P < 0.05$

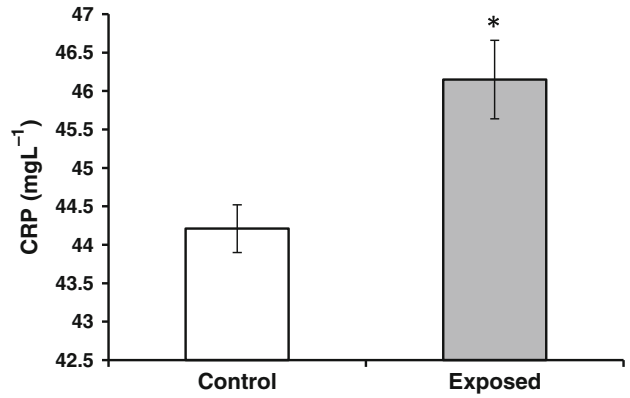

Fig. 3 CRP of control and fipronil exposed *C. carpio* fry at the end of 15 days exposure. Data represent the mean ± SE of six replicates. *Asterisks* indicate significant difference from control and fipronil exposure where *$P < 0.05$

in brain (Fig. 5). ALP activity in the liver and MDH activity in muscle were significantly ($P < 0.05$) decreased in the exposed group than the control group. However, there were no significant changes in ALT and LDH activity in both liver and muscle tissues as well as MDH activity in muscle tissue (Table 3).

Activity of catalase and SOD in the liver of fish exposed to fipronil were increased significantly ($P < 0.05$) whereas activity of catalase and SOD in gill did not vary significantly ($P > 0.05$) (Table 3).

Discussion

Cyprinus carpio, is the most important exotic carp species widely used in composite fish culture systems with Indian major carps. It also forms vital component of paddy cum fish integrated fish farming system due to its omnivorous feeding and hardiness to the fluctuating environmental situation. High growth potential coupled with consumer preference in domestic market has established C. *carpio* as one of the important freshwater species cultured not only in India but also in the other parts of the world.

According to environmental protection agency (EPA), fipronil is highly effective, broad-spectrum phenylpyrazole insecticide and is toxic to many fishes [13]. Sublethal dose

Table 3 Changes in the different enzyme parameters in *C. carpio* fry treated with sublethal concentration of fipronil

Parameters	Control	Exposed
AST (liver)	2.67 ± 0.05	4.01 ± 0.21*
AST (muscle)	2.55 ± 0.34	3.45 ± 0.20*
ALT (liver)	1.94 ± 0.24	2.67 ± 0.23
ALT (muscle)	2.08 ± 0.31	2.56 ± 0.29
ALP (liver)	36.62 ± 3.90	23.89 ± 3.56*
LDH (liver)	1.18 ± 0.15	0.83 ± 0.14
LDH (muscle)	0.32 ± 0.02	0.33 ± 0.03
MDH (liver)	0.10 ± 0.03	0.07 ± 0.02
MDH (muscle)	0.07 ± 0.01	0.03 ± 0.01*
Catalase (Liver)	5.35 ± 0.80	8.79 ± 0.51*
Catalase (gill)	5.15 ± 0.46	4.52 ± 0.25
SOD (liver)	81.96 ± 1.20	110.03 ± 1.40*
SOD (gill)	55.63 ± 4.80	51.45 ± 3.11

Enzyme activities are expressed as follows ALT: specific activities expressed as nano moles of sodium pyruvate formed/mg protein/min at 37 °C. AST specific activities expressed as nano moles of oxaloacetate released/min/mg protein at 37 °C. LDH: specific activity expressed as U min^{-1} mg^{-1} protein at 37 °C MDH: specific activity expressed as U min^{-1} mg^{-1} protein at 37 °C. nanomoles *p*-nitrophenol released/mg protein/min at 37 °C (ALP). Catalase: m moles H_2O_2 decomposed/min/mg protein at 37 °C; SOD: μ mol mg^{-1} protein/min at 37 °C

Values are represented as the mean ± SE of six replicates. Asterisks indicate significant difference from control and fipronil exposure where * $P < 0.05$

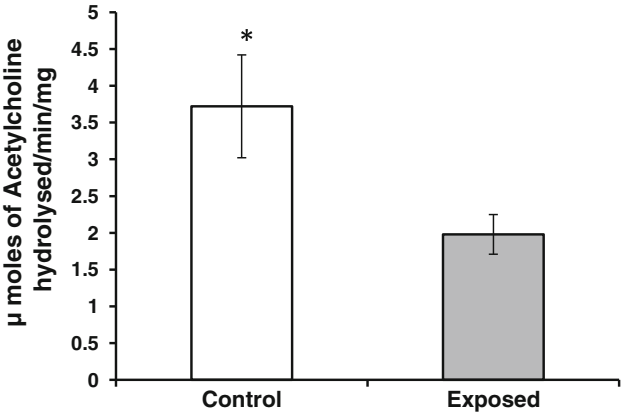

Fig. 5 Acetylcholine esterase (AChE) enzyme activity of control and fipronil exposed *C. carpio* fry at the end of 15 days exposure. Data represent the mean ± SE of six replicates. *Asterisks* indicate significant difference from control and fipronil exposure where *$P < 0.05$

is a potentially lethal dose of a substance which is not large enough to cause death. Therefore, sublethal fipronil concentration of 1/3rd of LC_{50} (0.142 mg L^{-1}) was selected for the study, which may produce considerable alterations in the haemato-biochemical responses, resulting into delayed adverse health effects and ultimate death of fishes. Thus, it may lead to heavy economical loss to the fish farmers.

Significant increase of WBC count in fipronil exposed group indicates the hypersensitivity of WBC to fipronil, which may be due to immunological reactions to produce antibodies to cope up with stress induced by fipronil. Increase in WBCs count can be correlated with an increase in antibody production which helps in survival and recovery of fish exposed to pesticides [3, 35]. Decrease in the Hb content in this study may be resulted from rapid oxidation of Hb to methaemoglobin or release of O_2^- radical due to the toxic stress of fipronil. It is well documented that xenobiotics capable of undergoing redox cycling can exert toxic effects via generation of oxygen free radicals. Results of the present study are in agreement with the findings of Matkovics et al. [36], who observed a quick decrease in Hb content in *C. carpio* due to paraquat toxicity. Although, there were no significant ($P > 0.05$) changes in the RBC count and Hct values in the fipronil treated group, but sublethal exposure of fipronil resulted in significant ($P < 0.05$) increase in the MCH values. The mechanism by which MCH values were elevated in exposed group is not known.

Significant increase in the blood glucose level in fipronil exposed group might have resulted from gluconeogenesis to provide energy for the increased metabolic demands imposed by fipronil stress. Changes in blood glucose have been suggested as sensitive indicator of stress in the teleost [37]. The stress related hyperglycemia reported in many species of teleost is mediated mainly by the effect of catecholamine on glucose release from the liver, the main carbohydrate store in fish, with epinephrine being more potent than nor-epinephrine [38].

There was significant decrease in total serum protein content of the *C. carpio* fry in the fipronil exposed group, which is in agreement with the finding of Jenkins et al. [39]. Fish utilizes protein mainly as a source of energy to meet the metabolic and physiological demand of the body, but on exposure to stressors more protein may be mobilized via oxidation of amino acids. Thus decreased serum protein level may be attributed to stress mediated mobilization to accomplish an increased energy demand by the fish to cope up with stress [39]. The depletion in total protein content may be due to enhanced proteolysis and possible utilization of their product for metabolic purposes as reported by Ravinder et al. [40]. However, Neff [41] has suggested that decline in protein content may be related to impaired food intake, increased energy cost of homeostasis, tissue repair and detoxification mechanism during stress.

Serum cortisol level is widely used as a primary stress response to exposure of pollutants, handling and space restriction such as confinement, air exposure and crowding

[42]. The hypothalamo-pituitary-interrenal axis of fish is activated to produce cortisol and other corticosteroid hormones for the maintenance of disturbed homeostasis [43, 44]. In the present study, plasma cortisol level increased from baseline control values of 123.73–156.55 ng mL^{-1} upon fipronil exposure. Elevated cortisol level upon different environmental stressors in teleost fishes has been reported by many workers [45–47]. Basal circulating cortisol levels show diurnal and seasonal fluctuations and interspecific differences and therefore the normal ranges are highly variable in aquatic vertebrates including fish.

CRP acts as secondary stress response parameter and is considered as reliable biomarker for aquatic toxicity studies [48]. Significant increase in CRP level in fipronil exposed group is in agreement with Sinha et al. [49] who observed induction of CRP level in *Labeo rohita* after exposure of mercury and cadmium stress. Toxic agents increase the non antibody proteins, in fishes as a first line defense towards environmental pollutant. This increased the presence of CRP in the acute phase response of invertebrates and some fishes which suggests that they may have an important role in overall host mechanism to toxicity exposure, directing attention to the apparently reciprocating relationship between immune and chemical detoxifying systems [49–51].

In the present experiment, significant inhibition of 47 % was observed in brain AChE due to fipronil stress. AChE is one of the most widely used enzyme known as a biomarker for environment pollution studies. In general, fishes can tolerate about 70–80 % inhibition of AChE activity before death as reported by Sarma [52], but Devraj et al. [53] showed 80 % inhibition of AChE activity in *Oreochromis mossambicus* without recording mortality; however both observed some behavioral changes like sluggish movement and loss of balance etc. According to them, this may be due to maximum inhibition of AChE activity in cerebellum, which controls the muscular co-ordination. Results of the current investigations are in agreement with the findings of Tejpal et al. [42] in *Cirrhinus mrigala*, Sarma et al. [54] in *Channa punctatus* and Akhtar et al. [55] in *L. rohita* on exposure to endosulfan.

Significant increase of AST activity in both liver and muscle tissues suggests that stress conditions in general induce elevation in transamination pathway [56]. Involvement of alternate pathways like aminotransferase reactions are also possible due to inhibition of oxidative enzymes like MDH, a situation also demonstrated by Ghosh [57] in *L. rohita* under cypermethrin toxicity. Transamination enzymes transfer amino group of an amino acid to a keto acid changing the latter into a new amino acid and the original amine into a new keto acid, thereby redistributing amino group among amino acids forming new amino acids. Amino acids are the major source of energy in teleosts [58]. Generally, stress hormone (cortisol) is responsible for mobilizing protein and lipid source for glucose synthesis. For this, amino acids are de-aminated to produce tricarboxylic acid (TCA) cycle intermediates resulting in the induction of elevation in transamination pathway.

ALP, a zinc-containing metallo-enzyme, plays an important role in phosphorus metabolism in the body. The activity of ALP in the liver reduced significantly in the fipronil exposed group. Inhibition of hepatic ALP activity in fipronil exposed group is consistent with other results previously observed in *C. punctatus* [54] and also in *C. carpio* fingerlings [59]. Such inhibition of ALP activity could possibly be an indication of role of fipronil in phosphorous metabolism. The MDH activity, an enzyme of TCA cycle, which is involved in the reversible conversion of L-malate and oxaloacetate, showed inhibitory effect in muscle due to fipronil exposure. The inhibition in the muscle MDH activity could either be due to changes in the conformation of active site, formation of enzyme-inhibitor complex leading to impairment of carbohydrate metabolism. Results of present investigations are in agreement with the findings of Sarma et al. [54] in *C. punctatus* on exposure to endosulfan toxicity and Verma et al., [59] in *C. carpio* on persistent sublethal exposure of chlorine.

The current study demonstrated significant increase in catalase and SOD activities in liver of exposed group. The increase in SOD activity in the liver of the fish may be due to the production of superoxide anions which led to the induction of SOD to convert the superoxide radical to H_2O_2. The increase in catalase activity in the liver may be a response to the hydrogen peroxide produced by SOD activity since catalase is responsible for the detoxification of hydrogen peroxide. Increase in the activity of catalase and SOD is usually observed in the face of environmental pollutants since SOD-CAT system represents the first line of defense against oxidative stress [60]. Furthermore, the increase in antioxidant enzymes in the liver demonstrates that liver has an important role in the detoxification of fipronil. This is also corroborated by the observations reported by Farombi et al. [61].

Conclusion

To summarize the above findings, haemato-biochemical parameters clearly indicated the fipronil induced impairment of metabolism as fish were observed to be under severe metabolic stress. The evaluated parameters like haematological alterations and variations in different enzyme activities can be used as good biomarkers of fipronil pollution in the water bodies. Cortisol, CRP and AChE are found to be indicator parameters for the pesticide to ascertain the toxicity of fipronil. Further research with toxicity testing methods in same or other species of fish would give more comprehensive picture which would be of great importance in monitoring possible ecotoxicological risk assessments of fipronil.

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References

1. Kilgore WW, Li M (1975) Environmental toxicology. In: Wilkinson CF Insecticide biochemistry and physiology, Olenum Press, New York, p 669
2. Bhatnagar MC, Bana AK, Tyagi M (1992) Respiratory distress to *Clarias batarachus* (Linn.) exposed to endosulfan: a histological approach. J Environ Biol 13:227–231
3. Prusty AK, Kohli MPS, Sahu NP, Pal AK, Saharan N, Mohapatra S, Gupta SK (2011) Effect of short term exposure of fenvalerate on biochemical and haematological responses in *Labeo rohita* (Hamilton) fingerlings. Pest Biochem Physiol 100:124–129
4. Kumar A, Prasad MR, Srivastava K, Srivastav SK, Suzuki N, Srivastav AK (2013) Cyto-histopathological alterations in the liver of azadirachtin treated catfish heteropneustes fossilis. Proc Nat Acad Sci India Sect B Biol Sci. doi:10.1007/s40011-013-0169-7
5. US Environmental protection Agency (USEPA) (1997) Environmental fate and effects Section 3 registration decision for fipronil: use on rice seed, D235912, Environmental Fate and Effects Division, Office of Pesticide Programs, Washington, DC
6. US Environmental protection Agency (USEPA) (2001) Fipronil environmental fate and ecological effects assessment and characterization for a Section 3 for broadcast treatment with granular product to control turf insects and fire ants (addendum), Environmental Fate and Effects Division, Office of Pesticide Programs, Washington, DC
7. Fenet H, Beltran E, Gadji B, Cooper JF, Coste CM (2001) Fate of phenylpyrazole in vegetation and soil under tropical field conditions. J Agric Food Chem 49(3):1293–1297
8. Schlenk D, Huggett DB, Allgood J, Bennett EJ, Rimoldi AB, Beeler D, Block D, Wolder AW, Hovinga R, Bedient P (2001) Toxicity of fipronil and its degradation products to *Procambarus* sp.: field and laboratory studies. Arch Environ Contam Toxicol 41:325–332
9. Walse SS, Pennington PL, Scott GI, Ferry JL (2004) The fate of fipronil in modular estuarine mesocosms. J Environ Monitor 6:58–64
10. Gupta SK, Pal AK, Sahu NP, Saharan N, Mandal SC, Chandraprakash, Akhtar MS, Prusty AK (2012) Dietary microbial levan ameliorates stress and augments immunity in *Cyprinus carpio* fry (Linnaeus, 1758) exposed to sublethal toxicity of fipronil. Aquacult Res. doi:10.1111/are.12030
11. Mulrooney JE, Wolfenbarger DA, Howard KD, Goli D, Goli D (1998) Efficacy of ultra low volume and high volume applications of fipronil against the boll weevil. J Cotton Sci 2:110–116
12. Colliot F, Kukorowski KA, Hawkins DW, Roberts DA (1992) Fipronil: a new soil and foliar broad spectrum insecticide. Brighton Crop Prot Conf-Pests Dis 2(1):29–34
13. US Environmental protection Agency (USEPA) (1996) New pesticide fact sheet, EPA 737-F-96-005, Office of Pesticide Programs, Washington, DC
14. APHA–AWWA–WPCF (1975) Bioassay for aquatic organisms. Standard methods for the estimation of water and waste water, 19th edn. American Public Health Association, Washington, p 800–869
15. Finney DY (1971) Probit analysis, 3rd edn. Cambridge University Press, London
16. Das BK, Mukherjee SC (2003) Toxicity of cypermethrin in *Labeo rohita* fingerlings: biochemical, enzymatic and haematological consequences. Comp Biochem Physiol 134(C):109–121
17. Blaxhall PC, Daisley KW (1973) Routine haematological methods for use with fish blood. J Fish Biol 5(6):771–781
18. Dacie JV, Lewis SM (2001) Practical haematology, 9th edn. Churchill Livingstone, London, p 633
19. Nelson-Somogii (1944, 1945) Cited by Oser BL (1965). In: Hawk's physiological chemistry, 14th edn. McGraw Hill Publication, New York, pp 113
20. Afonso LOB, Basu N, Nakano K, Devlin RH, Iwama GK (2003) Sex related differences in organismal and cellular stress response in juvenile salmon exposed to treated bleached kraft mill effluent. Fish Physiol Biochem 29(2):173–179
21. Stefansson SO, Nilsen TO, Ebbesson LOE, Wargelius A, Madsen SS, Bjornsson BT, McCormick SD (2007) Molecular mechanisms of continuous light inhibition of Atlantic salmon parr smolt transformation. Aquaculture 273(2–3):235–245
22. Fast MD, Hosoya S, Johnson SC, Afonso LOB (2008) Cortisol response and immune related effects of Atlantic salmon (*Salmo salar* L.) subjected to short and long term stress. Fish Shellfish Immunol 24(2):194–204
23. Reinhold JG (1953) Manual determination of serum total protein, albumin and globulin fractions by Biuret method. In: Reiner M (ed) Standard method of clinical chemistry. Academic Press, New York, p 88
24. Doumas BT, Watson WA, Biggs HG (1971) Albumin standards and measurement of serum albumin with bromocresol green. Clinica Chimica Act 31(1):87–96
25. Wroblewski F, Ladue JS (1955) LDH activity in blood. Proc Soc Exp Biol Med 90:210–213
26. Ochoa S (1995) Malic dehydrogenase and 'malic' enzyme. In: Coloric Kaplan SP (ed) Methods of enzymology I. Academic Press, New York, pp 735–745
27. Hestrin L (1949) modified by Augustinsson (1957) The reaction of acetyl choline esters and other carboxylic acid derivatives with hydroxylamine and its analytical application. J Biol Chem 180:249–261
28. Garen A, Levinthal CA (1960) A Fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characterization of alkaline phosphatase. Biochim Biophys Acta 38:470–483
29. Fiske CH, Subbarow Y (1925) The colorimetric determination of phosphorus. J Biol Chem 66:375–400
30. Wotton IDP (1964) Microanalysis. In: Coloric, Kaplan (eds) Medical biochemistry, vol 4. Churchill J & A, London, pp 101–107
31. Claiborne A (1985) Catalase activity. In: Greenwald RA (ed) CRC handbook of method in oxygen radical research. CRC Press, Boca Raton, pp 283–284
32. Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for super oxide dismutase. J Biol Chem 217(10):3170–3175
33. Lowry OH, Ronebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with folin phenol reagent. J Biol Chem 193:265–276
34. Baily NTJ (1959) Statistical methods in biology. The English University press, London
35. Joshi P, Deep H, Smith J (2002) Effect of lindane and malathion exposure to certain blood parameters in a freshwater teleost fish *Clarias batarachus*. Poll Res 21:55–57
36. Matkovics B, Witas H, Gabrielak T, Szabo L (1987) Paraquat an agent affecting antioxidant enzymes of common carp erythrocytes. Comp Biochem Physiol Part C 87(1):217–219
37. Nemcsok J, Boross L (1982) Comparative studies on sensitivity of different fish species to metal pollution. Acta Biol Acad Sci Hung 33(1):23–27
38. van Raaji MT, VandenThillart GE, Hallemeesch M, Balm PH, Steffens AB (1995) Effect of arterially infused catecholamines and insulin on plasma glucose and free acids in carp. Am J Physiol Regul Integr Comp Physiol 268:1163–1170

39. Jenkins F, Smith J, Rajanna B, Shameem U, Umadevi K, Sanhya V, Madhavi R (2003) Effect of sublethal concentration of endosulfan on hematological and serum biochemical parameters in the carp, *Cyprinus carpio*. Bull Environ Contam Toxicol 70(5): 993–997
40. Ravinder V, Suryanarayan N, Narayana G (1988) Decis induced biochemical alterations in a fresh water catfish, *Clarias batrachus*. Ind J Comp Anim Physiol 6:5–12
41. Neff JM (1985) Use of biochemical measurement to detect pollutant-mediated damage to fish. In: Cardwel RD, Purdy R, Bahner RC (eds) Aquatic toxicology and hazard assessment. American Society for Testing Materials, Philadelphia, pp 155–181
42. Tejpal CS, Pal AK, Sahu NP, Kumar JA, Muthappa NA, Vidya S, Rajan MG (2008) Dietary supplementation of L-tryptophan mitigates crowding stress and augments the growth in *Cirrhinus mrigala* fingerlings. Aquaculture 293(3–4):272–277
43. Gagnon A, Jumarie C, Hontela A (2006) Effects of Cu on plasma cortisol and cortisol secretion by adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicol 78(1):59–65
44. Blahova J, Dobsikova R, Svobodova Z, Kalab P (2007) Simultaneous determination of plasma cortisol by high performance liquid chromatography and radioimmunoassay methods in fish. Acta Vet Brno 76(1):59–64
45. Grant BF, Mehrle PM (1973) Endrin toxicosis in rainbow trout (*Salmon gairdneri*). J Fish Res Board Can 30(1):31–40
46. Brown SB, Eales JG, Evans RE, Hara TJ (1984) Interrenal thryroidal and carbohydrate responses of rainbow trout (*Salmo gairdneri*) to environmental acidification. Can J Fish Aquat Sci 41(1):36–45
47. Bleau H, Daniel C, Chevalier G, Van-Rra H, Hontela A (1996) Effect of acute exposure to mercury chloride and methylmercury on plasma cortisol, T3, T4, glucose and liver glycogen in rainbow trout (*Onchorynchus mykiss*). Aquat Toxicol 34(3):221–235
48. Dincel AS, Benli ACK, Selvi M, Sarıkaya R, Sahin D, Ozkule IA, Erkoc F (2009) Sublethal cyfluthrin toxicity to carp (*Cyprinus carpio* L.) fingerlings: biochemical, hematological, histopathological alterations. Ecotoxicol Environ Safe 72(5):1433–1439
49. Sinha S, Mandal C, Allen AK, Mandal C (2001) Acute phase response of C-reactive protein of *Labeo rohita* to aquatic pollutants is accompanied by the appearance of distinct molecular forms. Arch Biochem Biophys 396:139–150
50. Szalai AJ, Bly JE, Clem LW (1994) Changes in serum concentrations of channel catfish (*Ictalurus punctatus Rafinesque*) phosphorylcholine-reactive protein (PRP) in response to inflammatory agents, low temperature-shock and infection by the fungus *Saprolegnia* sp. Fish Shellfish Immunol 4(5):323–336
51. Paul I, Mandal C, Mandal C (1998) Effect of environmental pollutants on the C-reactive protein of a freshwater major carp *Catla catla*. Dev Comp Immunol 22(5–6):519–532
52. Sarma K (2003) Biochemical responses of *Channa punctatus* to endosulfan and its implication in environmental monitoring, PhD Dissertation, Central Institute of Fisheries Education, Mumbai
53. Devraj P, Selvarajan VR, Durairaj S (1991) Relationship between acetyl cholinesterase and monoamine oxidase in brain regions of *O. mossambicus* exposed to phosalone. Ind J Exp Biol 29: 790–792
54. Sarma K, Pal AK, Sahu NP, Ayyappan S, Baruah K (2009) Dietary high protein and vitamin C mitigates endosulfan toxicity in the spotted murrel, *Channa punctatus* (Bloch, 1793). Sci Total Environ 407(12):3668–3673
55. Akhtar MS, Pal AK, Sahu NP, Alexander CJ, Gupta SK, Choudhary AK, Jha AK, Rajan MG (2010) Stress mitigating and immuno-modulatory effect of dietary pyridoxine in *Labeo rohita* (Hamilton) fingerlings. Aquacult Res 41:991–1002
56. Awasthi M, Shah P, Dubale MS, Gadhia P (1984) Metabolic changes induced by organophosphates in the piscine organs. Environ Res 35(1):320–325
57. Ghosh TK (1989) Influence of cypermethrin on the oxidative metabolism of fish *Labeo rohita*. Proc Ind Natl Sci Acad B 55:115–120
58. Belinski E (1974) Biochemical aspects of fish swimming. In: Malins BC, Sargent JR (eds) Biochemical perspectives in marine biology. Academic press, New York, pp 239–288
59. Verma AK, Pal AK, Manush SM, Das T, Dalvi RS, Chandrachoodan PP, Ravi PM, Apte SK (2007) Persistent sub-lethal chlorine exposure elicits the temperature induced stress responses in *Cyprinus carpio* early fingerlings. Pest Biochem Physiol 87(3):229–237
60. Pandey S, Parvez S, Sayeed I, Haque R, Bin-Hafeez B, Raisuddin S (2003) Biomarkers of oxidative stress: a comparative study of river Yamuna fish *Wallago attu* (Bl. & Schn.). Sci Total Environ 309(1–3):105–115
61. Farombi EO, Ajimoko YR, Adelowo OA (2008) Effect of butachlor on antioxidant enzyme status and lipid peroxidation in fresh water African catfish, (*Clarias gariepinus)*. Int J Environ Res Public Health 5(5):423–427