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The role of vitamin C as antioxidant in protection of biochemical and haematological stress induced by chlorpyrifos in freshwater fish *Clarias batrachus*

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HIGHLIGHTS

- Modulatory and protective role of vitamin C on the toxicity of chlorpyrifos.
- Experiments include control group, E_1 and E_2 groups.
- The E_1 group showed less weight gain, survival rate and changes in other parameters.
- Potential impacts of Vitamin C on improvement of toxic stress are discussed.

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ABSTRACT

The study was conducted to explore the modulatory effects of chlorpyrifos and protective role of vitamin C in tissues of *Clarias batrachus*. Treatments include E_1 group (basal diet plus 1.65 mg L^{-1} CPF) and E_2 group (basal diet + 200 mg kg body weight vitamin C and 1.65 mg L^{-1} CPF) along with a control group of fishes (fed on basal diet only). After 1, 7, 15, and 30 d of treatment, fish tissues (brain, blood and liver) were used for the estimation of growth, biochemical and haematological parameters. The results of E_1 group indicated significantly lower weight gain and survival rate. Brain AChE activity was inhibited. The RBC, Hb, respiratory burst activity, total protein and HSI were also reduced whereas WBC count, plasma glucose and haematocrit were elevated. In contrast, liver glycogen content, lactate dehydrogenase, alkaline and acid phosphatase activities were inhibited and malate dehydrogenase, aspartate, alanine amino transferase were enhanced. The E_2 group of fish exhibited significant improvement in growth, survival, haematological indices, brain AChE, liver glycogen and oxidative enzyme activity. The findings support that dietary vitamin C supplementation might be helpful in abrogation of chlorpyrifos toxicity and improves growth, survival, biochemical and haematological conditions in fishes.

1. Introduction

Aquatic ecosystems are becoming more vulnerable to pesticide contamination, which is debilitating the sustenance of life. Pimentel (1995) reported that often less than 0.1% of an applied pesticide reaches the target pest, leaving 99.9% as an unintended pollutant in the environment. Presently, pesticides are found all over the aquatic habitats and even found their way into subsurface groundwater resources at varying concentrations due to direct overspray, drift, atmospheric transport, agricultural and residential runoff, individual misuse, and improper disposal (Gilliom and Hamilton, 2006; Singh and Singh, 2008).

Organophosphate chemicals are widely used as pesticides in residential settings and in agricultural practice to increase crop yields. The use of organophosphate pesticides have been remain pervasive in both developed and developing nations, concerns are increasing regarding the relative safety of these chemicals to the environment, wildlife and fish. One such organophosphate, is chlorpyrifos [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothionate] which is widely used throughout the world under the registered trademark (LORSBAN and DURSBAN). It has been detected in air, rain, and fog (Majewski and Capel, 1995), and very highly toxic to fish and has caused fish kills in waterways near treated fields (USEPA, 2000). Sun and Chen (2008) reported CPF

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effects in controlling arthropods in soil and foliage. Santerre et al. (2000) reported 11% of farm raised *Ictalurus punctatus* samples contained CPF residues ranging from 0.01 to 0.32 mg kg^{-1}.

Vitamins and minerals are included in fish food to promote optimal growth and health. Dietary vitamin C significantly enhanced growth, non-specific immunity and protection against infections (Zhou et al., 2012); however, high level was required to improve stress resistance of fish (Garcia et al., 2007). Vitamin C served as an antitoxic agent against pesticide stress (Vani et al., 2011) and enhanced nonspecific immune responses and disease resistance (Lin and Shi-Yen, 2005). It has been observed that increased tissue reserves of AA through dietary supplement helped *Heteropneustes fossilis* to counter stress induced by cypermethrin (Saha and Kaviraj, 2009). A low level (50 mg kg^{-1} bw) in the diet did not, but a high level of AA (100 mg kg^{-1} bw) removed stress induced by the pesticide fenvalerate (Datta and Kaviraj, 2003). Misra et al. (2007) reported 100 mg kg^{-1} body weight vitamin C stimulated immune response and growth in *Labeo rohita*.

Therefore, the present study evaluated (i) weight gain and survival performance of fish, (ii) hepatosomatic index (HSI), Ascorbic acid (AA) levels of liver and plasma, (iii) haematological parameters such as red blood cells (RBC), white blood cells (WBC), haemoglobin (Hb), hematocrit (Hct) levels, plasma proteins, glucose and respiratory burst activity (NBT), (iv) assay of liver enzymes; such as malate dehydrogenase (MDH), lactate dehydrogenase (LDH), acid and alkaline phosphatase (ACP, ALP) and aspartate and alanine aminotransferase (AAT, AlAT) and (v) and assay of brain acetylcholinesterase enzyme.

2. Materials and methods

2.1. Pesticide and chemicals

All the chemicals used in the present study were purchased from Sigma–Aldrich chemical company (Saint Louis, USA). The test compound chlorpyrifos (20% EC) was purchased from local market (NOCIL Bombay-India).

2.2. Animal maintenance

The freshwater catfish, *Clarias batrachus*, were obtained from a local supplier and were transported to the laboratory in large aerated drums. They were first given prophylactic dip in 2% salt solution for 1 min followed by oxytetracycline treatment (15 mg L^{-1}) for the first three days, and were then acclimatized to laboratory conditions for 4 weeks prior to the experiment, during which they were fed basal diet.

2.3. Experimental diet

Two types of feed was prepared, one is basal diet and the other is an extra vitamin C (200 mg kg^{-1} bw) (Table 1A). The source of vitamin C is L-ascorbyl 2-polyphosphate (Sisco Research Labs Pvt., Ltd, Mumbai-India). All ingredients were mixed thoroughly, and dough was prepared with required amount of water. The dough was kept for half an hour for proper conditioning followed by steaming for 20 min in a pressure cooker. After cooling, vitamin and mineral mix (Agrimin; Glaxo India Ltd, Mumbai-India) were added to the dough and pellets were made to feed E_2 group of fish.

Table 1A
Composition of the basal diet.

Ingredients (% dry weight)			
Rice bran	18.43	Fish meal	40.71
Wheat flour	18.43	Vitamin mix[a]	02.00
Mustard oil cake	18.43	Mineral mix[b]	02.00
Proximate composition (%)			
Dry matter	94.30	Crude protein	31.08
Lipid	15.00	Ash	11.32

[a] Vit mix (%): B_1, 7.14; B_2, 2.55; B_4, 10.2; B_6, 1.02; B_{12}, 0.012; Biotin, 0.025; Calcium Pantothenate, 2.55; Niacin, 76.3; Vit A, 0.10; Vit C, 0.103.
[b] Mineral mix (%): Cu, 3.12; Co, 0.45; Mg, 21.48; Fe, 10.8; I, 1.6; Zn, 21.30; Ca, 30.0; P, 8.25; Mn, 3.00.

2.4. Pesticide preparation and experimental design

The LC_{50} (96 h) value of CPF was determined in the laboratory using the semi-static method of Finney (1971) and 1/10th of LC_{50} (1.65 mg L^{-1}) was selected for the study. 72 healthy fishes (average weight 40 ± 5 g and 22 ± 2 cm length) were distributed in three different groups (Control, E_1 and E_2), of 24 fish per tank. Each group was maintained in 140 L of water (40 × 70 × 50). Control group was fed with basal diet and kept in pesticide free water, E_1 was fed with basal diet and exposed to 1.65 mg L^{-1} chlorpyrifos pesticide whereas E_2 was fed with basal diet and vitamin C (200 mg kg^{-1} body weight) and exposed to 1.65 mg L^{-1} pesticide. Fish were fed twice a day to a level of 2.5% body weight. Siphoning of uneaten feed and fecal matter was done daily evening.

The ambient condition of natural photoperiod was maintained (light:12, dark:12) throughout the experiment period. The average mean values of water quality during investigation are; temperature 25 ± 3 °C, pH 7.4 ± 0.4, dissolved oxygen 8.24 ± 0.22 mg L^{-1}, total hardness 415 ± 1.2 mg L^{-1} as $CaCo_3$, alkalinity 348 ± 1.6 mg L^{-1} as $CaCo_3$, and total chloride 245.57 ± 1.44 mg L^{-1}.

2.5. Sample preparation

At the end of the experimental period (interval of 1, 7, 15 and 30 days), six fish per group were sampled, weighed and anaesthetized on ice for 10 min and dissected, liver tissue was used for the estimation of different parameters. Brain was used for the assay of AChE enzyme. Blood was collected from the caudal vasculature by syringe from fish selected randomly and divided into two aliquots. The blood samples were transferred to tubes (contained 1.0% (v/v) of 15% EDTA), centrifuged (9000*g* for 10 min at 4 °C) and plasma was collected for haematological assay.

2.6. Survival and growth performance

$$\text{Weight gain}\,(\%) = \text{Final weight} - \text{initial weight}/\text{initial weight} \times 100$$

$$\text{Survival}\,(\%) = \text{Number of fish survived after 30 d}/\text{initial number of fish stocked} \times 100$$

2.7. Hepatosomatic index (HSI)

Whole liver was removed, weighed to calculate hepatosomatic index (HSI) according to equation of Adams and McLean (1985) (without gall bladder): Liver weight (g)/whole body weight (g) × 100.

2.8. Ascorbic acid analysis

The levels of AA in liver and blood were determined by the method of Roe and Keuther (1943) by using UV spectrophotometer (E-Merck, Germany) at 540 nm (using 2, 4-dinitrophenylhydrazine).

2.9. Haematological studies

The RBCs were measured using a Neubauer haemocytometer after dilution with Grower's solution (Voigt, 2000). The white blood cells were counted using a Neubauer haemocytometer after dilution with Dacie's solution (Dacie and Lewis, 2001). The haemoglobin (g dL^{-1}) content was determined by the method of cyanohaemoglobin. The micro hematocrit method was used for the determination of hematocrit level (Ren et al., 2005). Plasma protein was estimated by total protein kit (Biuret method using dye reagent, Qualigens Fine Chemicals, Mumbai, India). Plasma glucose was estimated using a commercially available kit (GOD-POD based kit for estimation of blood glucose procured from Diatek, Kolkata, India).

2.10. Respiratory burst activity

Respiratory burst activity was done by the method of Secombes et al. (1990) as modified by Stasiack and Bauman (1996) (OD/540 nm). Briefly; blood sample (50 μL) was placed into the wells of "U" bottomed microtitre plates and incubated at 37 °C for 1 h to facilitate cell adhesion. The supernatant was discarded and the plates were washed three times in PBS after which 50 μL of NBT (Nitroblue tetrazolium) was added and incubated for an hour. The cells were then fixed with 100% methanol for 2–3 min and again washed with 30% methanol. Plates were air-dried and 60 μL of potassium hydroxide plus 70 μL of dimethyl sulfoxide were added into each well. This dissolves the formazan blue precipitate which was read at 540 nm (ELISA reader).

2.11. Liver biochemical enzymatic analyses

The glycogen content was determined by the method of Carool et al. (1956). MDH was assayed according to Nachlas et al. (1960) and LDH (L-lactate, nicotinamide adenine dinucleotide (NAD) oxidoreductase) by Srikanthan and Krishnamurthy (1955) method. For the estimation of AAT and AlAT enzymes, 5% liver homogenate was prepared in 0.25 M ice cold sucrose solution and the assay was performed by the method of Reitman and Frankel (1957). The alkaline phosphatase activity was estimated by the method of Moss et al. (1986) and acid phosphatase by the method of Jabeen (1984). Brain tissue was homogenized in ice-cold 100 mM L^{-1} phosphate buffer pH 7.4 and centrifuged at 9000*g* for 30 min at 4 °C. AChE activity was measured by the method of Ellman et al. (1961).

2.12. Statistical analysis

The data were subjected to Statistical Package for the Social Sciences (SPSS, version 12.0, Inc, 233, South Wacker Drive, Chicago, IL, USA). Duncan's multiple range tests were used to determine the differences among treatment means at $p > 0.05$, and each value is the mean ± SE of six individual values ($n = 6$).

3. Results

3.1. Growth performance and survival

The sub-lethal effects of chlorpyrifos caused significant (over 5%) reduction in weight gain. The lowest weight gain was observed in E_1 group on 30 days (35%), and the highest in E_2 group (155%). The survival percentage also follows the same trend at the end of the experimental period (80% in E_1 and 100% in E_2 group) (Table 1B).

3.2. Ascorbic acid, liver HIS and plasma biochemistry

Table 2 explained the AA levels of fish exposed to CPF. Tissues ascorbic acid concentration increased significantly with the increasing days in E_2 group (56%, 99%) and decreased in E_1 group (20%, 28%). The hepatosomatic index also showed similar results as that of AA. Plasma glucose increased on all days in E_1 group (53%) and remained unaltered in E_2 group (below 10%). Plasma totals proteins are significantly ($p < 0.05$) lower (41%) after chlorpyrifos exposure (E_1) whereas increased in E_2 group on day 30 as compared to control.

3.3. Haematological characteristics

The haematological characteristics are presented in Table 3. A significant reduction in the total number of RBCs (32%) in E_1 whereas increased (22%) in E_2 compared to control at the end of experimental period. WBC count increased (33%) in E_1 and E_2 group count was near to the control. The haemoglobin (Hb) level is significantly decreased (34%) in E_1 and 30% increment observed in E_2 group as compared to control. The Hct (haematocrit) is significantly influenced in both groups (13%, 25%) and throughout the experimental period. The respiratory burst activity (NBT) was significantly (20%) reduced in E_1 group and elevated (26%) in E_2 group as compared to the control.

3.4. Liver biochemical enzymatic analyses

The AChE activity of brain tissue was inhibited significantly in E_1, maximum inhibition (50%) was recorded on day 30. where as in E_2 group the inhibition was reduced. The result revealed that vitamin C protects the inhibition effect of chlorpyrifos on AChE (Fig. 1A). Compared to the control, CPF treatment significantly reduced liver glycogen (54%) in E_1 and the opposite was observed in E_2 (Fig. 1B). The decreased LDH activity was found in E_1 group (39%), which was insignificantly different from the control. The E_2 group is nearby control (8.5%) on day 7 but at the end of day 30 it was recorded insignificant ($p > 0.05$) from the control (Fig. 2A). Maximum elevation in MDH activity was observed in E_1 group (21%) and in E_2 it did not differ from control group of fish (Fig. 2B). The AAT and AlAT activities of the E_1 group was found to be significantly ($p > 0.05$) higher (36%, 65%) whereas E_2 group differ (4%) from controls (Fig. 3A and B). Activities of both acid and alkaline phosphatase (ACP, ALP) decreased significantly in E_1 group (55%, 33%) and remains somewhat elevated in E_2 (2.3%, 4%) (Fig. 4A and B).

4. Discussion

The present study examined the anti-stressor potential of vitamin C in fish exposed to CPF. Zhou et al. (2012) reported that the dietary supplemented vitamin C significantly influenced the growth performance and immune response in juvenile *Rachycentron canadum*. Survival and growth performance was

Table 1B
Consequences of vitamin C supplementation on weight gain and survival performance of *Clarias batrachus*.

Days	Weight gain			Survival		
	Control	E_1	E_2	Control	E_1	E_2
1	0%	0%	0%	100%	100%	100%
7	30%	6%	36%	100%	90%	100%
15	70%	13%	90%	100%	90%	100%
30	96%	35%	155%	100%	80%	100%

Table 2
Effects of dietary vitamin C supplemented feed on the Ascorbic acid (AA) levels (plasma and liver), Hepatosomatic index (HSI), plasma glucose and total protein of *Clarias batrachus* exposed to 1.65 mg L^{-1} chlorpyrifos.

Period		Plasma (AA)	Liver (AA)	Liver (HSI)	Glucose (Plasma)	Proteins (Plasma)
1 d	Con	20.23 ± 2.41	42.46 ± 7.41	1.38 ± 0.12	12.85 ± 2.61	8.45 ± 1.05
	E_1	20.35 ± 2.42 [N]	42.43 ± 7.61[N]	1.32 ± 0.11[N]	14.51 ± 1.82	7.95 ± 0.65
	E_2	20.64 ± 3.02 [N]	42.55 ± 8.52[N]	1.36 ± 0.23[N]	15.12 ± 1.72[N]	8.15 ± 0.91[N]
7 d	Con	22.26 ± 3.42	45.65 ± 5.93	1.42 ± 0.25	12.54 ± 2.86	7.92 ± 0.95
	E_1	18.25 ± 2.53	36.34 ± 7.31	1.02 ± 0.21	7.34 ± 1.32	6.27 ± 0.66
	E_2	22.67 ± 3.44	55.32 ± 6.04	1.36 ± 0.12[N]	13.68 ± 1.68[N]	8.21 ± 0.73[N]
15 d	Con	25.26 ± 3.53	47.12 ± 6.94	1.48 ± 0.13	11.66 ± 2.37	7.65 ± 0.64
	E_1	17.05 ± 3.92	32.53 ± 6.41	0.98 ± 0.35	18.86 ± 1.48	5.92 ± 0.34
	E_2	28.85 ± 2.54	66.37 ± 5.52	1.48 ± 0.17	12.64 ± 1.67	8.38 ± 0.82
30 d	Con	26.05 ± 3.52	51.26 ± 8.42	1.52 ± 0.23	11.28 ± 2.64	7.37 ± 0.62
	E_1	16.76 ± 4.05	30.23 ± 9.94	0.96 ± 0.34	17.24 ± 1.75	4.32 ± 0.82
	E_2	32.85 ± 2.64	84.44 ± 8.25	1.63 ± 0.26	12.22 ± 1.48	8.64 ± 0.54

Ascorbic acid levels in plasma (mM L^{-1}), liver (mg g^{-1}), liver HSI (g kg^{-1}), glucose (mg dL^{-1}) and total proteins (μg mL^{-1}). Values are represented (mean ± SE) of six individual values.
[N] Indicate values that are significantly different from control and exposure ($p < 0.05$).

Table 3
Consequence of dietary vitamin C supplemented feed on the haematology biochemistry of *Clarias batrachus* exposed to sub-lethal (1.65 mg L^{-1}) chlorpyrifos.

Period		RBC	WBC	Hb	Hct	NBT
1 d	Con	1.28 ± 0.08	185.2 ± 22.31	9.68 ± 1.34	31.20 ± 2.50	0.96 ± 0.08
	E_1	1.12 ± 0.07	202.5 ± 20.64	8.65 ± 1.05	29.24 ± 4.21	0.81 ± 0.04
	E_2	1.18 ± 0.09	201.3 ± 20.15	8.71 ± 1.03	32.07 ± 3.64[N]	0.94 ± 0.10[N]
7 d	Con	1.31 ± 0.10	165.3 ± 20.54	8.26 ± 1.28	28.34 ± 4.11	1.05 ± 0.07
	E_1	1.02 ± 0.12	220.5 ± 22.55	7.64 ± 1.11	22.64 ± 3.81	0.86 ± 0.06
	E_2	1.29 ± 0.11[N]	189.8 ± 15.67	10.2 ± 0.92	29.67 ± 6.24[N]	0.98 ± 0.09
15 d	Con	1.32 ± 0.08	159.5 ± 21.61	8.94 ± 1.54	30.24 ± 4.51	0.95 ± 0.05
	E_1	0.92 ± 0.08	215.8 ± 19.84	6.28 ± 0.98	32.82 ± 3.21	0.79 ± 0.03
	E_2	1.48 ± 0.15	165.5 ± 19.64	10.8 ± 0.85	32.45 ± 3.84	1.08 ± 0.08
30 d	Con	1.30 ± 0.12	165.8 ± 20.94	9.21 ± 1.27	30.57 ± 4.42	0.89 ± 0.09
	E_1	0.88 ± 0.07	212.8 ± 15.67	6.12 ± 0.92	34.57 ± 5.24	0.71 ± 0.06
	E_2	1.59 ± 0.13	192.2 ± 21.64[N]	11.9 ± 0.67	32.02 ± 3.64[N]	1.12 ± 0.07

RBC (10^6 cells μL^{-1}), WBC (10^3 cells μL^{-1}), Hb (g dL^{-1}), Hct (%), and NBT (OD/540 nm). Values are represented as (mean ± SE) of six individual values.
[N] Indicate values that are significantly different from control and exposure ($p < 0.05$).

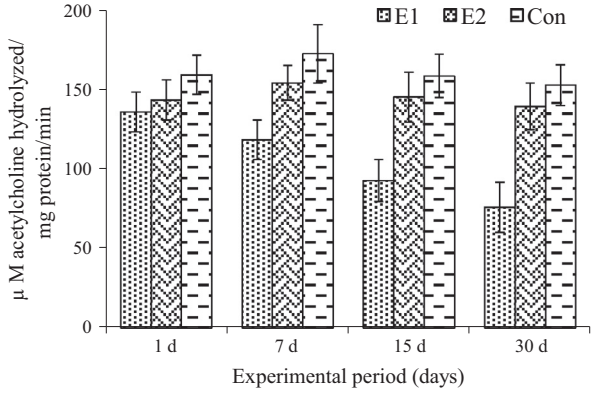

Fig. 1A. Effect of CPF on brain acetylcholine esterase and its amelioration performance with dietary vitamin C in *C. batrachus* for 30 days. Con: control; E_1: pesticide plus basal diet; E_2: pesticide plus diet with vitamin C. ($n = 6$).

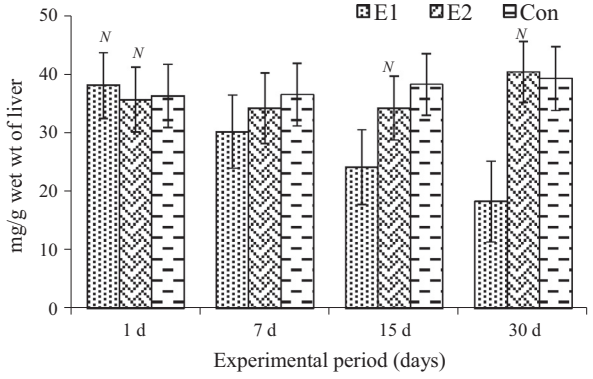

Fig. 1B. Effect of CPF on liver glycogen and its amelioration performance with dietary vitamin C in *C. batrachus* for 30 days. Con: control; E_1: pesticide plus basal diet; E_2: pesticide plus diet with vitamin C. [N] indicates not significant over control ($n = 6$).

found to be reduced in E_1 group whereas increased in E_2 group. The elevation of vitamin C in liver and blood concurred with better growth and survival. Similar results were observed in some fishes exposed to pesticides (Sarma et al., 2009; Saha and Kaviraj, 2012). Other researchers also observed a strong correlation between the growth and tissue levels of AA in fish Saha and Kaviraj, 2009; Goa et al., 2013).

Hepatosomatic index (HSI) is particularly used as an organ-level biomarker to identify possible contaminants of liver diseases and the nutritional state of the fish. Decreased HSI in E_1 group indicates that the energy drain imposed by CPF stress or depressed feeding due to toxicity, in turn is correlated with loss of energy stores (Heath, 1995). Ai et al. (2006) showed liver AA concentration as an indicator of tissues status in fishes. Our results indicated that dietary AA significantly increases AA concentration in blood and liver of *C. batrachus*.

In the present experiment plasma proteins were significantly reduced in E_1 group as compared to the control, it may be due to

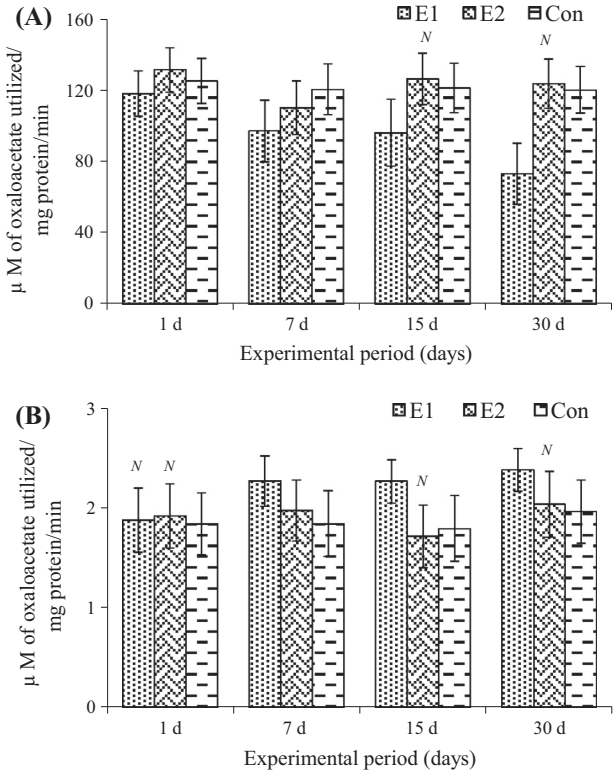

Fig. 2. (A and B) Effect of CPF on activities of LDH, MDH and its amelioration with dietary vitamin C in *C. batrachus* for 30 days. Con: control; E_1: pesticide plus basal diet; E_2: pesticide plus diet with vitamin C. ^{N}indicates not significant over control (n = 6).

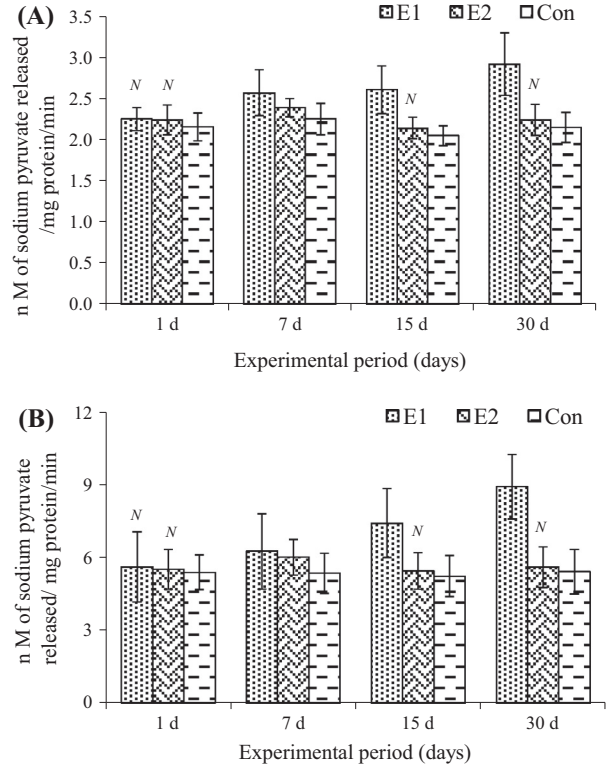

Fig. 3. (A and B) Effect of CPF on activities of AAT, AlAT and its amelioration with dietary vitamin C in *C. batrachus* for 30 days. Con: control; E_1: pesticide plus basal diet; E_2: pesticide plus diet with vitamin C; ^{N}indicates not significant over control (*n* = 6).

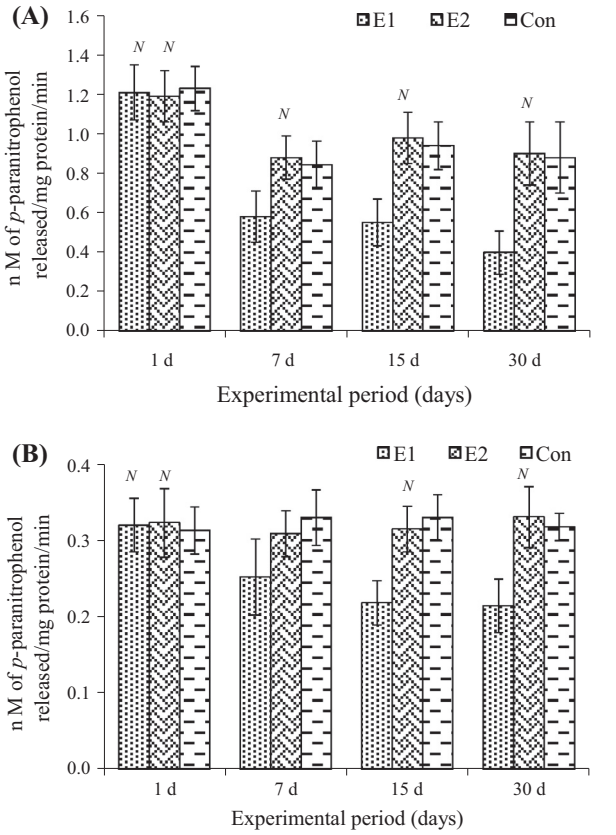

Fig. 4. (A and B) Effect of CPF on activities of ACP, ALP and its amelioration with dietary vitamin C in *C. batrachus* for 30 days. Con: control; E_1: pesticide plus basal diet; E_2: pesticide plus diet with vitamin C; ^{N}indicates not significant over control (*n* = 6).

breakdown for energy purpose to counter the stress effect of the CPF. Similar results were observed in fish *L. rohita* exposed to pesticides (Nayak et al., 2004) and this may lead to increased effects on immune system of the fish (Hussein et al., 1996). The increase in total protein in E_2 group indicates a reduction in enzymatic breakdown of protein for energy purpose. The improved level of total protein in E_2 group (200 mg kg^{-1} bw) was able to strengthen the immune system of fish under the stress of CPF.

The haematological results indicated significant decrease in RBC and Hb in the E_1 group, establishes a condition of erythropenia and hemolysis in *C. batrachus*. The reduction in RBC and Hb might be due to the inhibition of erythropoiesis, hemosynthesis, osmoregulatory dysfunction or due to increased rate of erythrocyte destruction in the hematopoietic organ (Jenkins et al., 2003). WBCs are involved in the control of immune functions and change in count after exposure to different toxicants may indicate a decrease in nonspecific immunity of organism. In the present study, WBC count was significantly increased in E_1 group throughout the exposure period which may be due to generalized immune response and a protective response to CPF stress.

In the present experiment, significant flection was observed in haematocrit and respiratory burst (DNT) in E_1 group whereas E_2 group is near to control, which suggests that the immune system has been compromised. Vitamin C is proved to increase phagocytic activity, natural complement and respiratory burst activity in gilthead sea bream *Sparus aurata*, for a short period after which it returns to normal level (Ortuño et al., 1999). They also suggested that vitamin C is highly interactive and may fortify antioxidant defense and enhance immune response directly by maintaining optimal vitamin C level. It is also an effective ameliorating agent

against stress and can enhance nonspecific immune response in Atlantic salmon exposed to 2 h confinement stress (Thompson et al., 1993). The findings of our results suggests that vitamin C (200 mg kg^{-1} bw) might be helpful in reducing the harmful effect of chlorpyrifos on RBC, Hb, WBC, Hct and NBT.

Olga et al. (2013) reported increased blood glucose in gold fish *Carassius auratus* exposed to herbicide 2, 4-dichlorophenoxyacetate. A significant reduction in liver glycogen level was recorded in freshwater fish species following exposure to CPF (Tripathi and Shasmal, 2011; Narra et al., 2012). Reduction of hepatic glycogen suggests expenditure of energy due to stress caused by CPF. Glycogen depletion is a regulatory step, which increases intermediary metabolism resulting in the protection of the hepatocytes under xenobiotic stress. Increases in plasma glucose level along with depletion of liver glycogen suggest that some of the hepatic glycogen is converted into glucose, which enters the circulation. This indicates enhanced glycogenolysis and inhibited glycolytic pathway. Saxena and Gupta (2005) reported that fish brain under stress condition secrete high amounts of catecholamine that deplete glycogen reserves. In the present study, the elevation of glucose and inhibition of total proteins and glycogen levels are ameliorated in the E_2 group fed with vitamin C (200 mg kg^{-1} body weight) supplemented diet, suggesting their anti-stress ability.

The results of the present study showed that brain AChE activity was significantly inhibited in chlorpyrifos exposed fish. Almeida et al. (2010) also reported inhibition of AChE in brain of European seabass *Dicentrarchus labrax* exposed to fenitrothion. The decrease in AChE activity could be due to the decrease of enzyme synthesis by the inhibitory nature of toxicant. In group E_2, supplementation of Vit C in the diet resulted significant alternation of AChE enzyme to normal. This could be attributed to the antioxidant property of vitamin C. In the present study, the liver LDH activity was inhibited maximum in the E_1 group fed with basal diet and exposed to CPF, whereas in the group fed with vitamin C diet (E_2) the activity was significantly increased. The results indicate that the dietary vitamin C supplementation can counter the LDH inhibition which might be due to higher glycolysis rate. The liver MDH activity, an enzyme of TCA (tricarboxylic acid) cycle, which is involved in the reversible conversion of L-malate and oxaloacetate, also showed reveres trend to LDH. The activity of MDH was elevated in the E_1 group fed with basal diet. These results agree with previous results (Amit et al., 2012).

The results of present findings indicate elevated aminotransferase activities in E_1 group. Similar results are reported in different fish species (Sancho et al., 2010; Narra et al., 2011; Amit et al., 2012). When fish were fed with diet vitamin C (E_2) no change was observed throughout the experiment. Alkaline phosphatase is a membrane bound enzyme, plays a major role in glycogen metabolism and is capable of inactivating phosphorylase enzymes thereby promoting glycogen synthesis. In the present investigation, ALP activity was significantly inhibited in E_1 whereas it remains insignificantly near to control in E_2 group. Inhibited ACP activity was observed in E_1 group, no change was noticed in E_2. Similar results were reported fishes exposed to pesticides, when fed with vitamin C to mitigates pesticide toxicity (Bhattacharya and Kaviraj, 2009; Sarma et al., 2009). The cell/organ has several ways to attenuate the toxic stress either by repairing the damage or by directly reducing the pro-oxidative state via enzymatic and non-enzymatic antioxidants (Korkmaz et al., 2009) Vit. C is an antioxidant agent, could reduce the initiation of ROS production which intern protect fish from pesticide stress.

In conclusion, dietary vitamin C supplementation is essential for normal growth and physiology of the fish. In the present study, a significant decrease in the AA level was observed in the liver of fish fed with basal diet and exposed to CPF. These symptoms lead to the utilization of vitamin C for detoxification process or for prevention of cell peroxidation. Our results indicate that requirement of AA (200 mg kg^{-1} body weight) of dietary supplementation to counter stress exerted by the CPF. The studied parameters are not sufficient to explain the exact mechanism behind the AA improving the health of CPF exposed fish. Further investigations are warranted to understand the exact role of AA in pesticide stress fishes. The data of such studies could be applicable in biomonitoring of aquatic environment. However, it can be said that at the end of culture period, feeding with AA diet can be beneficial in reducing the contaminant load in the muscle of the fish for a better growth, survival and market value.

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References

Adams, M., McLean, B., 1985. Estimation of the largemouth bass, *Micropterus salmoides* Lacepede, growth using the liver somatic index physiological variables. J. Fish Biol. 26, 111–126.

Ai, Q., Mai, K., Tan, B., Xu, W., Zhang, W., Ma, H., 2006. Effects of dietary vitamin C on survival, growth, and immunity of large yellow croaker, *Pseudosciaena crocea*. Aquaculture 261, 327–336.

Almeida, R., Oliveira, C., Gravato, C., Guilhermino, L., 2010. Linking behavioral alterations with biomarkers responses in the European seabass *Dicentrarchus labrax L.* exposed to the organophosphate pesticide fenitrothion. Ecotoxicol. 19 (8), 1369–1381.

Amit, K., Sharma, B., Ravi, P., 2012. Alterations in nitrogen metabolism in freshwater fishes, *Channa punctatus* and *Clarias batrachus*, exposed to a commercial-grade λ-cyhalothrin, REEVA-5. Int. J. Exp. Pathol. 93 (1), 34–45.

Bhattacharya, M., Kaviraj, A., 2009. Toxicity of the pyrethroid pesticide fenvalerate to freshwater catfish *Clarias gariepinus*: lethality, biochemical effects and role of dietary ascorbic acid. J. Environ. Sci. Health B 44, 578–583.

Carool, V., Longley, V., Roe, E., 1956. Glycogen determination in liver and muscle by the use of anthrone reagent. J. Biol. Chem. 220, 583–593.

Dacie, V., Lewis, M., 2001. Practical Haematology, ninth ed. Churchill Livingstone, London, p. 633.

Datta, M., Kaviraj, A., 2003. Ascorbic acid supplementation of diet for reduction of deltamethrin induced stress in freshwater catfish *Clarias gariepinus*. Chemosphere 53, 883–888.

Ellman, G., Courtney, K., Andres, V., Feather, R., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Finney, J., 1971. Probit Analysis, second ed. Cambridge University Press, London, UK.

Garcia, F., Pilarski, F., Onaka, M., de Moraes, R., Martins, L., 2007. Hematology of *Piaractus mesopotamicus* fed diets supplemented with vitamins C and E, challenged by *Aeromonas hydrophila*. Aquaculture 271, 39–46.

Gilliom, J., Hamilton, A., 2006. Pesticides in the nation's streams and ground water, 1992–2001. A summary fact sheet, (Reston: US Geological Survey).

Goa, J., Koshio, S., Ishikava, M., Yokoyama, S., Nguyen, T., Mamauag, E., 2013. Effect of dietary oxidized fish oil and vitamin C supplementation on growth performance and reduction of oxidative stress in Red Sea bream *Pagrus major*. Aquat. Nutr. 19, 35–44.

Heath, G., 1995. Water Pollution and Fish Physiology. CRC Press Inc., Boca Raton, Florida, p. 141.

Hussein, Y., El-Nasser, A., Ahmed, M., 1996. Comparative studies on the effects of herbicide Atrazine on freshwater fish *Oreochromis niloticus* and *Chrysichthyes auratus* at Assiut, Egypt. Bull. Environ. Contam. Toxicol. 57, 503–510.

Jabeen, K., 1984. Toxicological evaluations of some pesticides against chicken (*Gallus gallus domesticus*) and rat (*Rattus rattus norvegicus*) with special reference to haematology, blood and urine biochemistry. Ph.D thesis, Osmania University, Hyderabad, India.

Jenkins, F., Smith, J., Rajanna, B., Shameem, U., Umadevi, K., Sandhya, V., Madhavi, R., 2003. Effect of sub lethal concentration of endosulfan on hematological and serum biochemical parameters in the carp, *Cyprinus carpio*. Bull. Environ. Contam. Toxicol. 70, 993–997.

Korkmaz, N., Cengiz, E.I., Unlu, E., Uysal, E., Yanar, M., 2009. Cypermethrin-induced histopathological and biochemical changes in Nile tilapia (*Oreochromis niloticus*), and the protective and recuperative effect of ascorbic acid. Environ. Toxicol. Pharmacol. 28, 198–205.

Lin, M., Shi-Yen, S., 2005. Dietary L-ascorbic acid affects growth, nonspecific immune responses and disease resistance in juvenile grouper, *Epinephelus malabaricus*. Aquaculture 244, 215–221.

Majewski, S., Capel, D., 1995. Pesticides in the Atmosphere: Distribution, Trends, and Governing Factors. Ann Arbor, Chelsea, MI.

Misra, K., Das, K., Mukherjee, C., Pradhan, J., 2007. Effects of dietary vitamin C on immunity, growth and survival of Indian major carp *Labeo rohita* fingerlings. Aquat. Nutr. 13, 35–44.

Moss, W., Henderson, R., Kochmar, F., 1986. Enzymes: principles of diagnostic enzymology and the aminotransferases. In: Tietz, N.W. (Ed.), Text Book of Clinical Chemistry. Saunders, Philadelphia, P.A., pp. 663–678.
Nachlas, M., Margulius, P., Seligman, M., 1960. A colorimetric method for the determination of succinic dehydrogenase activity. J. Biol. Chem. 235, 499–504.
Narra, Madhusudan Reddy, Begum, G., Rajender, K., Rao, J.V., 2011. In vivo impact of monocrotophos on biochemical parameters of a freshwater fish during subacute toxicity following cessation of exposure to the insecticide. Z. Naturforsch. 66 (c), 507–514.
Narra, Madhusudan Reddy, Begum, G., Rajender, K., Rao, J.V., 2012. Toxic impact of two organophosphate insecticides on biochemical parameters of a food fish and assessment of recovery response. Toxicol. Ind. Health 28 (4), 343–352.
Nayak, K., Das, K., Kohli, S., Mukherjee, C., 2004. The immunosuppressive effect of α-permethrin on Indian major carp, rohu (*Labeo rohita* Ham.). Fish Shellfish Immunol. 16, 41–50.
Olga, K., Tetiana, A., Kenneth, S., Volodymyr, L., 2013. Goldfish can recover after short-term exposure to 2, 4-dichlorophenoxyacetate: use of blood parameters as vital biomarkers. Comp. Biochem. Physiol. C. 157, 259–265.
Ortuño, J., Esteban, A., Meseguer, J., 1999. Effect of high dietary intake of vitamin C on non-specific immune response of Gilt head sea bream (*Sparus aurata* L.). Fish Shellfish Immunol. 9, 429–443.
Pimentel, D., 1995. Amounts of pesticides reaching target pests: environmental impacts and ethics. J. Agri. Environ. Ethics 8, 17–29.
Reitman, S., Frankel, S., 1957. A colorimetric method for the determination of serum glutamic oxaloacetate and glutamic pyruvic transaminases. Am. J. Clin. Pathol. 289, 56–63.
Ren, T., Koshio, S., Teshima, S., Ishikawa, M., Alam, M.S., Panganiban, A., Moe, Y., Kojima, T., Tokumitsu, H., 2005. Optimum dietary level of L-ascorbic acid for Japanese eel, *Anguilla japonica*. J. World Aquacult. Soc. 36, 437–443.
Roe, H., Keuther, A., 1943. The determinations of ascorbic acid in whole blood and urine through the 2, 4-dinitrophenylhydrazine (DNPH) derivative of dehydroascorbic acid. J. Biol. Chem. 147, 399–407.
Saha, S., Kaviraj, A., 2009. Effects of cypermethrin on some biochemical parameters and its amelioration through dietary supplementation of ascorbic acid in freshwater catfish *Heteropneustes fossilis*. Chemosphere 74, 1254–1259.
Saha, S., Kaviraj, A., 2012. Dietary ascorbic acid as a means to counter the stress of cypermethrin on the growth of freshwater catfish *Heteropneustes fossilis*. Toxicol. Ind. Health 29, 468–473.
Sancho, E., Villarroel, J., Ferandez, C., Andreu, E., Ferrando, D., 2010. Short-term exposure to sublethal tebuconazole induces physiological impairment in male zebrafish (*Danio rerio*). Ecotoxicol. Environ. Saf. 73 (3), 370–376.
Santerre, R., Ingram, R., Lewis, W., Davis, T., Lane, G., Grodner, M., Wei, I., Bush, B., et al., 2000. Organochlorines, organophosphates and pyrethroids in Channel catfish, Rainbow trout and Red swamp crawfish from aquaculture facilities. J. Food Sci. 65, 231–235.
Sarma, K., Pal, K., Sahu, P., 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, 3668–3673.
Saxena, K., Gupta, P., 2005. Pyrethroid induced toxicity to muscle glycogen content in Channa punctatus (Bl.). Proc. Natl. Acad. Sci. India 75 (BII), 103–106.
Secombes, J., Stolen, J., Fletcher, C., Anderson, P., Roberson, S., Van, B., Winkel, M., 1990. Isolation of salmonid macrophages and analysis of their killing ability. In: Techniques in Fish Immunology. SOS Publication, New Jersey, pp. 137–152.
Singh, B., Singh, V., 2008. Pesticide bioaccumulation and plasma sex steroids in fishes during breeding phase from north India. Environ. Toxicol. Pharmacol. 25, 342–350.
Srikanthan, N., Krishnamurthy, R., 1955. Tetrazolium test for dehydrogenase. J. Sci. Ind. Res. 14, 206–213.
Stasiack, A.S., Bauman, C.P., 1996. Neutrophil activity as a potent indicator for concomitant analysis. Fish. Shellfish Immunol. 37, 539.
Sun, F., Chen, S., 2008. Monitoring of pesticide chlorpyrifos residue in farmed fish: investigation of possible sources. Chemosphere 71, 1866–1869.
Thompson, I., White, A., Fletcher, T.C., Houlihan, D.F., Secombes, C.J., 1993. The effect of stress on the immune response of Atlantic salmon (*Salmo salar* L.) fed diets containing different amount of vitamin C. Aquaculture 114, 1–18.
Tripathi, G., Shasmal, J., 2011. Concentration related responses of chlorpyriphos in antioxidant, anaerobic and protein synthesizing machinery of the freshwater fish, *Heteropneustes fossilis*. Pestic. Biochem. Physiol. 99, 215–220.
USEPA, 2000. Reregistration eligibility science chapter for chlorpyrifos. Fate & environmental risk assessment chapter (Revised June). <http://www.epa.gov/pesticides/op/chlorpyrifos/efedrra1.pdf>.
Vani, T., Saharan, N., Mukherjee, C., Ritesh, R., Rajesh, K., Brahmchari, K., 2011. Deltamethrin induced alterations of hematological and biochemical parameters in fingerlings of *Catla catla* (Ham.) and their amelioration by dietary supplement of vitamin C. Pestic. Biochem. Physiol. 101, 16–20.
Voigt, L., 2000. Hematology Techniques and Concepts for Veterinary Technicians. Iowa State University Press, Ames, p. 139.
Zhou, Q., Wang, L., Wang, H., Xie, F., Wang, T., 2012. Effect of dietary vitamin C on the growth performance and innate immunity of juvenile cobia (*Rachycentron canadum*). Fish Shellfish Immunol. 32, 969–975.