Dietary choline, betaine and lecithin mitigates endosulfan-induced stress in *Labeo rohita* fingerlings

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Abstract A five-week experiment was conducted to delineate stress-mitigating effects of three different methyl donors in *Labeo rohita* fingerlings subjected to endosulfan toxicity. Four iso-nitrogenous and iso-caloric feed were prepared with and without supplementation of methyl donors. The feed were basal or control diet (i.e., without methyl donor supplementation), feed supplemented with choline, feed supplemented with betaine and feed supplemented with lecithin. Two hundred and twenty-five fishes were distributed randomly in five treatment groups each with three replicates. The experimental setup were normal water (without endosulfan) and fed with control diet (control group), endosulfan-treated water and fed with control diet (T_1), endosulfan-treated water and fed with choline supplemented feed (T_2), endosulfan-treated water and fed with betaine supplemented feed (T_3) and endosulfan-treated water and fed with lecithin-supplemented feed (T_4). The level of endosulfan in endosulfan treated water was maintained at the level of 1/10 of LC_{50}, that is, 0.2 ppb. During the experiment, growth performances, metabolic enzyme activity and histological examination were done to assess the effect of treatments. The growth performance (percentage weight gain, feed conversion ratio, specific growth rate and protein efficiency ratio) and nutrient digestibility were significantly different ($P < 0.01$) in lecithin, betaine and choline fed group when compared to endosulfan-exposed group fed with basal diet. The liver LDH and MDH activity were significantly ($P < 0.01$) improved in the groups fed with methyl donor supplemented diet. The liver AST and ALT, brain AChE and muscle ALT did not change with supplementation in the diet, but muscle ALT and G6PDH significantly ($P < 0.01$) changed with supplementation. The gill and liver ATPase and intestinal ALP were significantly ($P < 0.01$) noticeably changed in supplemented group. After endosulfan exposure, histopathology alter like slight large vacuolation in hepatocyte and lipoid vacuole were observed and with supplementation normal appearance of liver were observed. The chromosome aberration (karyotype) was observed in endosulfan-exposed group. The result obtained in present study concluded that inclusion of methyl donors, particularly lecithin and betaine, in feed as nutritional supplements has a potential stress-mitigating effect in *L. rohita* fingerlings.

Keywords Endosulfan · Methyl donors · Growth · Metabolic enzymes · Histopathology · Karyotyping

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Introduction

Aquaculture is the fastest growing agricultural sector with a growth rate of about 8.8% in the world (FAO

2007). To sustain this growth rate and to accomplish the huge gap between demand and supply, there is a need to augment the aquaculture production. The indiscriminate use of antibiotics, synthetic growth promoters and pesticides has become a regular practice in aquaculture industry. This in turn leads to ecological imbalance and stressful conditions to the aquatic animals especially in fish (Elia et al. 2002). Kilgore and Mingyuli (1975) emphasized that concentration of pesticide residues is more in aquatic ecosystem than the terrestrial ecosystem. Pesticide like endosulfan, an organochlorine pesticide, is considered one of the most toxic pesticides for aquatic organisms, especially fish, and has been registered as a 'priority pollutant' by the US Environmental Protection Agency (Cengiz and Unlu 2002). Although it has been phased out in many Western countries, this pesticide is still extensively used on many agricultural crops in India and also in many parts of the tropical and sub-tropical regions (EFSA 2005). The endosulfan used in agricultural fields enters the aquatic environment generally as a consequence of runoff from the fields and/or accidental discharge and gets dispersed widely throughout the water system. In addition, elevated residue levels of endosulfan in plant ingredients have also been reported (Lorenzatti et al. 2004). The increasing use of these plant ingredients in aqua feeds in order to develop a more sustainable aquaculture system has led to an increased exposure of endosulfan to fish. This compound has been found to be moderately persistent in the environment and has a tendency to bioaccumulate in various aquatic organisms (Naqvi and Vaishnavi 1993). Hence, these chemicals pose a constant threat to the non-target aquatic organisms, and especially fish, since the pesticides are known to alter various biochemical and physiological functions.

Lecithin, a source of phospholipids (PL) in the diet, acts as a non-protein energy source. Also it acts as a feed attractants, increases resistance to stress and can reduce the oxidation (antioxidant) of vitamin A, C and E, and enhance the utilization of these vitamins in aquacultural species (ADM 2003). Benefits of lecithin are especially pronounced in the diets of young aquatic species as in early life stages, the digestive tracts have a very limited ability to synthesize adequate quantities of phospholipid to enhance growth and survival. Betaine has two major metabolic functions, as methyl donors and as an osmoprotectant. Betaine, being a compatible osmolyte, increases the water retention of cells, replaces inorganic salts and protects intracellular enzymes against osmotically or temperature-induced inactivation (Yancey et al. 1982). Choline is a precursor of betaine, acetylcholine (neurotransmitter) and phosphatidylcholine (PC). Choline is also an important component of some plasmalogens, sphingomyelins, and lecithin, and acts as a source of methyl groups, via betaine, for the synthesis of various methylated metabolites. Choline deficiency has been shown to produce poor growth and fatty liver (Ketola 1976), hepatic lipidosis and renal hemorrhage (Griffin et al. 1994) anorexia and hemorrhagic areas in kidney, liver and intestine (Wilson and Poe 1988). As a part of our main endeavor to study the effects of these methyl donors under conditions of environmental stress, which we believe will protect fish, we first evaluated growth performance, metabolic status nutrient digestibility, karyotyping and histopathology of *Labeo rohita* in response to supplementation of these compounds under stress husbandry conditions. Hence, the experiment was conducted to delineate the role of dietary lecithin, betaine and choline in mitigating endosulfan-induced stress in *L. rohita* fingerlings.

Materials and methods

Fish and experimental design

Fingerlings of *L. rohita* (7.96 ± 0.04 g, average weight ± SE) were procured from Prem Fisheries Consultancy, Gujarat, India, and transported in a circular container (500-l) with sufficient aeration to the experimental facilities at Central Institute of Fisheries Education, Mumbai, and were acclimatized to the experimental rearing conditions for 15 days. After acclimatization, fish were transferred to 15 uniform size experimental plastic tanks of 150-l capacity and reared for 37 days. Continuous aeration was provided throughout the experimental period. Fifteen fish of uniform size (initial weight 7.96 ± 0.04 g, average weight ± SE) per container were stocked in five distinct groups with three replicates for each treatment in plastic containers (80 × 57 × 42 cm) of 150-l capacity each following a completely randomized design. The fish were fed with the experimental diet twice daily (09:00 and 17:00 h) to approximate satiation for 37 days. Round-

the-clock aeration was provided to all the containers from a compressed air pump, and manual water exchange (two-third) was carried out at every alternate day. Four iso-nitrogenous and iso-caloric diets were formulated, that is, control diet or basal diet (without supplementation of methyl donor), choline-supplemented diet (1 g/kg), betaine-supplemented diet (5 g/kg) and lecithin-supplemented diet (20 g/kg) using soylecithin, betaine hydrochloride and choline chloride as a source of lecithin betaine and choline, respectively. The experimental setup were normal water (without endosulfan) and fed with control diet (control group), endosulfan-treated water and fed with control diet (T_1), endosulfan-treated water and fed with choline-supplemented feed (T_2), endosulfan-treated water and fed with betaine-supplemented feed (T_3) and endosulfan-treated water and fed with lecithin-supplemented feed (T_4). The endosulfan treatment was made at level of 1/10 of LC_{50} for all the treatment groups using technical grade endosulfan (99%; a: b ratio of 7:3) purchased from Excel Crop Care Limited, Hubli, Karnataka, India). Water-quality parameters were checked every week using the methods of APHA (1998) and were found to be within the recommended range for carp rearing.

Experimental diet

Four iso-nitrogenous and iso-caloric diets (composition: Table 1) were formulated, that is, control diet or basal diet (without supplementation of methyl donor), choline-supplemented diet (1 g/kg), betaine-supplemented diet (5 g/kg) and lecithin-supplemented diet (20 g/kg) using soylecithin, betaine hydrochloride and choline chloride as a source of lecithin betaine and choline, respectively. Soylecithin, betaine hydrochloride and choline chloride were procured from HIMEDIA (JTJ Enterprises, Mumbai, India) and SD Fine chemical Ltd. India. Betaine and choline chloride was first dissolved in water and incorporated along with vitamin mineral premix, whereas lecithin was mixed in oil. For formulation of pelleted diet, good-quality fish meal, soybean meal, sunflower meal, wheat flour, wheat bran and sunflower oil were procured from local market. Manually prepared vitamin and mineral mixture along with ascorbyl phosphate (SRL Ltd., Mumbai, India) as the source of vitamin C was used. The dough was mixed properly and was pelleted, air-dried and kept in hot air oven at 60°C until dry and was

Table 1 Ingredients and proximate composition of the different experimental diets during the experimental period of 37 days

Ingredients	Treatments			
	Control	Choline	Betaine	Lecithin
Soybean meal[a]	45.5	45.5	45.5	45.5
Fish meal[a]	10.00	10.00	10.00	10.00
Sunflower meal[a]	10.00	10.00	10.00	10.00
Wheat flour[a]	14.97	14.87	14.47	14.97
Wheat bran[a]	10.00	10.00	10.00	10.00
Sunflower oil[a]	4.00	4.00	4.00	2.50
Cod liver oil[a]	2.00	2.00	2.00	1.50
CMC[b] (Carboxyl methylcellulose	1.00	1.00	1.00	1.00
Vitamin + mineral mix[c] (manual prepared)	2.00	2.00	2.00	2.00
Vitamin C[d]	0.030	0.030	0.030	0.030
Chromic oxide	0.50	0.50	0.50	0.50
Soylecithin[b]	–	–	–	2.00
Betaine hydrochloride[b]	–	–	0.50	–
Choline chloride[d]	–	0.10	–	–
Total	100	100	100	100

[a] Procured from local market

[b] HIMEDIA (JTJ Enterprises, Mumbai, India)

[c] Prepared manually and all components from Himedia Ltd

[d] SD Fine Chemicals Ltd., India. Composition of vitamin mineral mix (quantity/250 g starch powder): vitamin A 55,00,00 IU; vitamin D_3 11,00,00 IU; vitamin B1:20 mg, vitamin B_2 2,00 mg; vitamin E 75 mg; vitamin K 1,00 mg; vitamin B_{12} 0.6 mcg; calcium pantothenate 2,50 mg; nicotinamide 1000 mg; pyridoxine: 100 mg; Mn 2,700 mg; I 1,00 mg; Fe750 mg; Zn 5,00 mg; Cu 200 mg; Co 45 mg; Ca 50 g; P 30 g; selenium 5 ppm

subsequently stored at 4°C until required for feeding. The digestibility studies of the experimental diet were carried out by indirect method using chromic oxide as marker. The experimental diets were prepared by incorporating 5 g kg^{-1} chromic oxide (Cr_2O_3) in the diet and fed till end of the experimental period.

Tissue homogenate preparation

The muscle, liver, gill and intestine of the fishes from the exposed all groups were dissected carefully and weighed. Tissues were homogenized separately to get 5% homogenate with chilled sucrose solution (0.25 M) in a glass tube using Teflon-coated

mechanical tissue homogenizer (MICCRA D-9, Dig-itronic, Germany). The tube was continuously kept in ice to avoid denaturation of the enzymes during the homogenization. The homogenates were centrifuged at 5,000 rpm for 20 min at 4°C in a cooling centrifuge machine and sample kept at −80°C until use.

Growth study

Fish were weighed at the start and every 15-day interval thereafter till 37th day. At the end of the experiment, fish were anesthetized with clove oil (50 μl/l) and were weighed individually. The growth performance of fingerling was evaluated in terms of percent weight gain (%), feed conversion ratio (FCR), protein efficiency ratio (PER) and specific growth rate (SGR).

activities were measured by the estimation of oxaloac-etate and pyruvate released, respectively, after incuba-tion of the reaction mixture at 37°C for 60 min (Wooten 1946). Lactate dehydrogenase (LDH) (L-lactate NAD1 oxidoreductase; E.C.1.1.1.27) was assayed 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 0.2mM sodium pyruvate as the substrate, and optical density (OD) was recorded at 340 nm (Wrob-leuiski and Ladue 1955). A similar reaction mixture was used for the estimation of malate dehydrogenase (MDH) (L-malate: NAD + oxidoreductase: E.C.1.1.1.37) except for the substrate (1 mg oxaloacetate/ml of chilled triple distilled water) (Ochoa 1955). Glucose-6-phos-phate dehydrogenase (G6PDH) (E.C.1.1.1.49) activity was measured by method of De Moss (1955), 1.5 ml of

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\begin{aligned}
\text{Weight gain}(\%) &= (\text{Final weight} - \text{Initial weight})/\text{Initial weight} \times 100 \\
\text{FCR} &= \text{Total dry feed intake}(\text{gm})/\text{wet weight gain} \\
\text{SGR} &= 100(\text{log}_e \text{ average final weight} - \text{log}_e \text{ average initial weight})/\text{number of culture days} \\
\text{PER} &= \text{Total wet weight gain}(\text{g})/\text{crude protein fed}(\text{g})
\end{aligned}
$$

Proximate analysis of feed

The proximate composition of the experimental diets was determined following the standard methods of AOAC (1995) and is presented in Table 1. The moisture content was determined by drying at 105°C to a constant weight. Nitrogen content was estimated by Kjeldahl (2200 Kjeltec Auto distillation, Foss Tecator, Hogonas, Sweden) method and crude protein was estimated by multiplying nitrogen percentage by 6.25. Ether extract (EE) was measured by solvent extraction method (1045 Soxtec extraction unit, Foss Tecator) using diethyl ether (boiling point, 40–60°C) as a solvent, and ash content was determined by incinerating the samples in a mu\e furnace at 600°C for 6 h. Total carbohydrate was calculated by differ-ence, that is, total carbohydrate %5100-(CP% EE% Ash%). The digestible energy value of experimental diets was calculated by Halver (1976).

Enzyme assays

Aspartate aminotransaminase (AST) (E.C.2.6.1.1) and alanine aminotransaminase (ALT) (E.C.2.6.1.2)

0.1 M Tris buffer (pH 7.8), 0.2 ml of 2.7 mM NADP, 0.1 ml of tissue homogenate, 1.05 ml of distilled water and 0.1 ml of 0.02 M glucose-6-phosphate (G6P), optical density (OD) was recorded at 340 nm. Total adenosine triphosphatase (ATPase) (E.C.3.6.1.3) was assayed as per the modified method of Post and Sen (1967). Alkaline phosphatase (ALP) (E.C.3.1.3.1) was determined by the method of Garen and Levinthal (1960). Acetylcholine esterase (AChE) (EC. 3.1.1.7) activity as measured by the change in OD at 540 nm using the method of Hestrin (1949) modified by Augustinsson (1957).

Preparation of liver tissue samples for histopathology

The liver tissue of fish was taken from all the experimental group control fed diet, endosulfan exposed but fed with control feed, choline-fed endo-sulfan-exposed, betaine-fed endosulfan-exposed and lecithin-fed endosulfan-exposed group and fixed in buffered formalin and was dehydrated in 90% alcohol for an hour and three times in absolute alcohol for 45 min each separately. The samples were then cleared

two times in xylene for 30 min each and embedded in paraffin for 45 min. The samples were then blocked, allowed to cool, cut on a rotatory microtome at 5 μm, and mounted sections were dewaxed in xylene and dehydrated serially in alcohol and then the slides were washed in tap water for 1 min, stained in hematoxylin for 12 min, washed with tap water, dipped in 2% acid alcohol and again washed in tap water. The section was dehydrated through 50, 70 and 90% alcohol for 2 min each and stained in eosin for 4 min and dipped in absolute alcohol for 1 min. Finally, the stained sections were cleared in xylene for 5 min and mounted with DPX. Prepared slides were examined under a light microscope (Olympus CX-31, Japan, $10\times$ $40\times$ and $100\times$ magnifications) and photographs were taken. Photographs of high quality only are presented in the plates.

Karyotyping

Preparation of reagents

Colchicine ($C_{22}H_{25}NO_6$)

(a) Stock solution: 5 mg of colchicine (Himedia) was dissolved in 10 ml of triple distilled water and stored at 4^0 C.
(b) Working solution: 0.5 ml of the stock solution was withdrawn and the final volume was made up to 5 ml by the addition of triple distilled water to obtain a final concentration of 0.05 mg/ml and injected to fish intramuscular at the rate of 0.05 mg/100 gram body weight, 4 h prior to killing the animals for tissue collection.

Hypotonic saline solution (0.075 M KCl)

This solution was prepared by dissolving 0.56 grams of KCl in 100 ml triple distilled water and was prepared fresh every time.

Tissue collection

The fishes were taken out of water and dried with the help of clean cotton cloth before dissecting fish. The abdominal area was disinfected with spirit. Kidney tissues were collected and immediately transferred to separate petridish containing freshly prepared KCl hypotonic solution. The tissues were finely grinded immediately after removing from the body.

Preparation of metaphase spread

Hypotonic treatment and fixation

The finely grinded tissue was kept in KCl hypotonic solution for 30 min at the room temperature. Then, these tissues along with hypotonic solution were transferred to centrifuge tubes and subjected to centrifugation for 10 min at 1,000 rpm. Then, the supernatant was discarded. A freshly prepared fixative [methanol: absolute acetic acid (3:1)] of about 5 ml was added to each tube and contents were resuspended and subjected to centrifugation for 10 min at 1,000 rpm. Then, supernatant was pipette out. The above step was repeated 2 or 3 times until a clear supernatant along with a mass of white cells as sediment at the bottom of the tube was obtained. The final volume of the fixative was adjusted based on the pellet size, and a uniform suspension was prepared. Four to five drops of cell suspension was allowed to fall on a clean cold slide and air-dried for one hour.

Staining of chromosome

4% Giemsa stain in Sorenson's phosphate buffer was used for staining the chromosome. The slide were stained for 30 min in Coplin jar and washed with distilled water. Then the slides were air-dried and finally mounted with DPX.

Statistics

The data were statistically analyzed by statistical package SPSS version 16 in which data were subjected to one-way ANOVA, and Duncan's multiple range tests were used to determine the significant differences between the means. Comparisons were made at the 5% and/or 1% probability level.

Result and discussion

Growth performance

The data pertaining to percentage weight gain%, FCR, PER and SGR are presented in Table 2. The weight

Table 2 Percentage body weight, FCR, PER and SGR of *L. rohita* fingerlings exposed to low-dose endosulfan and fed with dietary lecithin, betaine and choline during the experimental period of 37 days

Treatments	Non-stressor	Stressor (endosulfan)	Stressors (endosulfan exposed)			P-value
Diets	Control	Control	Choline	Betaine	Lecithin	
Weight gain%	$67.72^{ab} \pm 4.85$	$53.51^{a} \pm 2.80$	$71.61^{ab} \pm 8.22$	$68.70^{ab} \pm 3.95$	$79.05^{b} \pm 5.50$	$P < 0.05$
SGR	$1.48^{ab} \pm 0.08$	$1.22^{a} \pm 0.05$	$1.53^{b} \pm 0.07$	$1.49^{ab} \pm 0.09$	$1.66^{b} \pm 0.13$	$P < 0.05$
FCR	$1.69^{a} \pm 0.13$	$2.21^{b} \pm 0.18$	$1.57^{a} \pm 0.19$	$1.61^{a} \pm 0.09$	$1.37^{a} \pm 0.10$	$P < 0.05$
PER	$1.71^{ab} \pm 0.14$	$1.30^{a} \pm 0.10$	$1.86^{b} \pm 0.26$	$1.76^{ab} \pm 0.10$	$2.10^{b} \pm 0.15$	$P < 0.05$

Mean values bearing different superscripts (a, b, c) under each rows vary significantly ($P < 0.05$). Data expressed as mean $\pm$ SE, $n = 3$

FCR feed conversion ratio, *SGR* specific growth rate, *PER* protein efficiency ratio

gain percentage, protein efficiency ratio (PER) and specific growth rate (SGR) were significantly higher in lecithin-fed group. The low-dose endosulfan-exposed (1/10th of LC_{50}, 0.2 ppb) group had significantly lower weight gain percentage, PER and SGR than lecithin-fed endosulfan-exposed group. Similar trend was observed for FCR.

The growth performance in endosulfan-exposed group (1/10th of LC_{50}, 0.2 ppb) was significantly reduced in *L. rohita* fingerlings. Similar effects have been noted previously in *L. rohita* exposed to water-borne endosulfan (Ramaneswari and Rao 2000) and in Nile Tilipia exposed to sublethal level of two pesticides, Malathion and Dimethoate (Sweilum 2006). The decreased trend in the growth of *L. rohita* could be a consequence of altered metabolism, resulting from toxic stress (Adeyemo 2005). However, mainly dietary lecithin mitigated the negative effect of endosulfan toxicity evident from increased growth. Lecithin in the diet might have enriched the amino acid pool in the cells, and the non-essential amino acids act to produce substrate for gluconeogenesis, which might aid in

combating against stressors. There is no available literature on the effect of lecithin, betaine and choline on growth performance reared in endosulfan-exposed condition to substantiate our findings. Based on primary response parameters like percent weight gain SGR and FCR, it appears that supplementation of choline, betaine and lecithin brings back the growth parameter toward normal when compared to the endosulfan-treated group and fed with control diet.

Apparent nutrient digestibility study

The data pertaining apparent nutrient digestibility coefficient of protein, carbohydrate and lipids are presented in Table 3. The protein, lipid and dry matter digestibility was significantly highest ($P < 0.05$) in lecithin- and betaine-fed endosulfan-exposed group. The dietary lecithin supplied highest ($P < 0.05$) energy in comparison with all other groups especially control fed endosulfan-exposed *L. rohita*. The carbohydrate digestibility did not change in any group of *L. rohita* fingerlings.

Table 3 Apparent nutrient digestibility (in %) of *L. rohita* fingerlings exposed to low-dose endosulfan and fed with dietary lecithin, betaine and choline during the experimental period of 37 days

Treatments	Non-Stressor	Stressor (endosulfan)	Stressors (endosulfan exposed)			P-value
Diets	Control	Control	Choline	Betaine	Lecithin	
Dry matter	$71.25^{b} \pm 0.37$	$70.29^{b} \pm 0.31$	$66.94^{a} \pm 1.32$	$76.29^{c} \pm 0.63$	$78.62^{c} \pm 0.88$	$P < 0.01$
Protein	$81.14^{b} \pm 0.22$	$78.24^{a} \pm 0.19$	$78.69^{a} \pm 0.04$	$86.21^{c} \pm 0.26$	$86.77^{c} \pm 0.94$	$P < 0.01$
Lipid	$89.10^{b} \pm 0.64$	$89.48^{b} \pm 0.49$	$92.05^{c} \pm 0.38$	$90.78^{b} \pm 0.21$	$92.05^{c} \pm 0.32$	$P < 0.01$
Carbohydrate	71.59 ± 1.115	70.16 ± 1.12	69.76 ± 2.25	75.89 ± 0.295	76.32 ± 0.865	NS
Energy	$73.94^{ab} \pm 1.22$	$71.25^{b} \pm 0.23$	$68.78^{a} \pm 2.95$	$78.37^{b} \pm 0.66$	$80.95^{c} \pm 0.13$	$P < 0.01$

Mean values bearing different superscripts (a, b, c) under each rows vary significantly ($P < 0.05/0.01$). Data expressed as mean $\pm$ SE, $n = 3$

The high digestibility coefficient found in the present study indicates the positive effect of lecithin and betaine supplementation on digestibility in *L. rohita* fingerlings subjected to endosulfan stress. Beneficial effect of lecithin on the digestibility of both protein and energy was reported for juvenile Atlantic Salmon (Hung et al. 1997). Similarly, Poston 1990 attributed lecithin for the increase in protein and energy digestibility in rainbow trout. The increase in lipid digestibility in endosulfan-exposed lecithin-fed groups compared with other groups can be credited to emulsification properties of phospholipids and their role in lipid absorption and transport. However, dietary betaine-fed group has similar lipid digestibility as that of the control.

Enzymes assays

Lactate dehydrogenase (LDH) and of Malate dehydrogenase (MDH)

Effects of dietary choline, betaine and lecithin on the muscle and liver LDH and MDH activity of the experimental groups at the end of the experiment are shown in Table 4. Dietary betaine and lecithin had significant ($P < 0.01$) effect on LDH and MDH activity in liver of *L. rohita* fingerling. In liver, the highest LDH and MDH activity was found in groups exposed to endosulfan and the activity reduced in the betaine- and lecithin-fed endosulfan-exposed group.

The present study demonstrates that the inclusion of dietary lecithin, betaine and choline has significant

effect on various enzymes like LDH, MDH, ATPase, ALP, etc. Generally, LDH activity increases in stress (Vijayaraghavan and Rao 1986). In the present study, in liver LDH and MDH, activity increased significantly ($P < 0.05$) in endosulfan-exposed group, suggesting that the animals were under stress induced by endosulfan (Akhtar et al. 2009). Higher activity of MDH indicates greater activity of TCA cycle due to increased energy demands during stress. LDH and MDH activity decreased significantly ($P < 0.01$) in the groups fed with lecithin- and betaine-supplemented diets indicating the stress-mitigating effect of these methyl donors. This is supported by the findings of Das et al. (2006) who found elevated MDH activity in fish in stress. Lower activity of LDH and MDH in the treatment groups suggests that the supplementation of dietary lecithin and betaine may help in reducing energy demands in the *L. rohita* fingerlings. There is no available literature on effect of lecithin, betaine and choline on LDH and MDH activity to substantiate our findings.

Aspartate aminotransaminase (AST) and Alanine aminotransaminase (ALT)

Effects of dietary choline, betaine and lecithin on the muscle and liver AST and ALT activity of the experimental groups at the end of the experiment are shown in Table 4. Dietary choline, betaine and lecithin had no significant ($P > 0.05$) effect on muscle and liver ALT as well as AST activity in liver of *L. rohita* fingerling. Muscle AST had significant

Table 4 Metabolic enzymes of *L. rohita* fingerlings exposed to low-dose endosulfan and fed with dietary lecithin, betaine and choline during the experimental period of 37 days

Treatments	Non-stressor	Stressor (endosulfan)	Stressors (endosulfan exposed)			P-value
Diets	Control	Control	Choline	Betaine	Lecithin	
LDH Liver	$0.62^{ab} \pm 0.19$	$0.86^{b} \pm 0.09$	$0.66^{ab} \pm 0.09$	$0.54^{ab} \pm 0.10$	$0.34^{a} \pm 0.06$	$P < 0.05$
MDH Liver	$1.74^{bc} \pm 0.26$	$1.87^{c} \pm 0.17$	$1.24^{abc} \pm 0.17$	$0.85^{a} \pm 0.31$	$1.14^{ab} \pm 0.16$	$P < 0.05$
AST Muscle	$11.04^{a} \pm 1.51$	$17.89^{b} \pm 1.56$	$12.86^{a} \pm 2.11$	$9.46^{a} \pm 1.79$	$11.02^{a} \pm 1.36$	$P < 0.05$
AST Liver	7.92 ± 1.48	5.82 ± 1.06	6.93 ± 1.18	7.76 ± 1.09	5.23 ± 1.36	NS
ALT Muscle	15.98 ± 2.38	11.24 ± 1.38	14.99 ± 1.84	9.67 ± 1.41	10.9 ± 1.21	NS
ALT Liver	15.28 ± 1.84	6.79 ± 1.86	14.15 ± 1.60	12.55 ± 1.43	9.82 ± 3.11	NS

Mean values bearing different superscripts (a, b, c) under each rows vary significantly ($P < 0.05$). Data expressed as mean ± SE, $n = 6$. LDH (lactate dehydrogenase): specific activity expressed as Units/min/mg protein at 37°C, MDH (malate dehydrogenase): specific activity expressed as Units/min/mg protein at 37°C, ALT (Aspartate amino transferase): specific activities expressed as nano moles of sodium pyruvate formed/mg protein/minute at 37°C. AST (Alanine amino transferase): specific activities expressed as nano moles of oxaloacetate released/min/mg protein at 37°C

($P < 0.05$) effect on choline-, betaine- and lecithin-fed group, and AST activity was highest recorded in endosulfan-exposed group.

The higher activity of muscle AST indicates the mobilization of aspartate and alanine via gluconeogenesis for glucose production to cope with stress. Chatterjee et al. (2006) reported that elevated level of transaminase activity during stress would lead to increased feeding of keto acids into TCA cycle. In the present study, the activity of the muscle transaminases (AST) was minimum in endosulfan exposed and concurrent exposed to endosulfan, which was not fed with supplementation, and this may be attributed to the fact that lecithin, betaine and choline are required as a co-factor for the enzyme transaminases. Supplemented groups can be inferred that addition of lecithin, betaine and choline reduces the stress in *L. rohita* fingerlings.

Total adenosine triphosphatase (ATPase)

Data pertaining to the impact of dietary choline, betaine and lecithin on the ATPase activity of gill and liver of *L. rohita* fingerlings exposed to endosulfan-induced chronic stress are shown in Table 5. There was a significant ($P < 0.01$) effect of choline, betaine and lecithin fed group on ATPase gill and liver. Activity of ATPase was significantly lower ($P < 0.01$) in endosulfan-exposed group.

Adenosine triphosphatase (ATPase) is a membrane-bound enzyme responsible for the transport of ions through the membrane and immediate release of energy. As this enzyme is related to immediate release of energy, reduction in the activity of this enzyme might have significantly affected the fishes in terms of the energy balance and ion transport. This might have considerably affected the glucose metabolism in the liver and gill, thereby leading to hyperglycemia state in *L. rohita* fingerling. In present study, ATPase activity in liver and gill was reduced significantly in endosulfan-exposed group. The lecithin, betaine and choline supplementation ATPase activity in liver and gill was significantly higher. This suggested that lecithin, betaine and choline may help in providing more energy to *L. rohita*. Our results are in close agreement with the findings of Sharma (1988), who observed significant reduction of liver ATPase activity in C. gachua upon endosulfan exposure. Oruc et al. (2002) reported reduction of liver $Na^+ K^+$-ATPase activity in Tilapia zilli and O. niloticus and suggested that increased lipid peroxide formation could disturb the anatomical integrity of the biomembrane and diminish its fluidity leading to inhibition of several membrane bound enzymes including $Na^+ K^+$-ATPase. In the present study, reduction of gill and liver ATPase activity may be due to alterations in the structure and functions of the liver and gill plasma membrane or may be due to direct inhibition of endosulfan on the enzymes. Higher activity of ATPase in liver and gill treatment groups suggests that the supplementation of dietary lecithin, betaine and choline may help in reducing energy demands in the *L.*

Table 5 ATPase, ALP, G6PDH and AChE of *L. rohita* fingerlings exposed to low-dose endosulfan and fed with dietary lecithin, betaine and choline during the experimental period of 37 days

Treatments	Non-stressor	Stressor (endosulfan)	Stressors (endosulfan exposed)			P-value
Diets	Control	Control	Choline	Betaine	Lecithin	
ATPase Liver	$15.58^b \pm 0.91$	$9.54^a \pm 0.86$	$17.81^b \pm 1.12$	$22.17^c \pm 1.06$	$28.37^d \pm 1.16$	$P < 0.01$
ATPase Gill	$144.01^b \pm 1.19$	$106.98^a \pm 3.49$	$149.84^b \pm 2.76$	$158.81^c \pm 2.74$	$169.45^d \pm 2.64$	$P < 0.01$
ALP	$86.90^b \pm 3.74$	$69.24^a \pm 2.98$	$104.91^c \pm 1.17$	$113.37^d \pm 1.37$	$116.90^d \pm 1.18$	$P < 0.01$
G6PDH Muscle	$0.05^a \pm 0.02$	$0.09^b \pm 0.02$	$0.03^a \pm 00.1$	$0.03^a \pm 0.01$	$0.06^a \pm 0.01$	$P < 0.05$
AChE Brain	0.35 ± 0.07	0.30 ± 0.07	0.37 ± 0.08	0.40 ± 0.07	0.45 ± 0.10	NS

Mean values bearing different superscripts (a, b, c) under each *rows* vary significantly ($P < 0.05$). Data expressed as mean ± SE, $n = 6$

ATPase, adenosine triphosphatase: microgram phosphorus released/min/mg protein at 37°C; ALP alkaline phosphatase (ALP) enzyme activities expressed in mg p-nitro phenol released/mg protein/min at 37°C. Glucose-6-phosphate dehydrogenase (G6PDH): specific activities expressed as Δ 0.01 OD/min/mg protein. Acetyl choline esterase (AChE): μ moles of acetyl choline hydrolyzed/min/mg protein

rohita fingerlings. There is no available literature on the effect of lecithin, betaine and choline on ATPase activity to substantiate our findings.

Alkaline phosphatase (ALP)

Effects of dietary choline, betaine and lecithin on ALP activity of the experimental groups at the end of the experiment are shown in Table 5. The intestinal ALP activity was significantly higher ($P < 0.01$) in endosulfan-exposed group. The intestinal ALP activity was normal in supplementation with choline-, betaine- and lecithin-fed diet group.

ALP, a zinc-containing metallo-enzyme, plays an important role in phosphorus metabolism. Induced of intestinal ALP activity in group fed control diet and endosulfan exposed group (T1 group). These results are consistent with other results previously observed in *C. punctatus* (Sharma 1990) and in other species (Verma et al. 2007). Such induction of ALP could possibly be an indication of role of endosulfan in inhibiting protein synthesis (Verma et al. 2007). However, when fish were fed with diet containing lecithin and betaine ALP activity normal, which might be due to the easily liberate phosphate ions to combat stressful condition or higher metabolic rate. To the best of our knowledge, there is no such effect shown by betaine and lecithin by any such researcher in stress full condition.

Glucose-6-phosphate dehydrogenase (G6PDH)

Effects of dietary choline, betaine, and lecithin on the muscle and liver LDH and MDH activity of the experimental groups at the end of the experiment are shown in Table 5. Dietary choline, betaine and lecithin had significant ($P < 0.05$) effect on G6PDH activity in muscle and liver of *L. rohita* fingerling.

G6PDH catalyzes glucose-6-phosphate to 6-phosphogluconolactone using $NADP^+$ as coenzymes and releases NADPH. This enzymes activity may increase when requirements of NADPH increase in order to synthesize fatty acid, cholesterol steroids and sphingolipid (lipid anabolism). In endosulfan-exposed group, activity of G6PDH was significantly higher than control and supplemented group. Under stress condition NADPH produced and also required NADHP oxidase for producing superoxide anions for destroying phagocytosized materials. So, the enzymes activity may increase with increased phagocytosis (Das 2002).

Enzymes of neurotransmission (AChE)

Effects of dietary choline, betaine and lecithin on the brain AChE activity of the experimental groups at the end of the experiment are shown in Table 5. Dietary choline, betaine and lecithin were not significantly changed, but the level of AChE activity was less in endosulfan-exposed group and the level of AChE was higher in lecithin-, betaine- and choline-fed endosulfan-exposed group.

The AChE activity in brain of *L. rohita* fingerlings was assayed at the end of the experiment. The experimental group fed diet without supplementation showed a decrease in the level of AChE activity, which indicates stress in animals induced by endosulfan. Similar results were described by David et al. (1975), who observed marine teleost exposed to lethal and sublethal to 1,2-dibromo-2,2-dichloroethyl dimethyl phosphate in seawater, and by Campero et al. (2007), who showed that AChE activity was not affected in the endosulfan treatments.
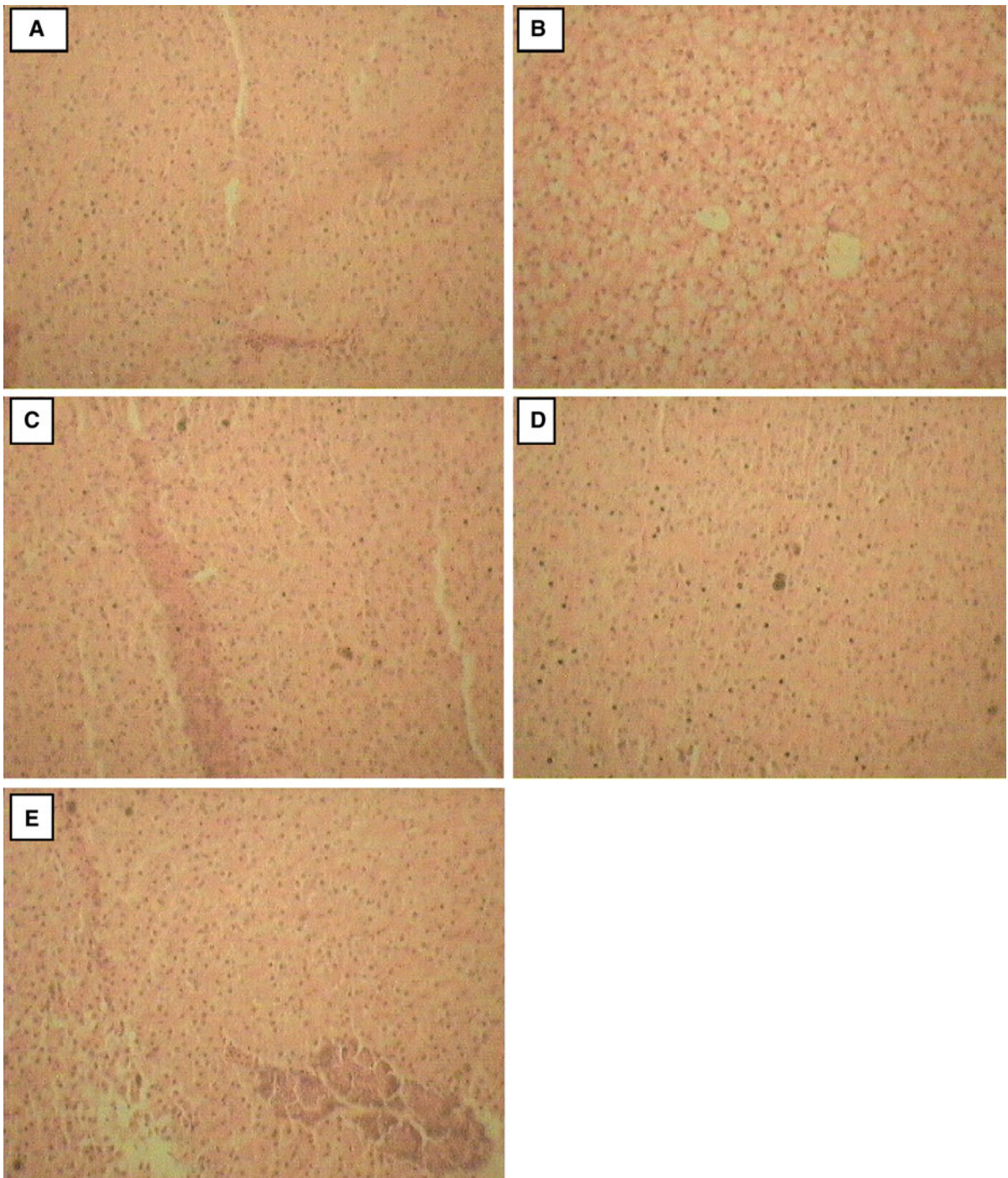
Histopathology

The structural details of the liver histopathological of *L. rohita* are shown in Fig. 1A–E. No recognizable changes (Normal hepatocyte) were observed in the liver of the control fed diet group, choline-, betaine- and lecithin-supplemented group (Fig. 1A, C, D, E). The liver of fish exposed to low-dose endosulfan (1/10th of LC_{50}) treatments for 37 days showed histopathological changes, and slightly large vacuolation in hepatocytes and lipoid vacuole were observed (Fig. 1B).

The liver is the primary organ for metabolism, detoxification of xenobiotics and excretion of harmful substances. The liver has the ability to degrade toxic compounds, but its regulating mechanisms can be overwhelmed by elevated concentrations of these compounds, which could subsequently result in structural damage (Brusle et al. 1996). The histopathological lesions in the liver observed in the present study were large vaculation and lipoid vacuole in endosulfan-exposed group. These histopathologies are consistent with those reported by Velmurugan et al. (2007).
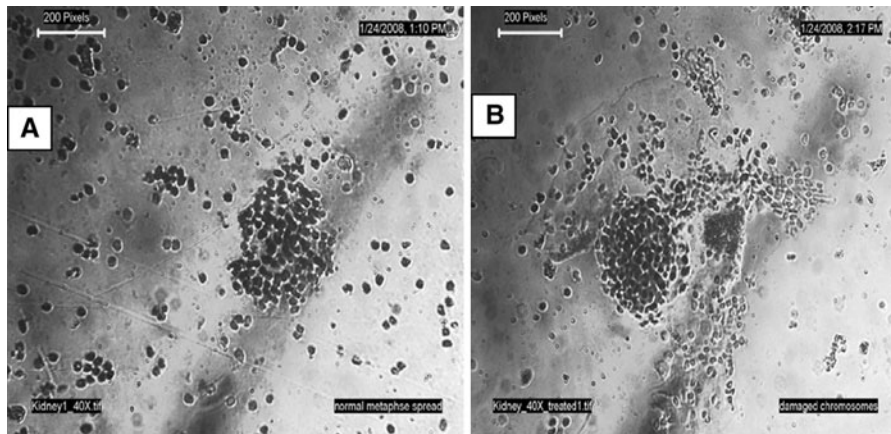
Karyotyping

The karyotype of kidney tissue of *L. rohita* is shown in Fig. 2A, B. The low-dose endosulfan (1/10th of

Fig. 1 Liver tissue of *L. rohita*. **A** Control: Normal hepatocytes. **B** Endosulfan exposed: Slightly large vacuolation and lipoid vacuole. **C** Choline fed endosulfan exposed: normal liver tissue shown (**D**). Betaine fed endosulfan exposed: no histopathological changes (**E**) Lecithin fed endosulfan exposed: normal liver tissue was observed. All figure are taken in 40× (**H & E**)

Fig. 2 Karyotyping of kidney tissue in *L. rohita*. **A** Control fed group: Normal chromosomes are shown (**B**). Endosulfan-exposed group: aberrated chromosomes are observed in endosulfan exposure condition

LC_{50})-exposed fishes had aberrated chromosome in kidney tissues, which are shown in Fig. 2B. Control fed groups had normal chromosome. Chromosome aberration in kidney upon exposure to different doses (0.019, 0.038, and 0.075 ppm) of mercuric nitrate on the *channa punctatus* was studied by Ansy and Jahageerdar (2003). Other study of chromosome aberration was reported by Jayasree et al. (2003) in different doses of aflatoxin-B1 (10, 33, and 100 μg/kg body weight) in *Clarias batrachus*.

Conclusion

The study concluded that dietary methyl donors particularly lecithin and betaine have mitigates endosulfan-induced stress properties in fish. These are revealed by growth performance, LDH, MDH, ALT, ATPase, ALP activities histopathology and karyotyping. Thus, methyl donors' supplementation in practical diets is beneficial for achieving a good health status that is found to be optimum to reduce stress during culturing of *L. rohita*.

References

Adeyemo OK (2005) Hematological and histopathological effects of Cassava mill effluent in *Clarias gariepinus*. Afr J Biomed Res 8:179–183

ADM (2003) Lecithin in aquaculture. Available at www.admworld.com/mktcolpdf/euLecithinsInAquaculture.pdf. Retrieved on 1 Aug 2007

Akhtar MS, Pal AK, Sahu NP, Alexander C, Gupta SK, Choudhary AK, Jha AK, Rajan MG (2009) Stress mitigating and immunomodulatory effect of dietary pyridoxine in *Labeo rohita* (Hamilton) fingerlings. Aquac Res: 1–12. doi: 10.1111/j.1365-2109.2009.02383.x

Ansy MNP, Shrinivas jahageerdar (2003) Effect of mercuric nitrate on the chromosome of *Channa punctatus* (Bloch, 1793). Fish Technol 40(2):77–82

AOAC (1995) Official methods of analysis of the association of official analytical chemists, vol. 1, 16th edn. In: Cunnif PA (ed) AOAC International, Arlington, pp. 31–65

APHA-AWWA-WEF (1998) Standard methods for the estimation of water and waste water, 20th edn. In: Clesceri LS, Greenberg AE, Eaton AD (eds). American Public Health Association, American Water Works Association, Water Environment Federation, Washington, DC

Augustinsson KB (1957) The reaction of acetyl choline esters and other carboxylic acid derivatives with hydroxylamine and its analytical application. J Biolog Chem 180: 249–261

Brusle J, Gonzalez I, Anadon G (1996) The structure and function of fish liver. In: Munshi JSD, Dutta HM (eds) Fish morphology. Science Publishers, New York

Campero M, Ollevier F, Stoks R (2007) Ecological relevance and sensitivity depending on the exposure time for two biomarkers. Environ Toxicol 22(6):572–581

Cengiz EI, Unlu E (2002) Histological changes in the gill of mosquito fish, Gambusia affinis exposed to endosulfan. Bull Environ Contam Toxicol 68:290–296

Chatterjee N, Pal AK, Das T, Manush SM, Sarma K, Venkateshwarlu G, Mukherjee SC (2006) Secondary stress response in Indian major carps *Labeo rohita* (Ham), *Catla catla* (Ham) and *Cirrhinus mrigala* (Ham) fry to increasing packing densities. Aquac Res 37:472–476

Das D (2002) Metabolism of proteins. In: Das D (ed) Biochemistry. Academic publishers, New York, pp 463–504

Das T, Pal AK, Chakraborty SK, Manush SM, Chatterjee N, Apte SK (2006) Metabolic elasticity and induction of heat

shock protein (hsp-70) in *Labeo rohita* acclimated to four temperatures. Asian Aust J Anim Sci 19:1033–1039

David L, Coppage, Matthews E (1975) Brain-acetylcholinesterase inhibition in a marine teleost during lethal and sublethal exposures to 1, 2-dibromo-2, 2-dichloroethyl dimethyl phosphate (naled) in seawater. Toxicol Appl Pharm 31(1): 128–133

DeMoss RD (1955) Glucose-6-phosphate and 6-phosphogluconic dehydrogenase from *Leuconostoc mesenteroides*. In: Colowick SP, Kalpan NO (eds) Methods in enzymology, vol I. Academic Press Inc, New York, pp 328–332

EFSA (2005) Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to endosulfan as undesirable substance in animal feed. EFS A J 234:1–29

Elia AC, Waller WT, Norton SJ (2002) Biochemical responses of bluegill sunfish (Lepomis macrochirus, Rafinesque) to atrazine induced oxidative stress. Bull Environ Contam Toxicol 68:809–816

FAO (2007) The state of world fisheries and aquaculture FAO fisheries and aquaculture department food and agriculture organization of the United Nations, Rome, Italy, 32 pp

Garen A, Levinthal CA (1960) Fine-structure genetic and chemical study of the enzyme Alkaline phosphatase of *E. coli*. l. Purification and characterization of Alkaline phosphatase. Biochem Biophys Acta 38:470

Griffin ME, Wilson KA, White MR, Brown PB (1994) Dietary choline requirement of juvenile hybrid striped bass. J Nutr 124:1685–1689

Halver JE (1976) The nutritional requirements of cultivated warm water and cold water fish species. In: Report of the FAO technical conference on aquaculture, Kyoto, Japan, 26 May–2 June 1976. FAO Fisheries Report No. 188 FI/R188 (En), p 9

Hestrin S (1949) Modified by Augustinsson (1957) The reaction of acetyl choline esters and other carboxylic acid derivatives with hydroxyline and its analytical application. J BioChem 180:249–261

Hung SSO, Berge GM, Stobebakken T (1997) Growth and digestibility effects of soya lecithin and choline chloride on juvenile Atlantic salmon. Aquac Nutr 3:141–144

Jayasree TP, Shrinivas, jahageerdar (2003) Cytogenetic study on effect of Aflatoxin-B1 in *Clarias batrachus* (Linn.) Appl Fish Aquacult III(1 and 2):27–31

Ketola HG (1976) Choline metabolism and nutritional requirement of lake trout (*Salvelinus namaycush*). J Anim Sci 43:474–477

Kilgore WW, Mingyuli (1975) Environmental toxicology. In: Wilkinson CF (ed) Insecticide biochemistry and physiology. Plenum Press, New York, 669 pp

Lorenzatti E, Altahus R, Lajmanovich R, Peltzer P (2004) Residues of endosulfan in soy plants in Argentina croplands. Fresenius Environ Bull 13:89–92

Naqvi SM, Vaishnavi C (1993) Bioaccumulative potential and toxicity of endosulfan insecticide to non-target animals. Comp Biochem Physiol C 105:347–361. doi:10.1016/0742-8413(93)90071-R

Ochoa S (1955) Malic dehydrogenase and 'Malic' enzyme. In: Coloric SP, Kaplan N (eds) Methods of enzymology, vol I. Academic Press, New York, pp 735–745

Oruc EO, Uner N, Tamer L (2002) Comparison of NA^+, K^+-ATPase activity and malondialdehyde contents in liver tissue for three fish species exposed to azinphosmethyl. Bull Environ Contam Toxicol 69:271–277. doi:10.1007/s00128-002-0057-y

Post RL, Sen AK (1967) Methods in enzymology. vol. 10. In: Colowick, SP, Kaplan NO (eds). Academic Press Inc., New York. pp. 762

Poston HA (1990) Performance of rainbow trout fed supplemental soya lecithin and choline. Progve Fish-Cult 52: 218–225

Ramaneswari K, Rao LM (2000) Bioconcentration of endosulfan and monocrotophos by *Labeo rohita* and *Channa punctatus*. Bull Environ Contam Toxicol 65:618–623

Sharma RM (1988) Effect of endosulfan on adenosine triphosphatase (ATPase) activity in liver, kidney and muscles of *Channa gachua*. Bull Environ Contam Toxicol 41:317–323. doi:10.1007/BF01688873

Sharma RM (1990) Effect of endosulfan on acid and alkaline phosphatase activity in liver, kidney and muscle of *Channa gachua*. Bull Environ Contam Toxicol 44:443–448

Sweilum MA (2006) Effect of sublethal toxicity of some pesticides on growth parameters, haematological properties and total production of Nile tilapia (*Oreochromis niloticus* L.) and water quality of ponds. Aquac Res 37:1079–1089

Velmurugan B, Selvanayagam M, Cengiz EI, Unlu E (2007) The effects of fenvalerate on different tissues of freshwater fish *Cirrhinus mrigala*. J Environ Sci Heal 42:157–163

Verma AK, Pal AK, Manush SM, Das T, Dalvi RS, Chandrachoodan PP (2007) Persistent sub- lethal chlorine exposure elicits the temperature induced stress responses in Cyprinus carpio early fingerlings. Pest Biochem Physiol 87:229–237

Vijayaraghavan S, Rao JVR (1986) Starvational stress effects on tissue lactate and lactate dehydrogenase activity in *Anabas scandens* (Cuvier). Comp Physiol Ecol 11:233–236

Wilson RP, Poe WE (1988) Choline nutrition of fingerling channel catfish. Aquaculture 68:65–71

Wooten IDP (1946) Microanalysis. In: Medical biochemistry, 4th edn. J. & A. Churchill, London, pp 101–107

Wrobleuiski L, Ladue JS (1955) LDH activity in blood. Proc Soc Exp Biol Med 90:210–213

Yancey PH, Clar ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evaluation of osmolyte system. Science 217:1214–1223