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Methyl donors potentiates growth, metabolic status and neurotransmitter enzyme in *Labeo rohita* fingerlings exposed to endosulfan and temperature

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Abstract A 2-month preliminary study was conducted to delineate the effect of dietary methyl donors (choline, betaine, and lecithin) on the growth performance and metabolic status of *Labeo rohita* fingerlings subjected to endosulfan alone and in combination with elevated temperature. Four iso-caloric and iso-nitrogenous diets viz. basal diet, betaine-supplemented diet, choline–supplemented diet and lecithin-supplemented diet were prepared and fed to the different experimental groups throughout the experimental period as per the design. Two hundred and seventy fingerlings (average weight 7.95 ± 0.04 g) were randomly distributed in six treatment groups each having three replicates. The experimental groups were as follows: fish subjected to normal water (without endosulfan) and fed with control diet (control group T_0), fish subjected to endosulfan-treated water and fed with control diet (T_1), fish subjected to concurrent exposure of endosulfan and elevated temperature and fed with control diet (T_2), fish subjected to endosulfan and elevated temperature and fed with choline-supplemented diet (T_3), fish subjected to endosulfan and temperature and fed with betaine-supplemented feed (T_4), and fish subjected to endosulfan and temperature and fed with lecithin-supplemented feed (T_5). The result shows that in both the groups, that is, endosulfan exposed and concurrent exposure to endosulfan and elevated temperature group of *L. rohita* the growth performance like percentage weight gain, feed conversion ratio and specific growth rates were significantly different ($P < 0.01$) when fed with supplemented diet compared with control fed group. The liver LDH and MDH activity were significantly lower in lecithin, betaine, and choline fed groups. The muscle AST as well as G6PDH, AST, and ALT did not vary but liver ALT, gill and liver ATPase, intestine ALP, muscle and liver glycogen varied significantly with dietary supplementation. The liver and gill glutathione-S-transferase (GST) activities were significantly lower in methyl donors-supplemented groups and brain AChE activity showed lower inhibition in supplemented groups in both endosulfan alone and concurrently exposed endosulfan and temperature groups. The result obtained in this study concludes that inclusion of methyl donors, particularly lecithin and betaine in feed as nutritional supplements have potential to improve growth and stress mitigating effect in *L. rohita* fingerlings.

Keywords Endosulfan · Temperature · Growth performance · Metabolic enzymes · AChE · Antioxidative status · *Labeo rohita*

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Introduction

Labeo rohita (Hamilton), one of the Indian major carps, is considered to be the major aquaculture candidate species in India as well as in South East Asian countries (FAO 2001) and provides livelihood to millions of people. But, global warming along with indiscriminate use of antibiotics, synthetic growth promoters, and pesticides like endosulfan is likely to potentially affect its productivity in wild fish populations as well as in aquaculture systems globally (Ficke et al. 2007). As fish is an ectothermal organism, any alterations in the water temperature also have a marked and direct effect on many of the key physiological processes and behavioral activities (Jonassen et al. 1999). Each species has a range of temperature over which it survives, and a narrower range where optimum growth occurs (Katersky and Carter 2007). However, temperature beyond optimum limits of a particular species adversely affects the health of aquatic animal due to metabolic stress and increases oxygen demand leading to susceptibility to diseases (Wedemeyer et al. 1999). Hence, thermal stress studies have gained significant attention among scientists to understand the impact of global warming on animals, including fish, as well.

Methyl groups are of vital importance for all animals, both terrestrial and aquatic. Moreover, animals cannot synthesize methyl groups and thus need to receive them in the diets (Kidd et al. 1997). The methyl group is used in methylation reactions to formulate useful compounds such as methionine, creatine, and phosphatidylcholine (PC) through the S-adenosyl methionine pathway (Bender 1992; NRC 1993). Metabolically, the major sources of methyl groups for practical diets, which support optimal animal performance are betaine, choline, methionine and lecithin, which is a nutritionally superior source of choline.

Lecithin, a source of phospholipids (PL) in the diet acts as a non-protein energy source, feed attractants beside this, it increases resistance to stress and can reduce the oxidation (antioxidant) of vitamin A, C, and E, and hence enhance the utilization of these vitamins in aquacultural species (ADM 2003). The 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) and also an important component of some plasmalogens, sphingomyelins, and lecithin. It acts as a source of methyl groups, via betaine, for the synthesis of various methylated metabolites. Choline deficiency has been shown to result in 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 experiment, we studied the effects of these methyl donors under conditions of environmental stress, which we believe will protect fish, we have evaluated the growth performance and metabolic status of *L. rohita* in response to supplementation of these compounds under stressed husbandry conditions.

Materials and methods

Fish and experimental design

Fingerlings of *L. rohita* (7.95 ± 0.02 g, average weight ± SE) were procured from Prem Fisheries Consultancy, Gujarat, India and transported in a circular container (150 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 18 uniform size experimental plastic tanks of 150 L capacity and reared for 65 days. Fifteen fish of uniform size (initial weight 7.95 ± 0.02 g, average weight ± SE) per container were stocked in six 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 throughout the experimental period. 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 two

alternate day. The experimental groups were as follows: fish subjected to normal water (without endosulfan) and fed with control diet (control group T_0), fish subjected to endosulfan-treated water and fed with control diet (T_1), fish subjected to concurrent exposure of endosulfan and elevated temperature (34°C) and fed with control diet (T_2), fish subjected to endosulfan and elevated temperature and fed with choline-supplemented diet (T_3), fish subjected to endosulfan and temperature and fed with betaine-supplemented feed (T_4), and fish subjected to endosulfan and temperature and fed with lecithin-supplemented feed (T_5). The endosulfan treatment was made at level of 1/10 of LC_{50} (0.2 ppb) 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) and 34°C temperature was maintained throughout the experiment using thermostatic water heaters (range up to 50°C from normal) with specification DTC-PID—50L × 51B × 52H were procured from General Trading Corporation, Mumbai, 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-caloric and iso-nitrogenous diets viz. basal diet and three supplemented diets as choline, betaine, and lecithin diet were prepared using choline chloride, betaine hydrochloride and soy lecithin as a source of and choline, betaine, and lecithin, respectively. Soy lecithin, betaine hydrochloride, and choline chloride were procured from HIMEDIA (JTJ Enterprises, Mumbai, India) and SD Fine chemical Ltd India. Betaine and choline were first dissolved in water and incorporated along with vitamin mineral premix, whereas lecithin was mixed in oil. For the 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 choline free 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 subsequently stored at 4°C until required for feeding.

Tissue homogenate preparation

The muscle, liver, gill, and intestine of the fishes from all the exposed 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, Digitronic, Germany). The tube was continuously kept in an ice to avoid denaturation of the enzymes during the homogenization. The homogenate was centrifuged at 5,000 rpm for 20 min at 4°C in a cooling centrifuge machine and the separated supernatant stored at −80°C until further use.

Growth study

Fishes were weighed at the start and every 15-day interval thereafter till 65th day. At the end of the experiment, fishes were anesthetized with clove oil (50 μL/L) and weighed individually. The growth performance of fingerlings was evaluated in terms of weight gain (%), feed conversion ratio (FCR) and specific growth rate (SGR).

$$\text{Weight gain } (\%) = \text{Final body weight (FBW)} - \text{Initial body weight (IBW)}/\text{Initial body weight (IBW)} \times 100$$

$$\text{FCR} = \text{Total dry feed intake (gm)}/\text{wet weight gain}$$

$$\text{SGR} = 100\,(\ln \text{FBW} - \ln \text{IBW})/\text{number of culture days}$$

Proximate analysis of feed

The proximate composition of the experimental diets was determined as per the standard methods of AOAC (1995) and presented in Table 1. Samples were analyzed for crude protein (CP), ether extract (EE), ash and total carbohydrate (TC). The digestible energy value of experimental diets was determined by following the method of Halver (1976).

Enzyme assays

Aspartate aminotransaminase (AST; EC.2.6.1.1) and alanine amino transaminase (ALT; EC.2.6.1.2) activities were measured by the estimation of oxaloacetate

Table 1 Ingredients and proximate composition of the different experimental diets during the experimental period of 65 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 choline free)	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
Soy lecithin[b]	–	–	–	2.00
Betaine hydrochloride[b]	–	–	0.50	–
Choline-chloride[d]	–	0.10	–	–
Total	100	100	100	100
Proximate composition of diets (%)				
Crude protein	35.40 ± 0.15	34.31 ± 0.68	34.89 ± 0.19	35.38 ± 0.21
Ether extract	9.98 ± 0.28	11.08 ± 0.17	9.06 ± 0.25	9.91 ± 0.15
ASH	9.04 ± 0.27	10.51 ± 0.56	10.33 ± 0.75	10.07 ± 0.52
Total carbohydrate	44.59 ± 1.15	43.94 ± 1.27	44.22 ± 1.51	40.53 ± 0.18
Digestible energy	409.79 ± 1.89	412.69 ± 2.50	405.59 ± 3.35	408.75 ± 0.87

Digestible energy (kcal 100 per g) = (% CP·4) + (% EE·9) + (TC·4), DM% = 100 − moisture%

[a] Procured from local market

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

[c] Prepared manually and all components from Himedia Ltd. 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_2 200 mg, vitamin E 75 mg, vitamin K 100 mg, vitamin B_{12} 0.6 mcg, calcium pantothenate 250 mg, nicotinamide 1,000 mg, pyridoxine 100 mg, Mn 2700 mg, I 100 mg, Fe 750 mg, Zn 500 mg, Cu 200 mg, Co 45 mg, Ca 50 g, P 30 g, selenium 5 ppm

[d] SD Fine Chemicals Ltd., India

and pyruvate released, respectively, after incubating the reaction mixture at 37°C for 60 min (Wooten 1964). Lactate dehydrogenase (LDH; L-lactate NAD1 oxidoreductase; EC.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 0.2 mM sodium pyruvate as the substrate and optical density (OD) was recorded at 340 nm (Wroblewski 1955). A similar reaction mixture was used for the estimation of malate dehydrogenase (MDH; L-malate: NAD+oxidoreductase: EC.1.1.1.37) except for the substrate (1 mg oxaloacetate/mL of chilled triple distilled water) (Ochoa 1955). Glucose-6-phosphate dehydrogenase (G6PDH; EC.1.1.1.49) activity was measured by method of De Moss (1955). The reaction mixture of 1.5 mL Tris buffer (0.1 M, 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) are mixed well and optical density (OD) was recorded at 340 nm. Gluthathione-S-transferase (GST; EC 2.5.1.18) was measured spectrophotometrically by the method of Habing et al. (1974). Acetylcholine esterase (AChE) (EC. 3.1.1.7) activity as measured by the change in OD at 540 nm using the method of Hestrin modified by Augustinsson (1949). 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; EC.3.1.3.1) was determined by the method of Garen and Levinthal (1960). Glycogen was estimated colorimetrically by the method described by Hassid and Abraham (1957).

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 was used to determine the significant differences between the means. Comparisons were made at the 5 and/or 1% probability level.

Results and discussion

Water quality parameter

The water quality parameters like temperature, pH, dissolved oxygen (DO), free carbon dioxide (CO_2), total alkalinity, total hardness, ammonia (NH_3), nitrite, and nitrate are presented in Table 2. Water quality parameters were checked every week and were found to be within the recommended range for carp rearing.

Growth performance

The data pertaining to weight gain (%), feed conversion ratio (FCR), and specific growth rate (SGR) are presented in Table 3. The weight gain (%) and SGR were significantly higher in lecithin, betaine, and choline fed group. The treatment groups exposed to low dose of endosulfan (1/10th of LC 50, 0.2 ppb) and concurrently exposed to endosulfan and elevated temperature (34°C) had significantly lower weight gain percentage and SGR than control group. This was in association with observed FCR trend.

The growth performance of *L. rohita* fingerlings was significantly lower in groups exposed to endosulfan and concurrently exposed to endosulfan (1/10th of LC 50, 0.2 ppb) and elevated temperature (34°C). Similar effects have been observed previously in *L. rohita* exposed to endosulfan (Ramaneswari and Rao 2000) and in Nile Tilipia (*Oreochromis mossambicus*) exposed to sublethal level of two pesticides namely malathion and dimethoate (Sweilum 2006). The decreasing trend in the growth of *L. rohita* could be a consequence of altered metabolism, resulting from toxic stress (Adeyemo 2005; Petri et al. 2006). However, dietary lecithin, betaine, and choline mitigated the negative effect of endosulfan toxicity and high temperature as evident from the increased growth. Lecithin, betaine, and choline in the diet might have enriched the amino acid pool in the cells and these non-essential amino acids act as substrates for gluconeogenesis, which might aid in combating against stressors. However, mainly dietary lecithin and betaine mitigated the negative effect of endosulfan toxicity and this is 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 (Kumar et al. 2011). Based on primary response parameters like weight gain %, SGR and FCR, it appears that supplementation of lecithin have beneficial effect on the growth of *L. rohita*

Table 2 Physico-chemical parameters of water during the experimental period of 65 days for different experimental groups

Treatments	Non-stressor	Stressor (endo-exposed)	Stressor [endo + temperature-exposed (E + T)]	Stressor [endo + temperature-exposed (E + T)]		
Diets	Control	Control	Control	Choline	Betaine	Lecithin
Temperature(°C)	26.5–28.6	26.7–28.4	33.8–34.2	33.7–34.2	33.6–34.1	33.8–34.2
pH	7.7–8.6	7.6–8.4	7.5–8.4	7.7–8.7	7.5–8.6	7.6–8.4
DO_2 (mg/L)	6.5–7.7	6.6–7.7	6.7–7.5	6.4–7.6	6.5–7.7	6.7–7.6
Free CO_2 (mg/L)	ND	ND	ND	ND	ND	ND
Hardness (mg/L)	236–244	234–245	235–244	238–243	237–244	236–243
Ammonia (mg/L)	0.21–0.27	0.20–0.25	0.20–0.24	0.21–0.23	0.19–0.22	0.18–0.23
Nitrite (mg/L)	0.001–0.002	0.002–0.003	0.003–0.005	0.001–0.004	0.002–0.003	0.001–0.003
Nitrate (mg/L)	0.002–0.04	0.06–0.07	0.05–0.07	0.04–0.06	0.03–0.05	0.02–0.04

Table 3 Percentage body weight, FCR and SGR of *L. rohita* fingerlings concurrently exposed to endosulfan and high temperature and fed with dietary lecithin, betaine and choline during 65 days

Treatments	Non-stressor	Stressor (endo-exposed)	Stressor [endo + temperature-exposed (E + T)]	Stressor (endo + temperature-exposed (E + T)]			
Diets	Control	Control	Control	Choline	Betaine	Lecithin	*P* value
Weight gain (%)	$77.71^{b} \pm 3.31$	$49.82^{a} \pm 0.58$	$41.45^{a} \pm 1.07$	$87.80^{bc} \pm 5.73$	$95.72^{c} \pm 7.06$	$109.01^{d} \pm 2.04$	$P < 0.01$
FCR	$1.92^{b} \pm 0.090$	$2.87^{c} \pm 0.04$	$3.35^{d} \pm 0.09$	$1.78^{ab} \pm 0.12$	$1.62^{a} \pm 0.14$	$1.51^{a} \pm 0.04$	$P < 0.01$
SGR	$0.88^{b} \pm 0.03$	$0.62^{a} \pm 0.01$	$0.53^{a} \pm 0.01$	$0.97^{bc} \pm 0.05$	$1.03^{c} \pm 0.06$	$1.13^{d} \pm 0.02$	$P < 0.01$

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

FCR Feed conversion ratio, *SGR* specific growth rate

fingerlings when fed practical diets. The weight gain % was significantly higher in the lecithin fed groups as compared to other groups. The beneficial effects of dietary lecithin on growth have been reported in larval and juvenile fish (Craig and Gatlin 1997). However, the effects of lecithin supplementation on growth of fish differ depending on the growth stages (Poston 1991). The inclusion of lecithin may increase growth by supplying phosphatidylcholine (PC) to the fish, thereby reducing energy normally expended in biosynthesis of PC (Craig and Gatlin 1997). The addition of betaine resulted in increased feed consumption and growth in rainbow trout fingerlings (Can and Sener 1992). Supplementation of dietary betaine has been found to improve growth in juvenile *Penaeus monodon* (Penaflorida and Virtanen 1996), *Penaeus indicus* (Jasmine et al. 1993) and *Macrobrachium rosenbergii* (Felix and Sudharsan 2004). Betaine (0.1–1%) has been shown to have no stimulating effect on feeding in gibel carp juveniles when fed with diets containing fish meal (Xue and Cui 2001). The growth result was well supported by the FCR and SGR values. The lower FCR in lecithin and betaine fed groups indicates better nutrient utilization in these groups. Lecithin supplementations have been reported to increase the feed efficiency of several fish species (Vijayaraghavan and Rao 1986). Thus, the results indicate the importance of lecithin in practical diet for efficient nutrient utilization in *L. rohita* fingerlings.

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 activities of the experimental groups at the end of the experiment are shown in Table 4. Dietary choline, betaine, and lecithin significantly ($P < 0.01$) effected LDH and MDH activities in liver and muscle of *L. rohita* fingerlings. In liver, considerably higher activities of LDH and MDH were found in groups exposed to endosulfan and were further increased in the group which was concurrently exposed to endosulfan and elevated temperature, while the reduced activities were recorded in betaine and lecithin fed groups.

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

Table 4 Metabolic enzymes of *L. rohita* fingerlings concurrently exposed to endosulfan and high temperature and fed with dietary lecithin, betaine, and choline during 65 days

Treatments	Non-stressor	Stressor (endo-exposed)	Stressor [endo + temperature-exposed (E + T)]	Stressor [endo + temperature-exposed (E + T)]			
Diets	Control	Control	Control	Choline	Betaine	Lecithin	*P* value
LDH-liver	$0.96^{a} \pm 0.23$	$1.36^{bc} \pm 0.07$	$1.94^{e} \pm 0.13$	$1.66^{bc} \pm 0.19$	$1.19^{ab} \pm 0.11$	$0.90^{a} \pm 0.13$	$P < 0.01$
MDH-liver	$0.89^{a} \pm 0.14$	$1.61^{b} \pm 0.14$	$1.71^{b} \pm 0.40$	$1.51^{b} \pm 0.14$	$0.71^{ab} \pm 0.16$	$0.57^{a} \pm 0.11$	$P < 0.01$
G6PDH-muscle	0.0094 ± 0.001	0.010 ± 0.002	0.012 ± 0.005	0.006 ± 0.00	0.0106 ± 0.03	0.007 ± 0.00	NS
G6PDH-liver	0.36 ± 0.09	0.48 ± 0.10	0.58 ± 0.05	0.27 ± 0.07	0.38 ± 0.06	0.34 ± 0.0	NS
AST-muscle	9.14 ± 0.66	9.53 ± 1.44	10.91 ± 0.94	10.85 ± 1.16	7.13 ± 2.00	9.08 ± 1.12	NS
AST-liver	8.34 ± 1.02	10.08 ± 0.29	10.71 ± 0.77	9.14 ± 0.80	8.30 ± 0.80	8.86 ± 2.19	NS
ALT-muscle	5.67 ± 0.90	8.35 ± 2.05	9.18 ± 1.61	6.95 ± 0.87	9.46 ± 1.17	5.31 ± 0.48	NS
ALT-liver	$7.61^{ab} \pm 0.95$	$8.13^{c} \pm 0.77$	$8.54^{e} \pm 0.90$	$6.91^{bc} \pm 0.70$	$5.14^{ab} \pm 0.97$	$4.22^{a} \pm 0.62$	$P < 0.01$

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, glucose-6-phosphate dehydrogenase: specific activities expressed as Δ 0.01 OD/min/mg protein

ALT (aspartate amino transferase): specific activities expressed as nano moles of sodium pyruvate formed/mg protein/min at 37°C. AST (alanine amino transferase): specific activities expressed as nano moles of oxaloacetate released/min/mg protein at 37°C

effect on various enzymes like LDH, MDH, ATPase, ALP etc. In the present study, LDH and MDH activities increased significantly ($P < 0.01$) in endosulfan exposed and concurrently exposed to endosulfan and temperature groups, suggesting that the animals were under stress induced by endosulfan and concurrent elevated temperature which is in agreement with previous observation that LDH activity generally increases in stress (Vijayaraghavan and Rao 1986). Higher activity of MDH indicates greater activity of TCA cycle due to increased energy demands during stress. LDH and MDH activities decreased significantly ($P < 0.01$) in the groups fed with lecithin, betaine, and choline-supplemented diets indicating the stress mitigating effect of these methyl donors. Similar stress mitigating role of methyl donor was reported by Kumar et al. (2011) when *L. rohita* exposed with endosulfan.

G6PDH (glucose-6- phosphate dehydrogenase)

G6PDH (glucose-6- phosphate dehydrogenase) muscle and liver, AST (aspartate amino transaminase) muscle and liver and ALT (alanine amino transaminase) activities in muscle of *L. rohita* fingerlings did not differ significantly ($P > 0.05$, Table 4) among different experimental groups.

Alanine amino transaminase (ALT)

Alanine amino transaminase (ALT) activity in liver of *L. rohita* fingerlings varied significantly ($P < 0.01$, Table 4) among different treatment groups and the highest activity of liver ALT was recorded in endosulfan-exposed group and group concurrently exposed to endosulfan and temperature which got reduced when fed with methyl donor-supplemented diet and the lowest ALT activity was observed in lecithin-supplemented group.

The higher activity of ALT in liver of fish exposed to endosulfan stress and combined stress of endosulfan and temperature fed with control diets, indicates the mobilization of aspartate and alanine via gluconeogenesis for glucose production to cope up with stress. Similarly, 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, inclusion of methyl donors reduces the ALT activity in liver which can be inferred

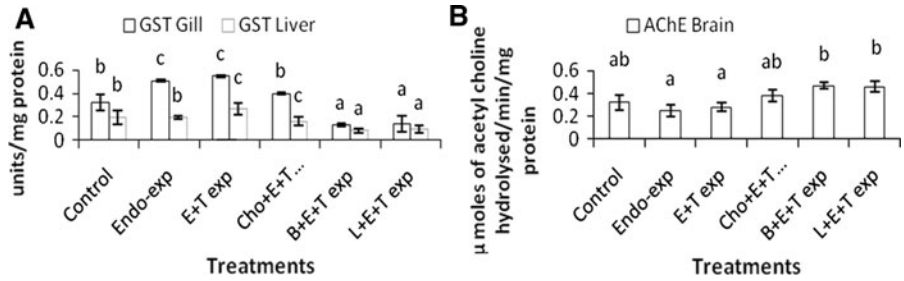

Fig. 1 Impact of dietary choline, betaine, and lecithin on brain AChE (acetyl choline esterase) and GST (glutathion-S-transferase) in gill and liver in response to concurrent exposure to endosulfan (1/10th of LC 50, 0.2 ppb) and elevated temperature (34°C) of *L. rohita* fingerlings at the experiment period of 65 days (values with different superscript differ significantly ($P < 0.01$), data expressed as Mean ± SE, ($n = 6$). (control T_0, endosulfan exposed = endo-exp T_1, concurrent exposed to endosulfan and temperature = E + T expo T_2, choline fed endosulfan and temperature-exposed group T_3, betaine fed endosulfan and temperature-exposed group T_4, lecithin fed endosulfan and temperature-exposed group T_5)

that inclusion of lecithin, betaine, and choline in diets reduces the stress born energy demand and stress in *L. rohita* fingerlings.

Antioxidant enzymes (glutathion-S-transferase)

Compared with endosulfan alone and concurrent exposure to endosulfan and temperature glutathione-S-transferase activity in gill and liver exhibited a significant ($P < 0.05$, Fig. 1a) decreases in betaine- and lecithin-supplemented group in gill as well as in liver.

The GST activity in gill and liver of *L. rohita* fed with methyl donors especially betaine- and lecithin-supplemented group showed decreased in the activity but endosulfan alone and concurrent exposure to endosulfan and temperature-exposed group were found to have higher GST activity. Endosulfan induces oxidative tissue damage resulting from the release of oxygen free radicals (OFRs) Hincal et al. (1995). Due to high reactivity of OFRs, most components of the cellular structure and function may become potential targets of oxidative damage. Reduced activities of the antioxidative enzymes, in supplemented group, indicate stabilization of the cellular structure from such oxidative damage due to supplementation of these methyl donors.

Neuro-transmission enzymes

Data pertaining to the impact of dietary pyridoxine on AChE activity in the brain tissue of *L. rohita* fingerlings exposed to endosulfan-induced chronic stress is shown in Fig. 1b.

The AChE activity in brain of *L. rohita* fingerlings was assayed at the end of the experiment. The experimental group fed diet without methyl supplementation showed a decrease in the activity, which indicates stress in animals induced by endosulfan alone and endosulfan- and temperature-exposed group. Similar results were described by Akhtar et al. (2009) who observed inhibition of AChE in *L. rohita* on exposure to endosulfan. It has also been reported that AChE is inhibited by organophosphorus compounds and some toxins (Gopal et al. 1985). In methyl donors-supplemented group especially betaine and lecithin, the enzyme activity was significantly more. This indicates the stress mitigating effect of dietary betaine and lecithin.

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 is shown in Table 5. There was a significant ($P < 0.01$) effect of dietary choline, betaine, and lecithin on ATPase activity of gill and liver. Activity of ATPase was significantly lower ($P < 0.01$) in endosulfan-exposed group and concurrently exposed to endosulfan and elevated temperature, compared with supplemented 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 fish in terms of

Table 5 ATPase, ALP and glycogen of *L. rohita* fingerlings concurrently exposed to endosulfan and high temperature and fed with dietary lecithin, betaine, and choline during 65 days

Treatments	Non-stressor	Stressor (endo-exposed)	Stressor [endo + temperature-exposed (E + T)]				
Diets	Control	Control	Control	Choline	Betaine	Lecithin	*P* value
ATPase-liver	$43.02^{bc} \pm 2.61$	$24.13^{a} \pm 1.47$	$35.38^{b} \pm 1.61$	$37.58^{b} \pm 2.58$	$53.35^{c} \pm 5.82$	$39.29^{b} \pm 6.07$	$P < 0.01$
ATPase-gill	$144.48^{c} \pm 3.85$	$132.90^{b} \pm 3.12$	$109.60^{a} \pm 1.23$	$151.36^{c} \pm 0.90$	$151.34^{c} \pm 5.47$	$173.97^{d} \pm 2.37$	$P < 0.01$
ALP-intestine	$114.49^{a} \pm 1.51$	$134.02^{bc} \pm 1.41$	$146.02^{c} \pm 0.56$	$155.53^{bc} \pm 1.46$	$99.69^{a} \pm 1.28$	$95.17^{a} \pm 1.62$	$P < 0.01$
Glycogen-muscle	1.08 ± 0.10	1.24 ± 0.26	1.33 ± 0.20	0.70 ± 0.14	0.45 ± 0.10	1.11 ± 0.15	NS
Glycogen-liver	3.54 ± 0.40	5.39 ± 0.21	5.34 ± 0.34	2.67 ± 0.45	3.33 ± 0.65	3.14 ± 0.54	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

Glycogen: mg glycogen/g tissue

the energy balance and ion transport. This might have considerably affected the glucose metabolism in the hyperglycemia state in *L. rohita* fingerlings. In present study, liver and gill thereby leading to ATPase activity in liver and gill was reduced significantly in endosulfan-exposed group and concurrently exposed to endosulfan and elevated temperature. The ATPase activity in liver and gill was significantly higher in group fed with lecithin-, betaine-, and choline-supplemented diets. This suggests 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 in liver ATPase activity in *Channa gachua* upon endosulfan exposure. In the present study, reduction in liver and gill 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 enzymes by endosulfan. Higher activity of ATPase in liver and gill the treatment groups suggests that the supplementation of dietary lecithin, betaine, and choline may help in reducing energy demands in the *L. rohita* fingerlings. Similar results were reported by Kumar et al. (2011) who find that lecithin, betaine, and choline enhance ATPase activity during endosulfan exposure to *L. rohita*.

Alkaline phosphatase (ALP)

The intestinal ALP activity was significantly higher ($P < 0.01$, Table 5) in endosulfan-exposed group and further elevated when concurrently exposed to endosulfan and elevated temperature. The intestinal ALP activity was normal in betaine- and lecithin-supplemented diet group.

ALP, a zinc-containing metallo-enzyme, plays an important role in phosphorus metabolism. ALP activity was found to be low in the fish exposed to endosulfan alone and was much higher compared with endosulfan in the group exposed concurrently to endosulfan and elevated temperature. These results are consistent with other results previously observed in *Channa punctatus* (Sharma 1990), and in other species (Verma et al. 2007). Such decreased ALP activity could possibly be an indication of role of endosulfan in inhibiting protein synthesis (Verma et al. 2007). However, when fishes were fed with diet containing lecithin and betaine, ALP activity was normal, which might be due to the easily liberated phosphate ions to

combat stressful condition or higher metabolic rate (Kumar et al. 2011).

Glycogen

Glycogen in muscle and liver of the experimental group at the end of experiment are shown in Table 5. No significant difference was observed in muscle and liver glycogen in *L. rohita* fingerlings. Higher level of muscle and liver glycogen was observed in endosulfan exposed and concurrently exposed to endosulfan and temperature groups as compared to control and choline, betaine, and lecithin fed groups.

Glycogen broke down into glucose in the early stage of stress (Barton and Iwama 1991). In the present study, no significant difference was observed in muscle and liver glycogen level but were higher in endosulfan-exposed group and concurrently exposed to endosulfan and temperature groups. It may be due to utilization of tissue glycogen reserve in initial stage of stress (Vijayan et al. 1997). Similar reports are available in common dentex (*Dentex dentex*) exposed to handling stress, glycogen reserve were not affected (Morales et al. 2005).

Conclusion

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

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