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Dietary pyridoxine potentiates thermal tolerance, heat shock protein and protect against cellular stress of Milkfish (*Chanos chanos*) under endosulfan-induced stress

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ABSTRACT

We herein report the protective role of pyridoxine in enhancing thermal tolerance of Milkfish *Chanos chanos* reared under endosulfan-induced stress. Four isocaloric and isonitrogenous diets were prepared with graded levels of pyridoxine (0, 50, 75 and 100 mg/kg). Two hundred and twenty five fishes were randomly distributed into four treatment groups in triplicate, reared under endosulfan-treated water, which were fed with pyridoxine supplemented diet, while the negative control group was reared without endosulfan-treatment and control fed. The concentration of endosulfan in treated water was maintained at a level of 1/40th of LC_{50} i.e. 0.52 μg/L. Dietary pyridoxine supplementation had significant ($p < 0.01$) effect on temperature tolerance viz. CTmax (Critical temperature maxima), LTmax (Lethal temperature maxima), CTmin (Critical temperature minima) and LTmin (Lethal temperature minima) of milkfish. The positive correlation was observed between CT max and LTmax ($Y = -1.54 + 15.6x$, R^2, 0.943) as well as CTmin and LTmin ($Y = -1.44 + 1.021x$, R^2, 0.941). At the end of the thermal tolerance study, antioxidative status and HSP 70 were significantly reduced in pyridoxine supplemented groups, whereas brain AChE was significantly ($p < 0.01$) elevated compared to positive and negative control. It is concluded that CTmax, LTmax, CTmin and LTmin, antioxidative status, neurotransmitter enzyme and HSP 70 strengthened the enhancement of thermal tolerance of Milkfish.

1. Introduction

The direct relationship between metabolic rate and life span is strongly supported from the temperature manipulation experiments in fish [1,2]. The aquatic ectotherms do not physiologically control their body temperature, but it is regulated by environmental temperature. Due to global warming increases in atmospheric and environmental temperature and its make aquatic ectotherms vulnerable to considerable thermal stress. Hence, thermal stress studies have gained significant attention among scientists to understand the impact of global warming on animals, including fish. Moreover, recent studies have also demonstrated the deleterious effects of various pesticides viz. fenvalerate [3] and cypermethrin [4] in *Labeo rohita*, fipronil in *Cyprinus carpio* [5] and endosulfan in *L. rohita* and *Oreochromis mossambicus* [6,7]. It has also been reported that, the presence of any kind of contamination reduces the thermal tolerance of fish [8]. However there is no information available about the concurrent effect of pesticide along with temperature in milkfish. Therefore, it is pertinent to explore the nutritional strategies as alternative methods to alleviate the deleterious effect of pesticides and thermal stress in aquaculture.

The pyridoxine is an active hypothermic agent [9], water soluble B complex vitamin and also called as vitamin B6. It includes pyridoxine, pyridoxal and pyridoxamine. It is an essential nutrient required to maintain normal physiological functions of animals and is also a precursor for coenzyme (pyridoxal 5-phosphate and pyridoxinamine 5-phosphate) which is required for more than 100

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enzyme reactions. The coenzyme of aminotransferase and decarboxylase in the reactions of amino acid and nitrogenous compounds are the most important ones [10]. Due to the multiple roles of pyridoxine at various metabolic levels, metabolic disturbances relating to pyridoxine have profound effects on physiological functions of animals. It participates in the protein and amino acid metabolism in the form of the prosthetic group of enzymes [11] and as a cofactor on erythropoiesis [12].

The coastal and estuarine water bodies are more productive and having more biological diversity compared to freshwater bodies. However, the coastal and estuarine water bodies are more susceptible to anthropogenic and natural stressors such as temperature and pollutant [13]. Temperature tolerance varies with species, acclimation temperature, acclimation duration and salinity [14,15]. Beyond the optimum temperature, the health of aquatic animals was adversely affected due to metabolic stress and increased oxygen demand leading to susceptibility to diseases [16]. The living organisms that live in these environments experience alterations in their biochemical, molecular and physiological processes related to homeostasis. Euryhaline species, *Chanos chanos* is one of the most important brackishwater fish in Southeast Asia and is widely cultured in the Philippines, Indonesia, India and Taiwan [17]. The production of *C. chanos* has been estimated to be 9, 43, 259 tons [18]. There are few studies in relation to toxicity and thermal tolerance of this species [1,19–21]. Therefore, the present experiment was conducted to delineate the possible protective role of dietary pyridoxine against thermal stress in endosulfan exposed *C. chanos*, one of the Indian brackishwater fish widely cultured in coastal area.

2. Materials and methods

2.1. Experimental design and conditions

Milkfish fingerlings were obtained from the Central Institute of Brackishwater Aquaculture (CIBA), Muttukudu Experimental Station, Chennai, India. The experimental animals were acclimatized in fibre reinforced plastic (FRP) tanks (Circular, 500 L) for a period of 15 days prior to the experiment. After that, the fish were randomly distributed into 15 FRP tanks (80 × 57 × 42 cm) of 150 L capacity and reared for five weeks. Fifteen fish of uniform size (12.65 ± 1.25 g) per tank were stocked in five different treatment groups in triplicates following a completely randomized design. The fish were fed with the experimental diet twice daily (10:00 a.m. and 17:00 p.m.) to satiation for five weeks. The aeration was provided round the clock and water temperature recorded to be in the range of 26.0–28.6 °C and 33–35 ppt salinity and manual water exchange (two third) was carried out at every second alternate day. The experimental setup consisted of normal water (without endosulfan) and fed with the control diet (control group, Ctr/Ctr), endosulfan-treated water & fed with the control diet (EE/Ctr), endosulfan treated water and fed with pyridoxine 50 mg/kg (EE/PY 50 mg), endosulfan-treated water & fed with pyridoxine 75 mg/kg (EE/PY 75 mg) and endosulfan-treated water and fed with pyridoxine 100 mg/kg (EE/PY 100 mg). The endosulfan level was maintained at 1/40 (0.52 μg/L) of 96 h LC_{50} 21.5 μg/L, (*C. chanos*, wt. 110 ± 5.65 g) [21], for all the treatment using technical grade endosulfan (99% pure; α: β ratio, 7:3) purchased from Excel Crop Care Limited, Hubli, Karnataka, India). The standard was kept in an airtight container at 4 °C. As endosulfan does not readily dissolve in water, a stock solution of 10 ppm was prepared in 5% ethanol solution (99.9% pure) as described in our earlier [6,7,21] report. The water quality parameters viz. dissolved oxygen and temperature (dissolved oxygen and temperature meter, Merck, Germany), pH (digital pH meter, LABINDIA, Mumbai), free carbon dioxide (titrimetric method, APHA [22], total hardness (carbonate hardness test kit, Merck, Germany), ammonia (at 635 nm by phenate method APHA [22], nitrite and nitrate (543 nm wavelength APHA [22] were recorded weekly/daily.

2.2. Experimental diet preparation

Four iso-caloric and iso-nitrogenous diets viz. basal diet and three supplemented diets at 50 mg/kg, 75 mg/kg and 100 mg/kg of pyridoxine were prepared using pyridoxine hydrochloride. Pyridoxine was procured from M/S HIMEDIA, Mumbai, India. For formulation of pelleted diet, quality fish meal, soybean meal, sunflower meal, wheat flour, wheat bran and sunflower oil were procured from local market. The pyridoxine free vitamin mineral mixtures were used (SuppleVitE-M, Zydus Animal Health Limited, Ahmedabad) along with ascorbyl phosphate (SD Fine Ltd., Mumbai, India) as the source of vitamin C. The dough was mixed properly, pelleted, air dried and kept in hot air oven at 60°C until dry and subsequently stored at 4 °C until required for feeding.

2.3. Proximate analysis of feed

The proximate composition of the experimental diets was determined following the standard methods of AOAC [23] and is presented in Table 1. The moisture content was determined by drying in hot air oven at 105 °C, until constant weight attained. 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. The 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 muffle furnace at 600 °C for 6 h. The total carbohydrate was calculated by difference, i.e. total carbohydrate % 100-(CP% + EE% + Ash%). The digestible energy of experimental diets was calculated by method describe by Halver [24].

2.4. Tissue homogenate preparation

Gill, liver and brain tissues of fish from all the groups were dissected and carefully weighed. Tissues were homogenized (5% w/v) separately in chilled sucrose solution (0.25 M) in a glass tube using Teflon coated mechanical tissue homogenizer (MICCRA D-9, Digitronic, Germany). The tube was kept on ice to avoid denaturation of the enzymes during the homogenization. The homogenates were centrifuged at 5000 rpm for 20 min at 4 °C in a cooling centrifuge (Remi, India). Protein contents in the supernatants were quantified following the method of Lowry et al. [25] using bovine serum albumin as a standard. The supernatants were collected and stored at −20 °C until further analysis.

2.5. Thermal tolerance experiment

Thermal tolerance was determined as described by Beitinger et al. [26], Dalvi et al. [27], and Kumar et al. [1]. The fish were deprived of feed for one day before performing the thermal tolerance study. Four fish (two for CTmin & LTmin and two for CTmax & LTmax, separately) were randomly selected from each replicate for a particular treatment group and fish from each treatment group were shifted to separate thermostatic water bath aquaria of 52 L water capacity, sensitivity ±0.2 °C for temperature tolerance study. The temperature and endosulfan concentration of water in the thermostatic aquaria were maintained similar to the experimental groups and the dissolved oxygen concentration was maintained at

Table 1
Diet composition and proximate analysis of the experimental diets (% dry matter (DM) basis) fed to *C. chanos* fingerlings during the experimental period.

Ingredient	Pyridoxine			
	Control	PY 50 mg	PY 75 mg	PY 100 mg
Soybean meal[a]	45.5	45.5	45.5	45.5
Fish meal[a]	10	10	10	10
Sunflower meal[a]	10	10	10	10
Wheat flour[a]	14.97	14.92	14.895	14.87
Rice bran[a]	10	10	10	10
Sunflower oil[a]	4.5	4.5	4.5	4.5
Cod liver oil[a]	2	2	2	2
CMC[b]	1	1	1	1
Vitamin + mineral mix[c]	2	2	2	2
Vitamin C[d]	0.03	0.03	0.03	0.03
Pyridoxine[b]	0	0.05	0.075	0.1
Total	100	100	100	100
Proximate Analysis of Experimental feed				
CP[1]	34.87 ± 0.78	34.92 ± 0.98	35.22 ± 0.97	34.23 ± 1.08
EE[2]	11.23 ± 0.34	11.09 ± 0.56	11.33 ± 0.53	11.44 ± 0.58
ASH	9.48 ± 0.13	9.64 ± 0.38	9.43 ± 0.02	9.95 ± 0.13
TC[3]	44.42 ± 0.98	44.34 ± 0.96	44.01 ± 1.43	44.38 ± 1.07
OM[4]	90.52 ± 0.13	90.36 ± 0.38	90.57 ± 0.02	90.05 ± 0.13
DM[5]	92.90 ± 0.62	92.85 ± 0.80	92.45 ± 0.40	93.31 ± 0.06
DE[6]	418.24 ± 2.08	416.92 ± 3.01	418.94 ± 2.73	417.39 ± 3.36

Vitamin A 50,00,00 IU; Vitamin D_3 10,00,000 IU; VitaminB1:20 mg, Vitamin B_2: 2 g; Vitamin E 750 units, Vitamin K 1 g; Vitamin B_{12} 6 mg; Calcium Pantothenate 10 g, Nicotinamide 6 mg; Mn 27.5 g; I 1 mg; Fe7.5 g; Zn 15 g; Cu 2 g; Co 0.45 g; Ca 750 g.
* Digestible energy (K cal/100 g) = (% CP × 4) + (% EE × 9) + (TC × 4).
CP[1]- Crude Protein; EE[2]- Ether extract; TC[3]-Total Carbohydrate; OM[4]-Organic Matter, DM[5]: Dry matter.
DE[6]- Digestible Energy.
Data expressed as Mean ± SE, n = 3.
[a] Procured from local market.
[b] HIMEDIA (JTJ Enterprises, Mumbai, India), components from Himedia Ltd.
[d] SD Fine Chemicals Ltd., India.
[c] Composition of pyridoxine free vitamin mineral mix (quantity/2.50 kg).

6.5 ± 0.5 mg/L throughout the temperature tolerance study by continuous aeration using a 2-HPcentralized air blower. The water temperature in the aquarium was increased/decreased at a constant rate of 0.30 °C/min, until the loss of equilibrium (LOE) is reached, which was designated as the CTmax/CTmin [26]. The lethal thermal maxima (LTmax)/lethal thermal minima (LTmin) were determined by further increasing/decreasing the temperature until the opercular movement was ceased [28,29]. The LOE, indicates a physiological condition where the brain fails to maintain balance, but somehow try to survive by taking oxygen through opercula whereas loss of opercular movement is a condition where fish even fails to move its opercula and ultimately about to die. The thermal tolerance is established as a powerful tool for studying the thermal tolerance in fish [30] and this technique is critically evaluated in freshwater [14] and in brackishwater [1]. For the analysis of enzymes and Hsp 70, the liver, gill and brain tissue collected after lethal temperature minima (LTmin) and lethal temperature maxima (LTmax).

2.6. Measurement of antioxidant enzymes

2.6.1. Superoxide dismutase (SOD)

SOD (EC 1.15.1.1) activity was measured by the method of Misra and Fridovich [31]. The assay is based on the oxidation of epinephrine-adrenochrome transition by the enzyme. The reaction mixture of 50 µl tissue homogenate, 1.5 ml phosphate buffer and 0.5 ml epinephrine was used and read at 480 nm for 3 min.

2.6.2. Catalase (CAT)

CAT (EC 1.11.1.6) activity was measured by the method of Takahara et al. [32]. The reaction mixture of 2.45 ml phosphate buffer (50 mM; pH-7), 50 µl tissue homogenate and 1 ml of Hydrogen peroxide solution was used and decrease in absorbance was read at 240 nm for 3 min.

2.6.3. Glutathione-S-transferase (GST)

GST (EC 2.5.1.18) was measured spectrophotometrically by the method of Habing et al. [33] using S-2, 4-dinitrophenyl glutathione (CDNB) as substrate. The method is based on the principle of formation of adduct of CDNB, S-2, 4-dinitrophenyl glutathione, which is monitored by measuring the increase in absorbance at 340 nm against the blank.

2.7. Acetylcholine esterase (AChE)

AChE (EC. 3.1.1.7) activity was measured by the change in OD at 540 nm using the method of Hestrin modified by Augustinsson [34]. Acetylcholine iodide and dithiobisnitrobenzoic acid were used as substrate and activity was measured at 412 nm.

2.8. Heat shock protein (HSP 70)

The liver and gill HSP-70 (EIA kit, catalog no. EKS-700B) were determined as per the manufacturer's instructions (Bioguenix/Enzo Life Science, Mumbai, India). The absorbance was read in the ELISA plate reader (Biotek India Pvt. Ltd.).

2.9. Statistical analysis

The data were statistically analyzed by Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS, Chicago, IL), in which data were subjected to one way ANOVA followed by Duncan's multiple range tests were used to determine the significant differences between the means and comparisons were made at the 1% probability level.

3. Results and discussion

3.1. Thermal tolerance (CTmax, LTmax and CTmin and LTmin) and HSP 70

In the present study, dietary pyridoxine noticeably ($p < 0.01$) enhanced the thermal tolerance of *C. chanos* as shown by the decreased CTmin and LTmin (Fig. 1) and increased CTmax and LT max (Fig. 2). CTmax and LTmax were remarkably ($p < 0.01$) enhanced in the group fed with pyridoxine diets compared to negative (unexposed to endosulfan and fed with control diet) and positive control (endosulfan exposed group and fed to control diet). The positive correlations were observed between CTmax and LTmax ($Y = -1.54 + 15.6x$, R^2, 0.943) as well as between CTmin and LTmin ($Y = -1.44 + 1.021x$, R^2, 0.941). The LTmax was found maximum in the group fed with pyridoxine @ 100 mg/kg (45.70 ± 0.06 °C) diet followed by 75 mg/kg (45.03 ± 0.09 °C) and 50 mg/kg (44.17 ± 0.20 °C) in *C. chanos*. Similarly, the dietary pyridoxine significantly ($p < 0.01$) reduced the lower thermal tolerance limits (CTmin and LTmin) (Fig. 1) and maximum reduction (both in CT min and LTmin) was observed in the group fed with pyridoxine 100 mg/kg diet followed by 75 mg/kg and 50 mg/kg pyridoxine diet.

The gill and liver HSP 70 after CTmin and LTmin as well as CTmax and LTmax of *C. chanos* fingerlings under endosulfan exposure for five weeks are presented in Fig. 3. The levels of gill and liver HSP 70 were significantly ($p < 0.01$) higher in positive and negative control in comparison to pyridoxine supplemented group. The level of gill and liver HSP 70 were significantly reduced in the treatment groups fed with pyridoxine 100 and 75 mg/kg of diet.

Thermal tolerance depends on a variety of factors like temperature, size, condition factor [30], toxic chemical [26], species [14] and aquatic environment (coastal, estuaries and freshwater). In this study, the value of CTmax and LTmax of the control feed fed diet group were found to be 41.83 ± 0.20 °C and 43.43 ± 0.18 °C, whereas, the group fed with control diet, but exposed to endosulfan, showed reduced CTmax and LTmax drastically to 39.33 ± 0.20 °C and 40.87 ± 0.32 °C respectively, compared to pyridoxine supplemented group. Akhtar et al. [36] also found similar results with supplementation of 50, 75, 100 and 200 mg/kg of pyridoxine on *Labeo rohita*. Similarly, CTmin (15.37 ± 0.12 °C) and LTmin (14.27 ± 0.20 °C) values of control fed group were at similar with Kumar et al. [1]. But, after supplementation of pyridoxine @ 100 mg/kg, we observed significant reduction in CTmin (13.63 ± 0.09 °C) and LTmin (12.37 ± 0.15 °C), followed by pyridoxine 75 mg/kg diet in CTmin (14.47 ± 0.12 °C) and LTmin (13.47 ± 0.15 °C). The enhancement of thermal tolerance might be due to the hypothermic character of pyridoxine as described by Lindseth and Hicks [9], who found that hypothermia (reduction in core body temperature) occurs in rats when 100 mg/kg pyridoxine

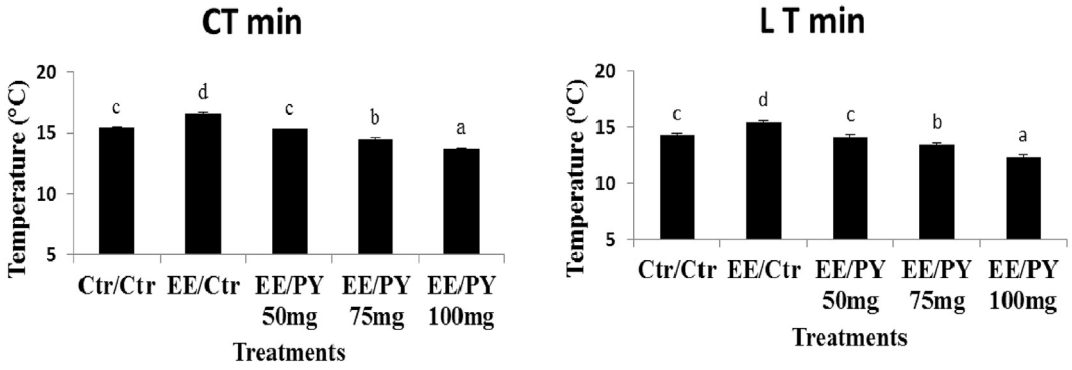

Fig. 1. Effect of dietary pyridoxine on critical temperature minima (CTmin) and lethal temperature minima (LTmin) of *C. chanos* fingerlings under endosulfan exposed for five weeks. Values in the same series with different superscript (a, b, c, d) differ significantly ($p < 0.01$). Abbreviations for exposure/diet treatments of fish: Ctr/Ctr, fish group reared in normal/control (Ctr) water and fed control (Ctr) feed; EE/Ctr, endosulfan-exposed (EE) and control feed (Ctr) fed group; EE/PY 50 mg, endosulfan exposed and supplemental 50 mg pyridoxine (PY) fed group; EE/PY 75 mg, endosulfan exposed and supplemental 75 mg pyridoxine fed group; EE/PY 100 mg, endosulfan exposed and supplemental 100 mg pyridoxine fed group. Values are expressed as mean ± SE, n = 6. Bars bearing different letters (a, b, c, d) indicate significant differences.

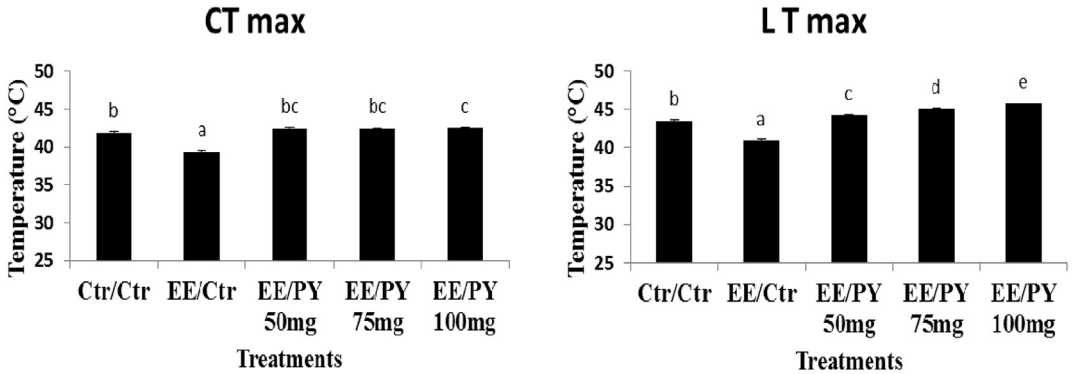

Fig. 2. Effect of dietary pyridoxine on critical temperature maxima (CTmax) and lethal temperature maxima (LTmax) of *C. chanos* fingerlings under endosulfan exposed for five weeks. Values in the same series with different superscript (a, b, c, d, e) differ significantly ($p < 0.01$). Abbreviations for exposure/diet treatments of fish: Ctr/Ctr, fish group reared in normal/control (Ctr) water and fed control (Ctr) feed; EE/Ctr, endosulfan-exposed (EE) and control feed (Ctr) fed group; EE/PY 50 mg, endosulfan exposed and supplemental 50 mg pyridoxine (PY) fed group; EE/PY 75 mg, endosulfan exposed and supplemental 75 mg pyridoxine fed group; EE/PY 100 mg, endosulfan exposed and supplemental 100 mg pyridoxine fed group. Values are expressed as mean ± SE, n = 6. Bars bearing different letters (a, b, c, d) indicate significant differences.

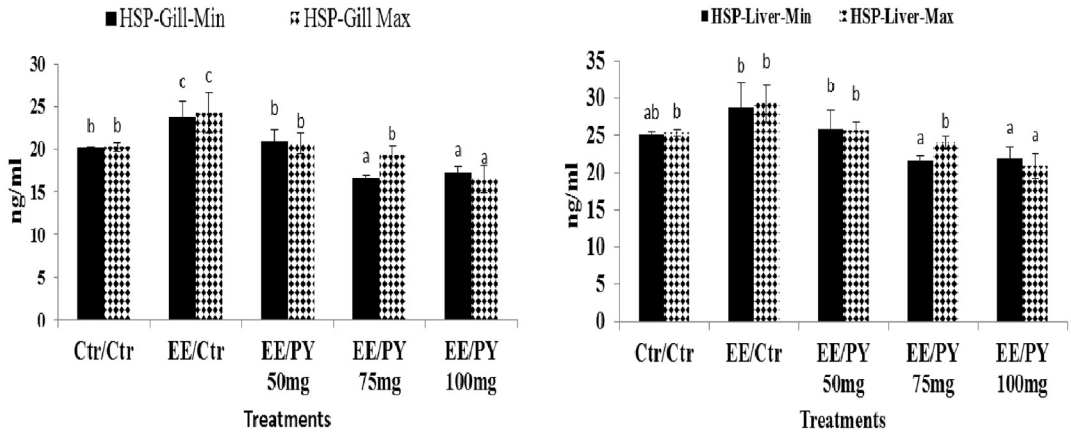

Fig. 3. Effect of dietary pyridoxine on gill and liver HSP 70 after lethal temperature minima (LTmin) and lethal temperature maxima (LTmax) of *C. chanos* fingerlings under endosulfan exposed for five weeks. Values in the same series with different superscript (a, b, c) differ significantly ($p < 0.01$). Abbreviations for exposure/diet treatments of fish: Ctr/Ctr, fish group reared in normal/control (Ctr) water and fed control (Ctr) feed; EE/Ctr, endosulfan-exposed (EE) and control feed (Ctr) fed group; EE/PY 50 mg, endosulfan exposed and supplemental 50 mg pyridoxine (PY) fed group; EE/PY 75 mg, endosulfan exposed and supplemental 75 mg pyridoxine fed group; EE/PY 100 mg, endosulfan exposed and supplemental 100 mg pyridoxine fed group. Values are expressed as mean ± SE, n = 6. Bars bearing different letters (a, b, c, d) indicate significant differences.

was administered through drinking water. Similar results were found by Akhtar et al. [35] for *Labeo rohita* fed to pyridoxine at 50, 75 and 100 mg/kg diet. To the best of our knowledge, there is no report on such effect of pyridoxine in thermal tolerance of brackishwater and marine fish. However, it is possible that dietary pyridoxine may have controlled the expression of heat shock proteins (HSPs), which is revealed by our HSP 70 values. Inside the cell, HSPs has been found to repair and prevent damage from cellular stress associated with protein denaturation at high and low temperatures [36]. In the present study, enhancement of thermal tolerance due to pyridoxine in *C. chanos* might have resulted from the establishment of homeostasis with increased stimulation of the nonspecific defense mechanism by pyridoxine. However, more elaborate investigations need to be carried out to understand the actual mechanism of action of dietary pyridoxine in enhancing thermal tolerance of fish.

3.2. Antioxidative enzymes

The activities of CAT, SOD and GST in liver and gill of *C. chanos* fingerlings fed with pyridoxine 50, 75 and 100 mg/kg, determined at the end of the thermal tolerance (maximum and minimum temperature), are presented in Tables 2 and 3. Activities of CAT, SOD and GST were also higher in different level of pyridoxine supplemented groups but it was lower than negative control (unexposed to endosulfan and fed to control diet). The activities of SOD in liver and gill was noticeably ($p < 0.01$) lower in pyridoxine supplemented groups in compared to positive (endosulfan exposed and fed to control diet) and negative control during lethal temperature minima, whereas during lethal temperature maxima the SOD in liver was significantly lower ($p < 0.01$) in 75 and 100 mg/kg pyridoxine supplemented groups in compared to all others treatments and in case of gill SOD 100 mg/kg pyridoxine supplemented group was significantly lower activities. During lethal temperature minima the catalase in liver was remarkable lower in 100 mg/kg of pyridoxine supplemented groups but in gill all treatments were lower activities in compared to positive controls. Furthermore, the liver and gill catalase activities were significantly lower in 75 and 100 mg/kg of pyridoxine supplemented groups in compared to all others treatments during lethal temperature maxima. In case of liver GST in 75 and 100 mg/kg of pyridoxine and gill GST in 100 mg/kg of pyridoxine supplemented groups were noticeably lower ($p < 0.01$) in compared to all other treatments during lethal temperature minima, whereas, at the time of lethal temperature maxima the 50, 75 and 100 mg/kg of pyridoxine supplemented groups were significantly ($p < 0.01$) lower activities in compared to positive control.

Increased temperature severely affects the membrane fluidity and phospholipid bilayer dynamics, leading to membrane breach, improper folding and production of free radicals. So most of the

Table 2
Effect of different level of dietary pyridoxine on SOD, catalase and GST in liver as well as gill after lethal temperature minima (LTmin) of *C. chanos* fingerlings under endosulfan exposed for five weeks.

Treatments	SOD		Catalase		GST	
	Liver	Gill	Liver	Gill	Liver	Gill
Ctr/Ctr	$27.13^{b} \pm 1.10$	$23.58^{c} \pm 1.37$	$1.07^{a} \pm 0.08$	$1.73^{a} \pm 0.32$	$0.26^{b} \pm 0.01$	$0.33^{b} \pm 0.01$
EE/Ctr	$33.35^{c} \pm 1.90$	$32.55^{d} \pm 0.55$	$2.09^{c} \pm 0.17$	$3.06^{b} \pm 0.40$	$0.38^{c} \pm 0.02$	$0.41^{c} \pm 0.02$
EE/PY 50 mg	$19.26^{a} \pm 0.45$	$20.45^{b} \pm 0.25$	$1.66^{b} \pm 0.07$	$1.76^{a} \pm 0.05$	$0.27^{b} \pm 0.01$	$0.30^{b} \pm 0.01$
EE/PY 75 mg	$19.42^{a} \pm 0.72$	$19.37^{b} \pm 0.87$	$0.89^{a} \pm 0.14$	$1.01^{a} \pm 0.06$	$0.20^{a} \pm 0.02$	$0.30^{b} \pm 0.01$
EE/PY 100 mg	$19.78^{a} \pm 0.18$	$16.16^{a} \pm 0.30$	$0.76^{a} \pm 0.09$	$1.02^{a} \pm 0.15$	$0.17^{a} \pm 0.02$	$0.25^{a} \pm 0.01$
P-Value	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$

Abbreviations for exposure/diet treatments of fish: Ctr/Ctr, fish group reared in normal/control (Ctr) water and fed control (Ctr) feed; EE/Ctr, endosulfan-exposed (EE) and control feed (Ctr) fed group; EE/PY 50 mg, endosulfan exposed and supplemental 50 mg pyridoxine (PY) fed group; EE/PY 75 mg, endosulfan exposed and supplemental 75 mg pyridoxine fed group; EE/PY 100 mg, endosulfan exposed and supplemental 100 mg pyridoxine fed group.
Super oxide dismutase (SOD), catalase and glutathione-s-transferase (GST): Units/mg protein.
Values are expressed as mean ± SE, n = 6. Bars bearing different letters (a, b, c, d) indicate significant differences.

Table 3
Effect of different level of dietary pyridoxine on SOD, catalase and GST in liver as well as gill after lethal temperature maxima (LTmax) of *C. chanos* fingerlings under endosulfan exposed for five weeks.

Treatments	SOD		Catalase		GST	
	Liver	Gill	Liver	Gill	Liver	Gill
Ctr/Ctr	$26.70^{c} \pm 1.03$	$26.92^{b} \pm 0.94$	$1.69^{b} \pm 0.04$	$1.86^{b} \pm 0.03$	$0.29^{ab} \pm 0.01$	$0.30^{a} \pm 0.02$
EE/Ctr	$33.50^{d} \pm 2.57$	$37.40^{c} \pm 2.73$	$3.11^{c} \pm 0.45$	$3.15^{c} \pm 0.42$	$0.43^{c} \pm 0.04$	$0.40^{b} \pm 0.01$
EE/PY 50 mg	$24.17^{bc} \pm 1.63$	$23.48^{b} \pm 0.10$	$1.79^{b} \pm 0.13$	$1.83^{b} \pm 0.05$	$0.30^{b} \pm 0.01$	$0.31^{a} \pm 0.03$
EE/PY 75 mg	$21.23^{ab} \pm 0.45$	$23.20^{b} \pm 0.37$	$0.79^{a} \pm 0.20$	$1.15^{a} \pm 0.05$	$0.25^{ab} \pm 0.02$	$0.27^{a} \pm 0.03$
EE/PY 100 mg	$19.33^{a} \pm 0.49$	$18.13^{a} \pm 1.56$	$0.59^{a} \pm 0.06$	$1.17^{a} \pm 0.16$	$0.23^{a} \pm 0.01$	$0.26^{a} \pm 0.01$
P-Value	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$

Abbreviations for exposure/diet treatments of fish: Ctr/Ctr, fish group reared in normal/control (Ctr) water and fed control (Ctr) feed; EE/Ctr, endosulfan-exposed (EE) and control feed (Ctr) fed group; EE/PY 50 mg, endosulfan exposed and supplemental 50 mg pyridoxine (PY) fed group; EE/PY 75 mg, endosulfan exposed and supplemental 75 mg pyridoxine fed group; EE/PY 100 mg, endosulfan exposed and supplemental 100 mg pyridoxine fed group.
Super oxide dismutase (SOD), catalase and glutathione-s-transferase (GST): Units/mg protein.
Values are expressed as mean ± SE, n = 6. Bars bearing different letters (a, b, c, d) indicate significant differences.

stressors like high temperature, pesticides and other xenobiotics alone or in combination lead synergistic impact and would damage the normal cellular functioning along with DNA and biomembranes. Fish adaptation to changing temperature involves adjustments of both density and functional properties of the mitochondria, thus affecting ROS (Reactive oxygen species) generation and antioxidant defenses [37]. The level of SOD could be a safeguard against changes in temperature to which these species are naturally exposed [38]. Abele and Puntarulo [39] indicated that life under permanent cold water conditions in polar habitats causes reduced activity, lower metabolic rates and lower rates of ROS formation in marine invertebrates and finfishes. However, according to these authors, cellular ROS production could actually be higher in cells of polar ectotherms under environmental stress. If higher mitochondrial densities are common feature of cold water adaptation in polar invertebrates and fishes, these mitochondria might actually produce more ROS under stress. To neutralize the impact of OFRs, both enzymatic and non-enzymatic antioxidants are activated [40]. The detoxification of endosulfan to endosulfan ether mainly occurs in liver [41]. Thus anti-oxidant enzyme activities that protect liver from oxidative damage are sensitive indicators of endosulfan toxicity. Study conducted by Tellez-Banuelos et al. [42]; Kumar et al. [6], after exposure to endosulfan, the activities of catalase, SOD and GST were higher in Tilapia and also in our earlier study, we found that after exposure to endosulfan the activities of catalase, SOD and GST in *Labeo rohita* were higher [1,43] but after 2% lecithin supplementation, catalase, SOD and GST were remarkably reduced. In another study by Kumar et al. [20], it was found that after pyridoxine supplementation the antioxidative enzymes were significantly reduced in endosulfan exposed in *C. chanos*. Similarly, Gupta et al. [44] reported that, decreased level of antioxidant enzyme with levan feeding in C. *carpio* fry exposed to sublethal dose of fipronil. In our earlier studies [45,46], we have reported that the use of nutritional supplementation to fish improved the immunity against stress conditions (endosulfan and concurrent exposure to endosulfan and temperature).

3.3. Enzyme of neurotransmitter

The brain AChE activity analyzed at the end of the thermal tolerance (minimum and maximum temperature), in *C. chanos* are presented in Fig. 4. The level of brain AChE in *C. chanos* during minimum and maximum thermal tolerance were significantly elevated ($p < 0.01$) in 75 and 100 mg/kg of dietary pyridoxine supplemented groups compared to negative (unexposed to endosulfan and fed to control diet) and positive control (exposed to endosulfan and fed to control diet) group after endosulfan exposure

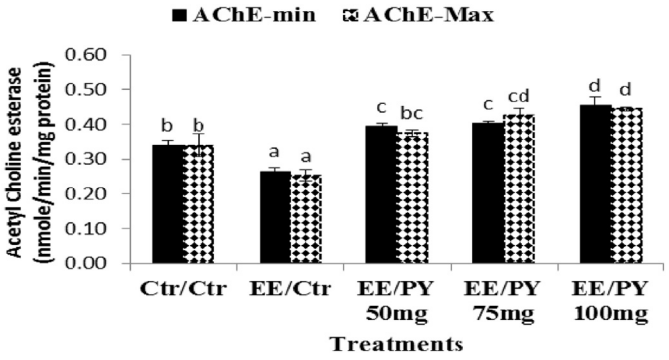

Fig. 4. Effect of dietary pyridoxine on brain AChE activity after lethal temperature minima (LTmin) and lethal temperature maxima (LTmax) of *C. chanos* fingerlings under endosulfan exposed for five weeks. Values in the same series with different superscript (a, b, c, d) differ significantly ($p < 0.01$).; Abbreviations for exposure/diet treatments of fish: Ctr/Ctr, fish group reared in normal/control (Ctr) water and fed control (Ctr) feed; EE/Ctr, endosulfan-exposed (EE) and control feed (Ctr) fed group; EE/PY 50 mg, endosulfan exposed and supplemental pyridoxine (PY) fed group; EE/PY 75 mg, endosulfan exposed and supplemental pyridoxine fed group; EE/PY 100 mg, endosulfan exposed and supplemental pyridoxine fed group. Values are expressed as mean ± SE, n = 6.

to five weeks and also minimum and maximum thermal tolerance temperature.

AChE is the most widely-used enzyme as a biomarker for environmental pollution. AChE inhibition occurs in organophosphate toxicity [47]. In general, fish can tolerate about 70–80% AChE inhibition before death. Kumar et al. [6], observed 80% AChE inhibition in *Oreochromis mossambicus* with behavioral changes such as sluggish movement, loss of balance, etc., but without any mortality. According to them, it may be due to maximum inhibition of AChE activity in the brain, cerebellum, since the cerebellum controls muscular coordination and any change in its activity causes changes in the behavior of the organism. Similar results were observed in our previous finding [43], in *C. chanos* fed with lecithin @ 1–2% improved thermal tolerance along with enhanced activities of neurotransmitter enzyme AChE. We also [6] found reduction in brain AChE activity in *Oreochromis mossambicus* when exposed to endosulfan for 96 h and significant enhancement in the AChE activity, after lecithin supplementation [45]. Mutthappa et al. [48], also reported that exposure to endosulfan resulted in significant reduction in AChE activity in *Labeo rohita* and supplementation of lecithin showed significantly higher AChE activity. In an another study carried out by Kumar et al. [20], also showed that after supplementation with pyridoxine, the activity of AChE was significantly enhanced in endosulfan exposed *Labeo rohita*.

3.4. Conclusion

The fluctuation of temperature and contamination in brackishwater and marine system is more when compared to freshwater system. The presence of any kind of contamination reduces the thermal tolerance of fish, therefore, it was very important to find out effective nutritional supplements which could enhance the thermal tolerance of fish. In the present study, the protective role of pyridoxine as an effective nutritional supplement has been reported in enhancing thermal tolerance of Milkfish *C. chanos* reared under endosulfan induced stress. These data could be useful in formulating suitable feed for culturing brackishwater and marine candidate species under thermal and pesticide induced stresses. However, the further work on the elucidation of mode of action of pyridoxine in enhancing thermal tolerance in fish could be of immense use in aquaculture.

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